Mesenchymal Stem Cells Instruct Oligodendrogenic Fate Decision on Adult Neural Stem Cells

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Key Words. Adult stem cells • Transcription factor • Somatic stem cells
Real-time reverse transcription-polymerase chain reaction • Oligodendrocytes • Enhanced green fluorescent protein
Differentiation

ABSTRACT

Adult stem cells reside in different tissues and organs of the adult organism. Among these cells are MSCs that are located in the adult bone marrow and NSCs that exist in the adult central nervous system (CNS). In transplantation experiments, MSCs demonstrated neuroprotective and neuroregenerative effects that were associated with functional improvements. The underlying mechanisms are largely unidentified. Here, we reveal that the interactions between adult MSCs and NSCs, mediated by soluble factors, induce oligodendrogenic fate decision in NSCs at the expense of astrogenesis. This was demonstrated (a) by an increase in the percentage of cells expressing the oligodendrocyte markers GalC and myelin basic protein, (b) by a reduction in the percentage of glial fibrillary acidic protein (GFAP)-expressing cells, and (c) by the expression pattern of cell fate determinants specific for oligodendrogenic differentiation. Thus, it involved enhanced expression of the oligodendrogenic transcription factors Olig1, Olig2, and Nkx2.2 and diminished expression of Id2, an inhibitor of oligodendrogenic differentiation. Results of (a) 5-bromo-2′-deoxyuridine pulse-labeling of cells, (b) cell fate analysis, and (c) cell death/survival analysis suggested an inductive mechanism and excluded a selection process. A candidate factor screen excluded a number of growth factors, cytokines, and neurotrophins that have previously been shown to influence neurogenesis and neural differentiation from the oligodendrogenic activity derived from the MSCs. This work might have major implications for the development of future transplantation strategies for the treatment of degenerative diseases in the CNS.

INTRODUCTION

Different sources of adult stem cells are currently explored for their potential use to repair degenerated central nervous system (CNS) tissue. Due to their high degree of accessibility and plasticity, bone marrow-derived MSCs might have the potential to be used for this purpose, given that they promoted regeneration and provided neuroprotection and functional recovery after transplantation in the lesioned CNS [1–5]. Several MSC-associated activities are discussed to be at the source of these observations. First, MSCs promoted axonal growth [2–4]. Second, MSCs bear a neurogenic potential and differentiate into neuronal-like cells [6–10]. Third, MSCs enhanced the proliferation and neuronal differentiation of endogenous neural progenitors [1], suggesting that there might be a crosstalk between different stem cell populations. The latter underscores the necessity to deepen our understanding of interactions between progenitors cells of different origins, which could contribute to future strategies to restore the integrity and function of damaged neural tissues.

Here, we analyzed the effects of MSCs on proliferation and cell fate of adult NSCs in cocultures and in experiments using soluble factors derived from MSCs. Cell fate determination and differentiation were analyzed (a) by quantitative reverse transcription-polymerase chain reaction (RT-PCR)-based expression profiling of NSCs for oligodendrogenic, neurogenic, and neural stem cell determinants and (b) by immunocytochemistry using neural cell type-specific antibodies. Moreover, the combination of 5-bromo-2′-deoxyuridine (BrdU) pulse-labeling,

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identification of pycnotic nuclei, and subsequent immunocytochemistry using neural differentiation markers was used to study proliferation, cell death, and cell fate of NSCs.

MATERIALS AND METHODS

MSC and NSC Cultures and Fluorescence-Activated Cell Sorting Analysis

See supplemental online data.

Cocultures of MSCs and NSCs

MSCs (passage 3–4) were plated on polyornithine (250 μg/ml) and laminin (5 μg/ml)-coated glass coverslips at a density of 2,000–4,000 cells per cm^2. Twelve to 24 hours later, NSCs isolated from transgenic rats ubiquitously expressing green fluorescent protein (GFP) [11] were plated over the MSC layer at a density of 10,000 cells per cm^2 in α-minimal essential medium (MEM)-10% fetal bovine serum (FBS) and cocultured for 7 days. Medium was refreshed on the third day. Cells were fixed for 30 minutes with phosphate-buffered 4% (wt/vol) paraformaldehyde (37°C, pH 7.4) and then processed for immunofluorescence staining.

Preparation and Use of Conditioned Media

MSC-conditioned medium (CM): MSCs were plated at 12,000 cells per cm^2 and incubated in α-MEM-10% FBS. After 3 days, the MSC-CM was collected and filtered using a 0.22-μm-pore filter. In some experiments, heat-sensitive factors were inactivated by heating the MSC-CM in boiling water for 10 minutes. Hematopoietic lineage enriched cells (HLC)-CM: HLCs were separated from the adherent cells (MSCs) and cultured in α-MEM-10% FBS for 3 days at the same density as the MSCs. Cells were centrifuged, and the supernatant was collected and filtered. Astrocyte-CM: CM from the astrocytic cell line CTX-TNA2 (American Type Culture Collection, Manassas, VA, http://www.atcc.org) was prepared by growing the cells (10,000 cells/cm^2) for 3 days in α-MEM-10% FBS, and supernatant was collected and filtered. NSCs were plated on polyornithine (250 μg/ml) and laminin (5 μg/ml)-coated glass coverslips at a density of 8,000–10,000 cells per cm^2 in α-MEM-10% FBS for 12–24 hours. Thereafter, medium was replaced with CM or with control α-MEM-10% FBS. After 3 or 7 days, cells were fixed for 30 minutes with phosphate-buffered 4% (wt/vol) paraformaldehyde (pH 7.4) and processed for immunofluorescence staining.

Treatment of NSCs with Candidate Factors

NSCs were plated on polyornithine (250 μg/ml) and laminin (5 μg/ml)-coated glass coverslips at a density of 8,000–10,000 cells per cm^2 in α-MEM-10% FBS for 12–24 hours. Then, NSCs were incubated for 7 days in MSC-CM or α-MEM-10% FBS containing 20 ng/ml human recombinant insulin-like growth factor (IGF)-1, 600 ng/ml human recombinant IGF binding protein-1 (IGFBP-1), 20 ng/ml IGF-1 plus 600 ng/ml IGFBP-1, 10 ng/ml human recombinant transforming growth factor (TGF)-β-1, 20 ng/ml ciliary neurotrophic factor (CNTF), 10 ng/ml neurotrophin (NT)-3, 10 ng/ml brain-derived neurotrophic factor (BDNF), 100 ng/ml nerve growth factor (NGF), 20 ng/ml fibroblast growth factor (FGF)-2 or 200 ng/ml vascular endothelial growth factor (VEGF), or MSC-CM containing 20 ng/ml human recombinant IGF-1, 600 ng/ml IGFBP-1, or 20 ng/ml IGF-1 and 600 ng/ml IGFBP-1. All factors were from R&D Systems Europe Ltd (Abingdon, Oxfordshire, UK, http://www.rndsystems.com). At the end of the experiment, cells were fixed for 30 minutes with phosphate-buffered 4% (wt/vol) paraformaldehyde (pH 7.4) and processed for immunofluorescence staining.

Immunofluorescence Analysis and Quantification

Fixed cells were washed in Tris-buffered saline (TBS) (0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5), then blocked with a solution composed of TBS, 0.1% Triton-X100 (only for intracellular antigens), 1% bovine serum albumin (BSA), and 0.2% Teleostean gelatin (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) (fish gelatin buffer [FGB]). The same solution was used during the incubations with antibodies. Primary antibodies were applied overnight at 4°C. Fluorochrome-conjugated species-specific secondary antibodies were used for immunodetection.

The following antibodies and final dilutions were used. Primary antibodies: rabbit anti-glial fibrillary acidic protein (GFAP) (1:1,000; Dako Denmark A/S, Glostrup, Denmark, http://www.dako.dk); rabbit anti-GalC (1:200; Chemicon International, Temecula, CA, http://www.chemicon.com); rabbit anti-NG2 chondroitin sulfate proteoglycan (1:200; Chemicon International); immunoglobulin M mouse anti-A2B5 (1:200; Chemicon International); mouse anti-rat nestin (1:500; BD PharMingen, San Diego, http://www.bdbiosciences.com); mouse anti-Map 2a+2b (1:250; Sigma-Aldrich); mouse anti-βIII-tubulin (1:500; clone 5G8; Promega, Madison, WI, http://www.promega.com); mouse anti-myelin basic protein (MBP) (1:750; SMI-94; Sternberger Monoclonals Incorporated, Lutherville, MD, http://home.att.net/~sternbmonoc); mouse anti-RIP (1:200; gift from Norbert Weidner); goat anti-GFP (1:1,000; Rockland Immunochemicals, Inc., Gilbertsville, PA, http://www.rockland-inc.com); and rabbit anti-Olig2 (1:50; gift from Magdalena Götz). Secondary antibodies: donkey anti-goat, -mouse, -rabbit, or -rat conjugated with Alexa Fluor 488 (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), rhodamine X (RHOX) 1:500 (Dianova, Germany); goat anti-mouse, -rabbit, or -rat conjugated with Alexa Fluor 488, RHOX (1:500). In cases of detergent-sensitive antigens (i.e., GalC, A2B5, and NG2), Triton X-100 was omitted from FGB buffer. Nuclear counterstaining was performed with 4′, 6′-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) at 0.25 μg/μl (Sigma-Aldrich). Specimens were mounted on microscope slides using a Prolong Antifade kit (Invitrogen). Epifluorescence observation and photodocumentation were realized using a Leica microscope (Leica, Wetzlar, Germany, http://www.leica.com) equipped with a Spot digital camera (Diagnostic Instrument, Inc., Sterling Heights, MI, http://www.diaginc.com).

For each culture condition, 10 randomly selected observation fields, containing a total of 500–1,000 cells, were photographed for cell fate analysis. The expression frequency of selected cell type markers was determined for every condition in two to four independent experiments.

Analysis of Cell Proliferation and Cell Survival

Cell proliferation was assessed by addition of 10 μM BrdU in the culture medium for a period of 24 hours starting on day 2 or

6 after MSC-CM stimulation. Then, cells were washed and further cultured under the respective conditions or fixed in 4% paraformaldehyde and processed. Fixed cells were incubated with primary antibodies against A2B5, Map2ab, GalC, and GFAP as described above. Cells were then postfixed for 30 minutes in 4% paraformaldehyde and incubated with 2 N HCl for 30 minutes at 37°C, washed with borate buffer 0.5 M (pH 8.5) for 10 minutes at room temperature, washed four times with TBS buffer, and blocked with FGB. Finally, cells were incubated overnight with a rat anti-BrdU antibody (1:500; Oxford Biotechnology, Kidlington, Oxfordshire, UK, http://www.oxfordbiotechnology.com). Detection with fluorochrome-conjugated secondary antibody was performed as described above.

To detect dying cells, 50 μg/ml propidium iodide (PI) (Sigma-Aldrich) was added in the culture medium on day 3 or 7 of the stimulation paradigm. After 10 minutes of incubation, cells were washed three times with TBS and fixed with phosphate-buffered 4% paraformaldehyde. Cells were stained for A2B5, Map2ab, GalC, and GFAP as described above. The fraction of BrdU- and PI-positive cells was determined for the total cell population as well as for each subpopulation expressing A2B5, Map2ab, GalC, or GFAP.

Quantitative RT-PCR

NSCs were plated on polyornithine (250 μg/ml) and laminin (5 μg/ml)-coated glass coverslips at a density of 8,000–10,000 cells per cm^2 in α-MEM-10% FBS for 12–24 hours. Then, NSCs were incubated in either α-MEM-10% FBS or MSC-CM. Medium change defines day 0. RNA was extracted at day 0, 3, or 7 with the RNeasy kit (QIAGEN GmbH, Hilden, Germany, http://www1.qiagen.com), and cDNA was synthesized using the RETRO Script kit (Ambion, Austin, TX, http://www.ambion.com). Expression analysis was performed by real-time quantitative PCR with the Rotor Gene 2000 (Corbett Research, Australia) with the QuantiTect SYBR Green PCR kit (Qiagen GmbH) using specific primers (supplemental online Table 1). As an internal reference, the rRNA primer pair QuantumRNA Universal 18S Internal Standard (Ambion) was used. For quantification, a standard curve was established by amplification of serial dilutions of brain cDNA obtained from rats of embryonic day 14 and postnatal day 9. The following temperature profile was used: activation of polymerase: 95°C, 15 minutes; denaturing: 94°C, 20 seconds (45 cycles); annealing: 56°C, 30 seconds; and elongation: 72°C, 60 seconds. The quality of the products was controlled by a Melt curve. For IGF-1, conventional RT-PCR was performed with an annealing temperature of 58°C.

Statistics

Statistical analyses were performed using parametric two-tailed *t* test and one-way analysis of variance (ANOVA). Averages are expressed with their standard deviations. For time-course statistical analysis, two-way ANOVA was used. Statistical analysis was performed using SYSTAT 11 (Systat Software, Inc., Point Richmond, CA, http://www.systat.com).

RESULTS

Cocultures of MSCs and NSCs: Effects on Differentiation of NSCs

Initial experiments tested and confirmed the stem or progenitor character and the multipotent differentiation fate of the adult MSCs and NSCs used in the present study. MSC cultures were generated using the differential adherence procedure to enrich for MSCs. Fluorescence-activated cell sorting analysis using an antibody against the hematopoietic lineage marker CD45 demonstrated that the MSC cultures were virtually pure and contained less than 1% hematopoietic lineage cells (supplemental online Fig. 1). MSCs were at least bipotent and differentiated into adipocytes and osteoblasts (supplemental online Fig. 2). NSCs proliferated, expressed the NSC markers nestin and in the majority of the cases (>90%) the neural progenitor marker A2B5, and differentiated into neurons, astroglia, and oligodendroglia (supplemental online Fig. 2).

Cocultures of MSCs and NSCs were established to study the interactions between the two different kinds of progenitor cells. For controls, NSCs and MSCs were cultured separately (Fig. 1A, 1B). To distinguish NSCs from MSCs in cocultures, NSCs were derived from transgenic rats expressing the GFP reporter gene under a ubiquitous promoter [11]. Thus, NSCs can be identified in cocultures (Fig. 1C) by the expression of GFP (Fig. 1D).

Expression of neural markers was analyzed after 7 days of culture. In NSC-derived cells (GFP-expressing), single-cultured or cocultured with MSCs, expression of markers for the three mature neural lineages could be detected: Map2ab for neurons (Fig. 1E–1H), GFAP for astrocytes (Fig. 1I–1L), and GalC for oligodendrocytes (Fig. 1M–1P). Cells that were devoid of GFP signal did not express these markers. Coculture of NSCs with MSCs significantly increased the proportion of NSC-derived cells expressing GalC, as compared with NSCs grown alone (Fig. 1Q). Similarly, we observed a higher percentage of NSC-derived cells expressing the RIP antigen, a marker for oligodendrocytes, in the coculture condition (data not shown). A lower percentage of NSC-derived cells expressed GFAP in cocultures (Fig. 1Q). In contrast, the proportion of Map2ab-expressing NSC-derived cells remained the same in two culture conditions (Fig. 1Q). In summary, the data suggest that in cocultures with MSCs, NSCs differentiate preferentially into oligodendrocytes.

Oligodendrogenic Effect of MSCs on NSCs Is Mediated by Soluble Factor(s)

To evaluate whether soluble factors secreted by MSCs were responsible for the increased percentage of NSC-derived oligodendrocytes, NSCs were incubated for 7 days in MSC-CM and subsequently analyzed for changes in cell morphology and for the expression of neural markers. Unconditioned medium, HLC-CM, astrocyte-CM, and heat-inactivated MSC-CM were used as control.

NSCs treated with MSC-CM for 7 days displayed multipolar processes with several secondary branches and phase-bright cell bodies (Fig. 2B). This was in contrast to NSCs exposed to unconditioned medium, which displayed fewer processes and phase-dark somata (Fig. 2A). An analysis of markers for mature neural cell types expressed in NSC-derived cultures revealed a significantly higher proportion of GalC-positive cells among

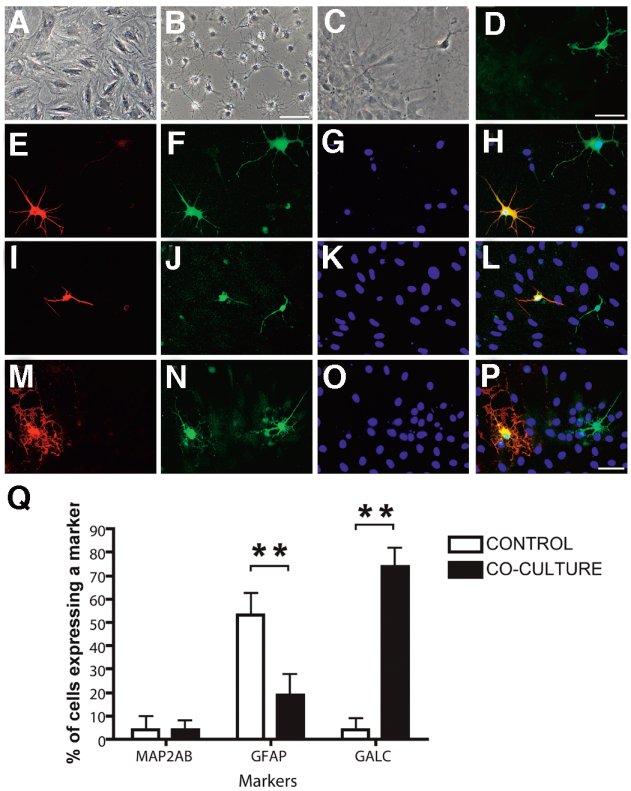

Figure 1. Cocultures of MSCs and NSCs promote oligodendrogenesis of NSCs. MSCs and NSCs (derived from green fluorescent protein [GFP] rats) were cultured in single **(A, B)** and in coculture **(C–P)** conditions for 7 days. **(A):** Phase-contrast images of MSC-only cultures. **(B):** Phase-contrast images of NSC-only cultures. MSC-NSC coculture in **(C)** phase-contrast and the same field in **(D)** fluorescence (green; GFP). Note that GFP-expressing NSC-derived cells can be identified. **(E–P):** Fluorescence images of expression of neural markers in cocultures. Map2ab **(E–H)**, GFAP **(I–L)**, and GalC **(M–P)** expression: cell type-specific markers in red, GFP in green, Dapi in blue; merged pictures are on the right. Note that expression of the neural markers is restricted to NSC-derived cells. **(Q):** Quantitative analysis of the expression of neural differentiation markers in control (NSC-only) and in cocultures. The percentage of GFP-positive cells expressing GFAP, GalC, or Map2ab was determined. Five hundred to 1,000 GFP-positive cells were analyzed in randomly chosen fields. Experiments were done in triplicate. Data represent means ± SD. Student's *t* test was used for statistical analysis; ** = $p < .01$. Scale bars: 100 μm **(B)**, 50 μm **(D, P)**. Abbreviations: GFAP, glial fibrillary acidic protein; MSC, mesenchymal stem cell; NSC, neural stem cell.

cultures containing MSC-CM as compared with those in control conditions (Fig. 2C, 2D, 2K). Similarly, we observed a significantly higher percentage of cells expressing MBP, a mature oligodendrocyte marker, in NSC cultures exposed to MSC-CM compared with control medium (Fig. 2I–2K). There was a lower frequency of GFAP-expressing cells in NSC cultures that were treated with MSC-CM (Fig. 2E, 2F, 2I–2K). Finally, the occurrence of Map2ab expression, which was very low in general, was not significantly affected by the presence of MSC-CM (Fig. 2G, 2H, 2K). The effects of MSC-CM on marker expression were specific for MSCs, given that astrocyte-CM did not induce similar changes (Fig. 2K). Because MSCs contained a minor contamination (<1%) of CD45-positive cells (supplemental online Fig. 1), we included HLC-CM in the experiment. HLC-CM induced a minor, but not significant, increase in the proportion of GalC-positive cells (Fig. 2K), making it unlikely that the MSC-CM effect is derived from the CD45-positive contaminating cell population. In an additional experiment, we tested the dose-dependency of the MSC-CM oligodendrogenic effect by a titration experiment. The results indicate that the oligodendrogenic activity present in the MSC-CM acts in a dose-dependent manner (supplemental online Fig. 3). The activity present in the MSC-CM was partially heat-sensitive; heating of MSC-CM in boiling water for 10 minutes partially blocked the effects on GalC expression (Fig. 2K). In summary, these results suggest that soluble factors released from MSCs promote, specifically and in a dose-responsive manner, the generation of mature oligodendrocytes in NSC cultures at the expense of the astrocytic lineage.

MSC Soluble Factors Instruct Neural Progenitors to the Oligodendrogenic Fate in NSC Cultures

To determine whether soluble factors released by MSCs have an instructive and/or selective activity on neural progenitors cells, we (a) monitored the effects of MSC-CM on NSC morphology and marker expression over time and (b) analyzed proliferation and death of cells in NSC cultures that were exposed to MSC-CM. At day 0 (cells were seeded the day before, day −1), NSCs displayed mostly bipolar morphologies with phase-dark cell bodies and few processes (Fig. 3A, 3D). Three days later, some cells displayed multipolar morphologies with secondary processes, an effect more pronounced in MSC-CM (Fig. 3B, 3E). After 7 days, most of the cells in MSC-CM had secondary processes reminiscent of oligodendrocyte morphology and phase-bright somata (Fig. 3F). NSCs incubated in control medium, however, were multipolar with few secondary branches and flat cell soma reminiscent of astrocytic morphology (Fig. 3C).

A significant time-dependant decrease in the percentage of A2B5-positive cells was observed in both culture conditions. The presence of MSC-CM did not influence the kinetics of the decrease of A2B5 expression (Fig. 3G). The analysis of GalC expression kinetics revealed a significant time-dependant increase in the proportion of GalC-positive cells when NSC cultures were incubated in MSC-CM compared with cultures maintained in the control medium (Fig. 3H). The Map2ab expression pattern remained the same over time, and there was no difference between the two culture conditions employed (Fig. 3I). The proportion of GFAP-positive cells increased in both conditions. However, compared with control medium, a lower percentage of cells expressed GFAP in MSC-CM over time (Fig. 3J). In summary, these data suggest that MSC-CM triggered A2B5-positive progenitor cells to differentiate into GalC-expressing oligodendrocytes at the expense of differentiation into GFAP-expressing cells.

In the next experiments, we tested whether the oligodendrogenic effect of the MSC-CM was due to a selection process. Therefore, we analyzed proliferation and death of cells in NSC cultures that were exposed to MSC-CM. Proliferation and cell death rates were measured by BrdU and PI incorporation, respectively, at days 3 and 7 of MSC-CM treatment for the total cell population as well as for the different cell types present in the cultures. The proliferation rates of most cell types were not modified by the presence of MSC-CM, as compared with con-

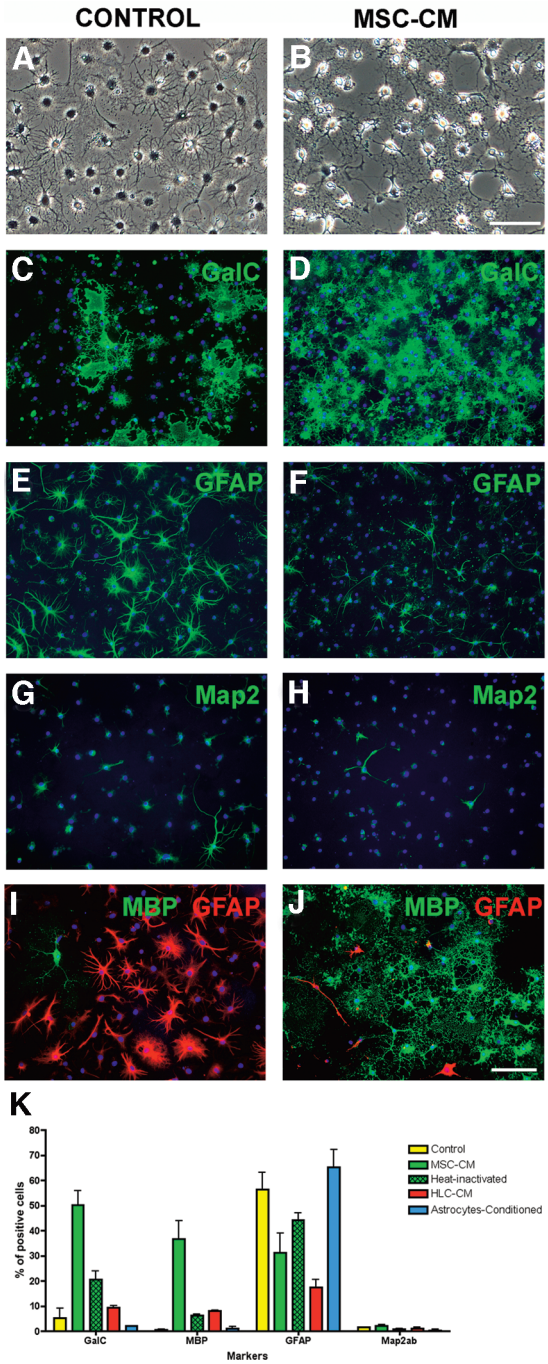

Figure 2. MSC soluble factors induce the expression of oligodendrocyte markers in NSCs. **(A–J):** NSCs were incubated for 7 days in control medium (left panel, **[A, C, E, G, I]**) and in MSC-CM (right panel, **[B, D, F, H, J]**), photographed, and processed for immunecytology. **(A, B):** Phase-contrast images for NSCs incubated in control conditions **(A)** and in MSC-CM **(B)**. **(C–J):** Fluorescence images for GalC (green)/Dapi (blue) **(C, D)**, GFAP (green)/Dapi (blue) **(E, F)**, Map2ab (green)/Dapi (blue) **(G, H)**, and MBP (green)/GFAP (red)/Dapi (blue) **(I, J)**. Note the higher number of GalC and MBP and the lower number of GFAP-expressing cells in MSC-CM. **(K):** Quantitative analysis of the effects of MSC-CM, heat-inactivated MSC-CM, astrocyte-CM, and HLC-CM on the expression of neural differentiation markers in NSC cultures. Note the significant increase in the percentages of GalC-positive ($p < .001$) and MBP-positive cells ($p < .001$) in MSC-CM compared with control medium and astrocyte-CM. A significantly lower percentage of cells expressed GFAP ($p < .05$) in MSC-CM compared with control medium. Experiments were done in triplicate. Results show means ± SDs. One-way analysis of variance-Tukey post hoc was used for statistical analysis. Scale bars: 100 μm **(B, J)**. Abbreviations: CM, conditioned medium; GFAP, glial fibrillary acidic protein; HLC-CM, hematopoietic lineage enriched-conditioned medium; MBP, myelin basic protein; MSC-CM, mesenchymal stem cell-conditioned medium; NSC, neural stem cell.

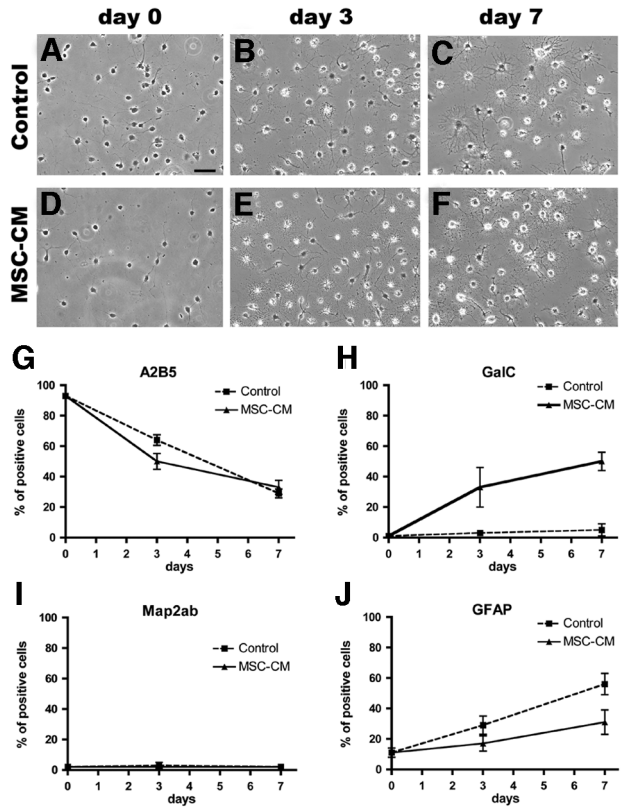

Figure 3. Temporal pattern of changes in morphology and marker expression in NSCs induced by MSC-derived soluble factors. NSCs were incubated for up to 7 days in control medium or in MSC-CM. **(A–F):** Cell morphology was analyzed at days 0, 3, and 7 on phase-contrast images. Scale bar = 100 μm. Note the acquisition of an oligodendroglial morphology in MSC-CM conditions. **(G–J):** The temporal changes in percentage of cells expressing different neural markers (A2B5, GFAP, GalC, and Map2ab) in control-treated and MSC-CM-treated NSCs is shown. **(G):** Significant decrease in A2B5 in control and MSC-CM conditions ($p < .001$). No differences were found between the two conditions ($p > .05$). **(H):** Significant temporal increase in GalC in MSC-CM and compared with control ($p < .005$). **(I):** No changes in Map2ab ($p > .05$). **(J):** GFAP expression pattern: Although the percentage of GFAP-positive cells increased in both conditions over time, a lower percentage of cells expressed GFAP over time in MSC-CM compared with control condition ($p < .05$). Results are shown as mean ± SDs. Experiments were done in triplicate. For statistical analysis, two-way analysis of variance was used. Abbreviations: GFAP, glial fibrillary acidic protein; MSC, mesenchymal stem cell; MSC-CM, mesenchymal stem cell-conditioned medium; NSC, neural stem cell.

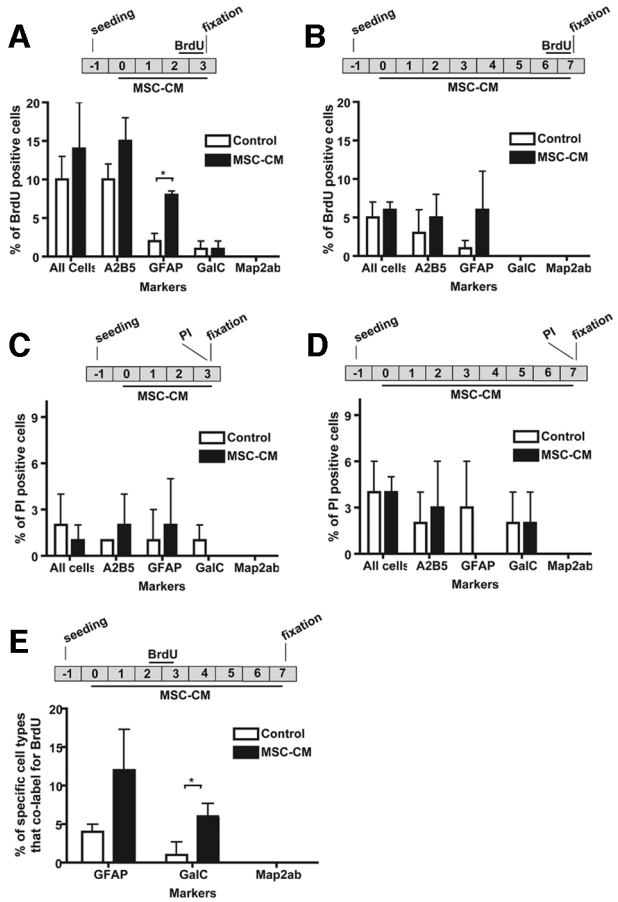

Figure 4. Effects of MSC-CM on proliferation and survival of NSCs and on specific cell types in NSC cultures. **(A, B):** Cell proliferation: NSCs were in control medium or in MSC-CM for up to 7 days. Ten micromolar BrdU was added at day 2 **(A)** or day 6 **(B)**, and 24 hours later, cells were processed for immunofluorescence analysis for BrdU and different neural markers. The percentages of BrdU-labeled cells among the total cell population, the A2B5-, GFAP-, GalC-, and Map2ab-expressing cells, were determined. **(C, D):** Cell death: Cultures were as above, but 50 μg/ml of PI was added at day 3 **(C)** or day 7 **(D)** for 10 minutes. Cells were then immunostained for different neural markers. The percentages of PI-labeled cells among the total cell population, the A2B5-, GFAP-, GalC-, and Map2ab-expressing cells, were determined. The PI incorporation rate was not different for any cell type tested. Three hundred to 500 cells were counted for each experiment **(A–D)**. Experiments were done in triplicate. Errors bars represent SDs; statistical analysis was performed by Student's t test, * = $p < .05$. **(E):** Quantitative cell fate analysis: NSCs were incubated for 7 days in control or MSC-CM. A 24-hour pulse of BrdU was given at day 2. At day 7, cells were coimmunostaining for BrdU and GFAP, GalC, or Map2ab. The percentages of BrdU-positive cells among GFAP-, GalC-, or Map2ab-expressing cells were determined. Only in cells that expressed GalC at day 7 was the percentage that incorporated BrdU significantly higher in MSC-CM compared with control ($p < .05$). Experiments were done in tetraplicate. Results are shown as mean $\pm$ SD. For statistical analysis, Student's t test was performed. Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; GFAP, glial fibrillary acidic protein; MSC-CM, mesenchymal stem cell-conditioned medium; NSC, neural stem cell; PI, propidium iodide.

trol medium. This was evident for the total cell population, as well as for the A2B5-, GalC-, and Map2ab-positive cell subpopulations cultured for 3 or 7 days (Fig. 4A, 4B). In contrast, GFAP-positive cells cultured in the presence of MSC-CM for 3 days displayed a significantly higher proliferation rate compared with the control (Fig. 4A). A tendency toward an increase in the percentage of GFAP-positive cells that colabel for BrdU was still present at day 7; however, no statistically significant differences were detected at this time point (Fig. 4B). No differences in cell death rates were detected between NSC cultures treated with control media or MSC-CM. Hence, differences were observed neither in the total cell population nor when specific subpopulations were scrutinized (Fig. 4C, 4D). In summary, these data indicate that neither a selective proliferation nor selective death of one particular cell type could explain the oligodendrogenic effect of MSC-CM on NSC cultures and therefore suggest an instructive mechanism.

To confirm that a proliferating progenitor population gives rises to the oligodendrocytes under MSC-CM, a BrdU pulse-labeling experiment was performed. NSCs were incubated for 7 days in control medium or in MSC-CM, a pulse of BrdU was given between days 2 and 3 for 24 hours, and on day 7, cells were fixed and analyzed for the presence of BrdU in the different cell populations. The percentage of BrdU-positive cells among the total population of a specific cell type was determined. In this analysis, the total number of a specific cell type colabeling for BrdU should represent the sum of the number of cells expressing a specific marker that proliferated between day 2 and day 3 plus proliferating progenitors that by day 7 differentiated and expressed a cell type-specific marker. The percentage of GFAP-expressing cells that integrated BrdU was higher in MSC-CM compared with control medium; however, this was not statistically significant (Fig. 4E). In contrast, a significantly higher percentage of GalC-expressing cells colabeled with BrdU in the MSC-CM-treated cultures compared with the control (Fig. 4E). Therefore, the higher number of GalC-expressing cells under MSC-CM resulted from a differentiation effect on a proliferating cell population, whereas most of the astrocytes originated from proliferating GFAP-expressing cells.

MSC Soluble Factors Induce Oligodendrocyte Fate Decision in NSCs by Stimulating the Expression of Oligodendrogenic Transcriptional Factors and Repressing an Oligodendrogenic Inhibitor Factor

The experiments reported above demonstrated that MSC soluble factors induce an oligodendrogenic differentiation program in adult NSCs. As a consequence of the oligodendrogenic instructive nature of the MSC-CM, expression of transcriptional factors involved in progenitor fate commitment to various neural lineages was scrutinized on days 0, 3, and 7 of incubation with either control or MSC-CM using quantitative RT-PCR.

First, we quantified the level of expression of Olig1, Olig2, and Nkx2.2, which are known to be oligodendrogenic transcriptional factors [12–18]. After 7 days of incubation, we found that compared with the control medium MSC-CM induced an approximately 12-fold increase in the Olig1 mRNA (Fig. 5A, 5I) and a nearly sevenfold increase in Olig2 mRNA (Fig. 5B, 5I). In addition, there was a significant increase in the level of Nkx2.2 mRNA in MSC-CM with respect to the control (Fig. 5C, 5I). The time course analysis (Fig. 5A, 5B) indicates that the differences in Olig1 and Olig2 mRNA expression levels are derived from an upregulation in the MSC-CM-treated cells rather than a downregulation in the control. The differences in Nkx2.2

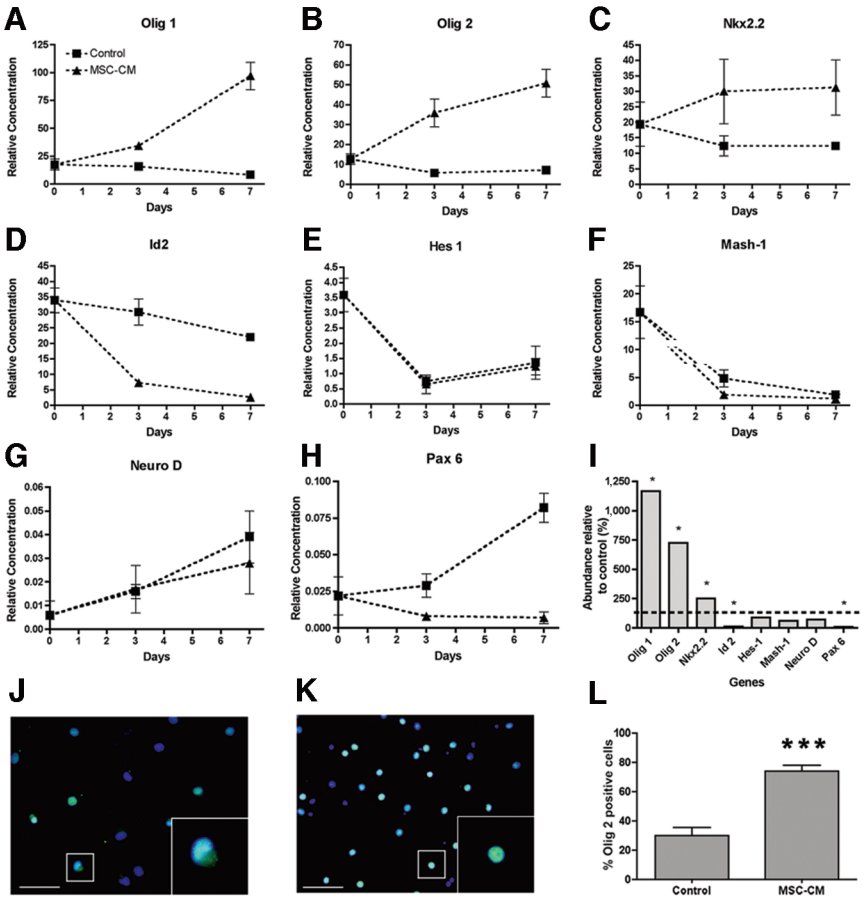

Figure 5. Temporal expression pattern of neural cell fate determinants in NSCs. NSCs were incubated in control medium or in MSC-CM for 0, 3, and 7 days. mRNA was prepared, and quantitative reverse transcription-polymerase chain reaction was performed. Data are expressed as relative mRNA concentrations with respect to a standard dilution curve versus time for Olig1 **(A)**, Olig2 **(B)**, Nkx2.2 **(C)**, Id2 **(D)**, Hes1 **(E)**, Mash1 **(F)**, NeuroD1 **(G)**, and Pax6 **(H)**. Significant changes were Olig1 ($p < .001$) and Olig2 ($p = .001$) over time, Nkx2.2 in MSC-CM compared with control ($p < .05$), Id2 in MSC-CM versus control over time ($p < .005$), Hes1 and Mash-1 over time ($p < .001$ and $p = .001$, respectively) without a difference between the conditions ($p > .05$), NeuroD1 levels over time ($p < .05$) but similar in the two conditions, and Pax6 in MSC-CM compared with control over time ($p < .005$). Experiments were performed in triplicate. Data represent means ± SD. Two-way analysis of variance was used for statistical analysis. **(I):** The relative amount of mRNA of 7-day MSC-CM-stimulated cells versus control medium-stimulated cells is shown for each gene. Asterisk indicates significant increase or decrease compared with control. **(J, K):** Photomicrographs of Olig2 immunostainings of 7-day control- **(J)** and MSC-CM- **(K)** treated NSCs. Inserts show higher magnification of cells included in the frame. Scale bars: 50 μm. **(L):** Quantitative analysis of the percentage of Olig2 immunoreactive cells in the 7-day control- and MSC-CM-treated NSCs. Experiments were done in triplicate. Results are shown as mean ± SD. For statistical analysis, Student's *t* test was performed. Abbreviations: MSC-CM, mesenchymal stem cell-conditioned medium; NSC, neural stem cell.

mRNA expression levels seem to result from a combination of upregulation in MSC-CM and downregulation in control-treated cells (Fig. 5C). Taken together, MSC-secreted soluble factors upregulated the expression of key transcriptional factors involved in the oligodendrogenic differentiation program in NSC cultures. To verify the differences on the protein level, we performed immunostainings for one of the most important transcription factor analyzed in the present study, Olig2. Quantitative analysis revealed an increased percentage of Olig2-expressing cells in the 7-day MSC-CM-treated NSCs compared with control (Fig. 5L). Although this is not quantitatively assessed, the immunostainings indicate a higher signal of Olig2 immunoreactivity at the individual cellular level (Fig. 5J, 5K). In addition, in MSC-CM-treated cells, Olig2 immunoreactivity is specifically confined to the nucleus, whereas under control conditions, the signal is more diffuse and to some level detected in the cytoplasm (Fig. 5J, 5K inserts).

Upregulation of specific oligodendrogenic transcriptional factors may not be sufficient for cell fate commitment into oligodendrocytes. Indeed, the presence of an inhibitory system limiting the activity of *olig* genes has been described [19]. The inhibitor of differentiation 2 (Id2), a key component in this system, could sequester olig proteins, thereby preventing them from entering the nucleus and thus inhibiting their activity [19]. The presence of this system promotes astrocytic differentiation of neural progenitors cells. The present analysis measured highest levels of Id2 mRNA on day 0 (Fig. 5D). After 7 days of incubation, although both conditions downregulated Id2 mRNA expression, cultures exposed to MSC soluble factors contained 10 times less Id2 mRNA as compared with those in control

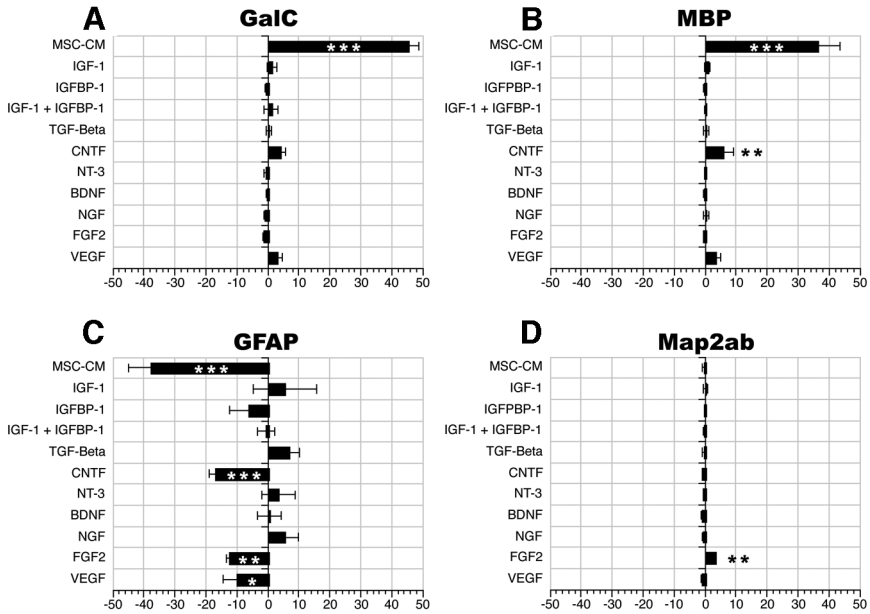

Figure 6. The oligodendrogenic effect of MSC soluble factors on NSCs: candidate factors. NSCs were incubated for 7 days in MSC-CM or in control containing IGF-1, IGFBP-1, IGF-1 plus IGFBP-1, TGF-β-1, CNTF, NT-3, BDNF, NGF, FGF-2, or VEGF and analyzed for expression of differentiation markers GalC **(A)**, MBP **(B)**, GFAP **(C)**, and Map2ab **(D)**. Three hundred to 500 cells were counted in random fields for each experiment. The percentage of cells expressing a specific marker normalized for the control condition is demonstrated. Experiments were done in triplicate. Results are demonstrated as means $\pm$ SD. One-way analysis of variance-Tukey-post hoc was used for statistical analysis. The posteriori analysis compared each condition with control ($* = p < .05$, $** = p < .01$, $*** = p < .001$). Abbreviations: BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; FGF, fibroblast growth factor; GFAP, glial fibrillary acidic protein; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; MBP, myelin basic protein; MSC, mesenchymal stem cell; MSC-CM, mesenchymal stem cell-conditioned medium; NGF, nerve growth factor; NSC, neural stem cell; NT, neurotrophin; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

medium (Fig. 5D, 5I). In summary, MSC-CM contained factor(s) promoting oligodendrogenic differentiation by enhanced expression of oligodendrogenic fate determinants and by reduced expression of oligodendrogenic inhibitors.

We further analyzed the temporal expression pattern of genes involved in determining stem cell and astroglial identity and in neuronal determination. The *Hes1* gene encodes for a transcription factor downstream of Notch-signaling, which is able to block neurogenesis and promote astrocytic differentiation [12]. Similarly to the Id2 mRNA, the highest levels of Hes1 mRNA were measured on day 0 (Fig. 5E). Thereafter, the levels of Hes1 mRNA significantly decreased over the next 7 days. However, no significant differences could be detected between the Hes1 mRNA levels measured in NSC cultures stimulated in MSC-CM versus control medium (Fig. 5E, 5I). *Mash-1*, *NeuroD1*, and *Pax-6* genes are involved at different levels of the neuronal determination and differentiation program [12, 20–24]. The highest levels of Mash-1 mRNA were detected on day 0 (Fig. 5F). Although the levels of Mash-1 mRNA significantly decreased over time, no difference in the Mash-1 mRNA levels was detected after 7 days of culture in MSC-CM compared with control medium (Fig. 5F, 5I). With respect to *NeuroD1* and *Pax6*, only very low levels of mRNA could be detected (Fig. 5G, 5H). Nevertheless, the amount of *NeuroD1* increased significantly over the 7 days in culture, but no differences were measured between the two culture conditions used (Fig. 5G, 5I). *Pax6* mRNA expression was significantly upregulated in control and significantly downregulated in MSC-CM-treated NSCs (Fig. 5H, 5I). In summary, soluble factors present in the MSC-CM did not specifically induce the neuronal fate in NSCs.

A Screen for Candidate Factors for the Oligodendrogenic Effect of MSC-CM on NSCs

Various growth factors, cytokines, and neurotrophic factors influence neurogenesis and the differentiation fate of NSCs [25]. We therefore scrutinized some of these factors as potential candidates that might exert the oligodendrogenic MSC-CM effect. We included the growth factors FGF-2, VEGF, and IGF-1, the cytokines TGF-β-1 and CNTF, and the neurotrophic factors NT-3, BDNF, and NGF in the study. IGF-1 was of particular interest because it was reported to be capable of inducing adult neural progenitor cells to differentiate into oligodendrocytes [26]. IGF-1 is to date the only potent oligodendrogenic factor described for adult neural progenitor cells. Similarly, IGF-1 was also described as a potent oligodendrogenic factor for O2A bipotent precursors, whereas IGFBP-1, -2, and -6 inhibit this activity [27, 28]. Moreover, expression of IGF-1 mRNA in the MSCs in culture could be documented via RT-PCR (data not shown).

To scrutinize the role of the various factors, we incubated NSCs for 7 days in MSC-CM or in control medium containing the various factors and processed for GalC, MBP, GFAP, and Map2ab immunofluorescence staining. The quantitative analysis of the percentage of marker-expressing cells demonstrates that none of the factors tested mimics the oligodendrogenic effects of the MSC-CM (Fig. 6). A minor, but significant,

inductive effect of CNTF on the expression of MBP, but not of GalC, was noticed. However, this effect was much lower than the one exerted by MSC-CM. We cannot rule out, nor can we proof at this point, that one or a combination of the factors tested contributes to the oligodendrogenic effect of MSC-CM. However, the data demonstrate that none of these factors alone is sufficient to explain the effect.

Because IGF-1 was to date the only potent oligodendrogenic factor described for adult neural progenitor cells [26], IGF-1 was scrutinized as a candidate factor by using the IGF-1-inhibiting molecule IGFBP-1. NSCs were stimulated in control medium or MSC-CM with IGF-1, IGFBP-1, or both and analyzed for expression of GalC, MBP, GFAP, and Map2ab. The treatments did not induce any significant differences, independently of whether the cells were in the control medium (Fig. 6) or in MSC-CM (data not shown). These observations suggest that IGF-1 is not involved in the oligodendrogenic effect on NSCs by MSC-CM.

DISCUSSION

In the present report, we investigated the effects of bone marrow-derived MSCs on stem and progenitor cells isolated from the adult brain, the NSCs. In summary, the following findings have to be considered: (a) proliferation of the A2B5 cell population was not significantly stimulated by MSC-CM (this population diminished over time but was not affected by enhanced cell death), (b) the GalC-expressing cell population did not proliferate, and proliferation was not stimulated by MSC-CM, but MSC-CM increased the percentage of cells expressing GalC without affecting the cell death rate of this population, and (c) the proliferation rate of GFAP-positive cells in MSC-CM was elevated, but fewer cells adopted a GFAP-expressing phenotype in MSC-CM compared with control medium, and the cell death rate of this cell population was not affected by MSC-CM. From these findings, we conclude that MSC-CM promoted differentiation of A2B5-positive cells to GalC-expressing oligodendrocytes and that MSC-CM stimulated proliferation of GFAP-expressing cells but prevents progenitor cells from acquiring a GFAP-expressing phenotype. Alternatively, proliferating GFAP-expressing cells might also contribute to the oligodendrocytes generated. This possibility is in agreement with the fact that some GFAP-positive astroglial cells represent the NSC population in vivo [23]. However, because the NSC cultures in the present study consisted of an almost pure population of A2B5-positive cells (94%), this possibility seems to be unlikely. The GFAP-expressing cell type might be only a transient phenotype and originate from the same A2B5 precursor as GalC-expressing cells. In summary, we concluded that soluble factors derived from MSCs induced oligodendrogenic fate decision in adult NSCs at the expense of astroglial differentiation by an instructive signal or signals and not by selection (Fig. 7).

MSC-derived soluble factors supported oligodendrogenic maturation. Hence, the expression of MBP, a functional marker for mature oligodendrocyte [29], was detected in a large proportion (approximately 70%) of the GalC-expressing cells after 7 days of incubation in MSC-CM. Thus, MSC-secreted factors did not simply induce aberrant expression of an oligodendroglial marker but contributed to the establishment of a bona fide genetic program. This was further supported by the expression pattern of oligodendrogenic fate determinants. Much like in the

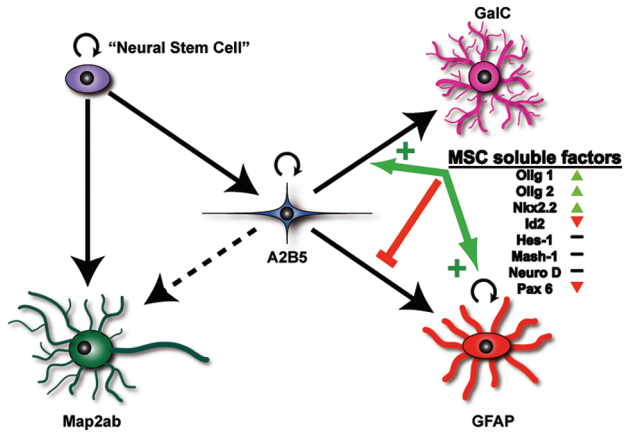

Figure 7. A model for the oligodendrogenic effect of MSCs on NSCs. Self-renewing and proliferative NSCs (violet cell) can give rise to the three major neural lineages: neurons (Map2ab, green cell), astrocytes (GFAP, red cell), and oligodendrocytes (GalC, pink cell). NSCs give rise to proliferative A2B5-expressing progenitor cells (blue cell). A2B5-positive cells are tripotent and differentiate to oligodendrocytes, astrocytes, and (to a lesser degree) neurons. When exposed to MSC-derived soluble factors, A2B5-expressing cells are induced to the oligodendrocyte (GalC) lineage, whereas the astrocytic differentiation pathway is partially inhibited. MSC soluble factors promote astrocyte proliferation; however, they impair astroglial differentiation. These effects are mediated by the regulation of cell fate-determining transcription factors. Oligodendrogenic transcription factors are upregulated (*Olig1*, *Olig2*, and *Nkx2.2*), and the oligodendrogenic inhibitor factor *Id2* and the neurogenic factor *Pax6* are downregulated, whereas no specific changes in *Hes 1*, *NeuroD*, and *Mash-1* mRNA are associated with the oligodendrogenic effect. Abbreviations: GFAP, glial fibrillary acidic protein; MSC, mesenchymal stem cell; NSC, neural stem cell.

developmental situation, in which the relative expression levels of basic helix-hoop-helix factors regulate oligodendrocyte determination and differentiation [19, 30], we demonstrate for the first time that oligodendrogenic differentiation of adult NSCs involved the balance of expression levels of the oligodendrogenic promoting *Olig* genes and the inhibitory *Id2* gene.

The most promising candidate factor by which the oligodendrogenic effect of MSCs on NSCs might be mediated was IGF-1. First, IGF-1 is to date the only known oligodendrogenic factor for adult NSCs [26]; second, it induces oligodendrocytic differentiation in oligodendroglial progenitors during development [27, 28]; and third, IGF-1 is produced and secreted by MSCs (our unpublished data and [31]). The present work excluded IGF-1 to be the factor responsible for the olidendrogenic effect. Hence, incubation of NSCs in the presence of IGF-1 did not lead to any significant increase in oligodendrocyte formation. Moreover, the addition of IGFBP-1 did not influence the MSC-CM-induced oligodendrogenesis. Differences in the experimental details and in the exact cell types used might account for this discrepancy. For example, the NSCs used in this study were pluripotent as demonstrated by their capacity to differentiate into neurons, astrocytes, and oligodendrocytes. It is, however, noteworthy that a large proportion (approximately 95%) of the neural progenitors expressed the marker A2B5. The latter was reported to be expressed by glial-restricted progenitors, although some neuronal differentiation potential of A2B5-expressing cells has also been reported [32, 33]. Future experiments might use homogenous NSC populations such as the

nestin-expressing cell population derived from transgenic mice expressing EGFP under control of the nestin enhancer element [34].

Developmental studies and investigations on Olig-mediated signal transduction have provided candidate factors for oligodendrogenic differentiation programs. For example, sonic hedgehog signaling might be one candidate mechanism because it participates in oligodendrocyte cell fate of precursors in spinal cord and telencephalon during development [35–38]. In addition, there might be one or more as-yet-undescribed soluble factors delivered by the MSCs responsible for the oligodendrogenic effect observed.

This study demonstrates the bivalent nature of adult bone marrow stromal cells; they can behave as stem cells differentiating under particular conditions and as stromal cells, producing different kind of signals affecting the biology of adult neural stem/progenitor cells, much as they do in the hematopoietic system in the bone marrow. This underscores the intricate interactions existing among the various progenitor cell populations and substantiates the need to consider the various systems, in particular the CNS, not as separated and closed units but as an integrated and communicative entity. Indeed, MSCs and NSCs might be in close contact in vivo; as a result of the dense network of capillaries present, any position in the brain is within 50 μm of the next capillary. Given that MSCs are found in the circulation, the spacing between NSCs and MSCs might be minimal. Moreover, after CNS lesions, the blood-brain barrier becomes leaky and MSCs as well as their secreted factors can penetrate the nervous tissue and act locally. Through their secreted factors at the lesion site, MSCs could locally induce resting progenitors to differentiate into oligodendrocytes and hence participate in a regenerative effort. For example, in an animal model of multiple sclerosis (experimental autoimmune encephalomyelitis [EAE]), lesions and inflammation were shown to induce the differentiation of neural progenitors into oligodendrocytes [39, 40]. Moreover, bone marrow stromal cell transplantations could improve functional recovery after EAE, probably via the stimulation of oligodendrogenesis [5].

CONCLUSION

Mesenchymal progenitors isolated from the bone marrow were shown here to secret factors profoundly modulating the differentiation of neural progenitors. One or more factors produced by the MSCs induced oligodendrocyte differentiation of NSCs in an IGF-1-independent manner. Identification of the molecules bearing this oligodendrogenic activity will open the way to studying the possible interaction of MSCs and NSCs in vivo and in particular during pathological processes.

ACKNOWLEDGMENTS

We thank Juergen Winkler, Beate Winner, and Norbert Weidner for critical and stimulating comments and Marco Mendez for statistical analysis support. We also thank Magdalena Götz for the Olig2 antibody and Norbert Weidner for the RIP antibody. This work was supported by Beca CONICYT (apoyo a realización de tesis doctoral, F.R.), CONICYT-DAAD (F.R.), DAAD (travel fellowship, L.A.), Volkswagen-Foundation, Hannover, Germany (L.A.), the ReForM program, School of Medicine, University of Regensburg (L.A.), and the Bayerische Forschungsstiftung, Munich, Germany (Grant PIZ 80/05, L.A.).

DISCLOSURES

The authors indicate no potential conflicts of interest.

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