Original Article

Bone Morphogenetic Protein-4 Influences Neural Differentiation of Induced Mouse Mesenchymal Stem Cells

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Abstract

Objective: Members of the transforming growth factor-β superfamily, including bone morphogenetic protein 4 (BMP4), have been implicated as regulators of neural differentiation. The aim of this study was to establish whether BMP4 could influence neuronal differentiation of mesenchymal stem cells (MSCs).
Materials and Methods: Therefore, neuronal differentiation of MSCs was induced by basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) and treatment. The expression of neuronal specific markers such as Nestin, MAP2, β-Tubulin III and NKX6.1 were detected by RT-PCR, flow cytometery and/or immunostaining.
Results: While the percentage of Nestin positive cells was increased significantly during treatment, the addition of BMP4 during the first 4 days of treatment with bFGF and EGF reduced Nestin expression as showed by flow cytometry. This observation was further confirmed by relative gene expression which showed the reduction in expression of neural markers such as Nestin, MAP2 and NKX6.1 following treatment with BMP4.
Conclusion: The results of this study suggest that BMP4 downregulates the neural fate of induced mouse MSCs.

Keywords: BMP4, Mesenchymal Stem Cells, Neural Differentiation

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Introduction

During Xenopus development and probably more generally in vertebrate development bone morphonetic protein 4(BMP4) behaves like a strong epidermal inducer in regions with low antagonist concentrations (ventral region of the ectoderm), while high levels of BMP4 antagonists in the dorsal region induce the formation of the neuroectoderm (1). This inhibitory model of the organizer action is described as the "neural default" model, according to which ectodermal cells will undergo neural differentiation unless BMPs are present (2). Moreover, most embryos that are homozygous null for BMP4 or its receptor (bmpr) fail to gastrulate owing to the loss of early mesoderm (3, 4). Consistent with these observations, exposure to BMP4 and other transforming growth factor β(TGFβ) family members can enhance the production of mesoderm during in vitro differentiation of mouse embryonic stem cells (ESCs) (5). These results argue strongly that BMP4 is involved in early mesodermal differentiation, but leave open the question of its role in early mammalian neural induction.

A review of the literature reveals that mesenchymal stem cells (MSCs) show high plasticity and are able to differentiate to different lineages, such as neural-like cells (Sanchez-Ramos) (6-8), chondrocytes and osteoblasts (9-11). Thus, we hypothesized that the neural fate of MSCs may

be influenced by BMP4. Therefore, in this study we evaluate the effect of BMP4 on mouse MSC-derived neural-like cells in which differentiation is induced by epidermal growth factor (EGF) and basic-fibroblast growth factor (bFGF). The results of this study reveal that addition of BMP4 during neural differentiation of MSCs can suppress neural fate.

Materials and Methods

Culture and expansion of MSCs

Mouse MSCs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, and 2mM L-glutamine. They were grown to confluency before being detached by Trypsin/EDTA treatment and were seeded at 1×10^4 cell/cm^2 in culture flasks before being evaluated for their multipotency while differentiating into adipocytes and osteoblasts.

Neuronal induction

Induction of neural differentiation was initiated by plating the cells on poly-L-lysine (Sigma, P4707)-coated glass cover slips at a concentration of 5000 cells/cm^2 in Neurobasal medium supplemented with 20 ng/ml EGF and 40 ng/ml bFGF (both from Sigma), 2% B27, 1% insulin-transferrin-selenite (ITS), 1% nonessential amino acid, 1% L-glutamine, 1% penicillin and streptomycin. The medium was changed once a week and growth factors were added twice a week for twelve days. All materials were purchased from Gibco except those mentioned.

Treatment with BMP4

MSCs were cultured on poly-L-lysine coated glass cover slips in differentiation medium supplemented with BMP4 (Sigma, B2680) at different concentrations (0, 50, 100, 150, 200 ng/ml) for the first 4 days (12).

Immunofluorescence staining

At day 12, the cells were rinsed twice with PBS-Tween 20 (0.05%), fixed with 4% paraformaldehyde (Sigma; P6148) in PBS at 4°C for 20 minutes. The cells were permeabilized with 0.2% Triton X100 in PBS. The fixed cells were blocked for 45 minutes at 37°C with 10% goat serum on PBS-Tween 20. Cells were incubated with anti-Nestin (Chemicon MAB353, 1:200), β-Tubulin III (β-Tub III, Sigma T8660, 1:200), and microtubule associated protein 2 (MAP2, Sigma M1406, 1:200) primary antibodies for 1 hour at room temperature. At the end of the incubation time, the cells were rinsed three times with PBS-Tween 20 (0.05%) and incubated with the FITC-conjugated secondary antibody (Chemicon AP 124F, 1: 50) for 1 hour at room temperature. After rinsing with PBS, the nuclei were counterstained with DAPI 100 ng/ml and the cells were then analyzed with a fluorescent microscope (Olympus, BX51, Japan).

Reverse transcription-polymerase chain reaction analysis

Total RNA was extracted from cultured cells using the RNeasy Mini kit (Qiagen, Spain). Prior to reverse transcription (RT), RNA samples were digested with DNase I (Fermentas; EN0521) to remove contaminating genomic DNA. Standard RT was performed using 2 μg total RNA, oligo (dT) and the RevertAid™ Minus First Strand cDNA Synthesis Kit (Fermentas; K1622) according to the manufacturer's instructions. The cDNA samples were subjected to polymerase chain reaction (PCR) amplification using mouse specific primers. Amplification conditions were as follows: Initial denaturation at 94°C for 5 minues, denaturation at 94°C for 30 seconds, annealing at 55-63°C for 45 seconds, extension for 45 seconds at 72°C followed by 35 cycles for neural markers and 25 cycle for β-tubulin a final polymerization at 72°C for 10 minutes. The PCRs were performed in triplicate. The PCR products were analyzed by gel electrophoresis on 1.7% Agarose and stained with Ethidium Bromide (0.5μg/ml) and visualized and photographed on UV transiluminator (SynGene,UK). Gel images were analysed using the GeneTools software (version 3.06) (SynGene,Cambridge,England). NKX6.1, Nestin and MAP2 were used as neural markers and PCRs were normalized by amplifying the same sample of cDNA with primers for β-tubulin as a house keeping gene before and after neural induction. The primer sequences are as follow: Nestin (Forward: 5'-TCGAGCAGGAAGTGGTAGG-3') and (Reverse: 5'-TTGGGACCAGGGACTGTTA-3'), β-tubulin III (Forward, 5'-TCACTGTGC CTGAACTTACC -3') and (Reverse, 5'-GGAACATAGCCGTAAACTGC-3'), MAP2: (Forward, 5'- GCGGAAAACCACAGCAGCAAG -3') and (Reverse, 5'-TTGGAGGAGTGCGGATGATGG -3'), NKX6.1 (Forward, 5'-GACAAAGATGGGAAGAGAAAAC-3') and (Reverse, 5'-GGTCCAGAGGTTTGTTGTAATC-3').

Flow cytometry analysis

At day 7, the cells were washed twice in staining buffer and fixed in 4% paraformaldehyde. For permeabilization Triton X-100 0.2% was used.

Non-specific antibody binding was blocked with 10% goat serum in staining buffer, and 1-1.5 $\times 10^5$ cells were used per sample. Cells were incubated with appropriate primary antibodies anti-Nestin (MAP353, 1:100) and appropriate IgG negative control (Chemicon, CBL600, 1:200). The cells were washed twice with staining buffer and incubated for 60min at 4°C with FITC-conjugated secondary antibody (Chemicon, AP 124F, 1:100). After washing, flow cytometric analysis was performed with a BD-FACS Calibur Flow Cytometer (Becton Dickinson). The experiments were replicated at least three times and the acquired data were analyzed using WinMDI (2.9) software.

Statistical analysis

The data were expressed as mean ± SD (standard deviation). Paired t tests were used to analyze group differences in the data collected from flow cytometry analysis. A difference between groups was considered as statistically reliable if the $p<0.05$.

Results

To convert MSCs into cells with the characteristics of neural-like cells, MSCs at passages 4-6 (Fig 1A) were cultured in Neurobasal medium in the presence of EGF and bFGF for 12 days. Following this treatment, the cells acquired a neural appearance (Fig 1B and 1C). The observed morphological changes during neural induction were similar to those reported previously (13), in which the cytoplasm in the flat cells was initially retracted toward the nucleus, forming a contracted multipolar cell body leaving membranous processes like peripheral extensions. Later, the cell bodies became increasingly spherical and retractile, exhibiting a typical neuronal perikaryal appearance (Fig 1B and 1C). After 12 days, immunoflourecence staining showed the expression of Nestin (Fig 1D), β-Tubulin III (Fig 1E), and MAP2 (Fig 1F) in treated MSCs.

Flow cytometry results revealed only 11% of the MSCs were positive for Nestin before neural induction (Fig 2A and 2D), while 62 ± 0.05% of cells expressed Nestin 7 days post neural induction (Fig 2B and 2E).

The expression of neuronal specific genes such as NKX6.1, Nestin, and MAP2 were also studied by RT-PCR analysis (Fig 3A) in which they were found to be weakly expressed in MSCs. The expression of neuronal markers in mouse bone marrow-derived MSCs has been reported in the past where the MSCs were found to express certain neuronal phenotype markers spontaneously in the absence of specialized induction reagents in culture. These cells were believed to be probably "primed" toward a neuronal fate by the constitutive expression of neuronal antigens and seemed to respond with an appropriate neuronal pattern of differentiation, when exposed to the environment of the developing brain (14).

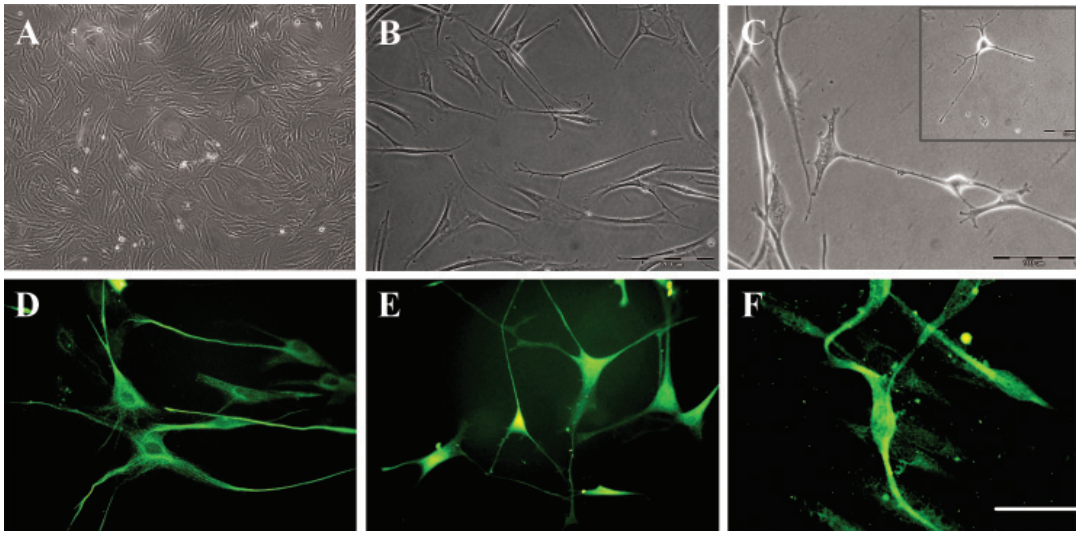

Fig 1: Induction of neural differentiation in mouse MSCs by treating with EGF and bFGF. (A) undifferentiated MSCs. MSCs comprised a morphologically heterogeneous population of cells including spindle-shaped cells, large flat cells, and small round cells (B) MSC-derived neuronal-like cells after 7 days (B) and 12 days displaying distinct neuronal morphologies ranging from simple bipolar to large, extensively branched multipolar cells making connections via their processes (C). Immunoflourescence of MSC-derived neuronal-like cells after 12 days were positive with anti-Nestin (D), anti-β-Tubulin-III (E), and anti-MAP2 (F). Bar =100 μm.

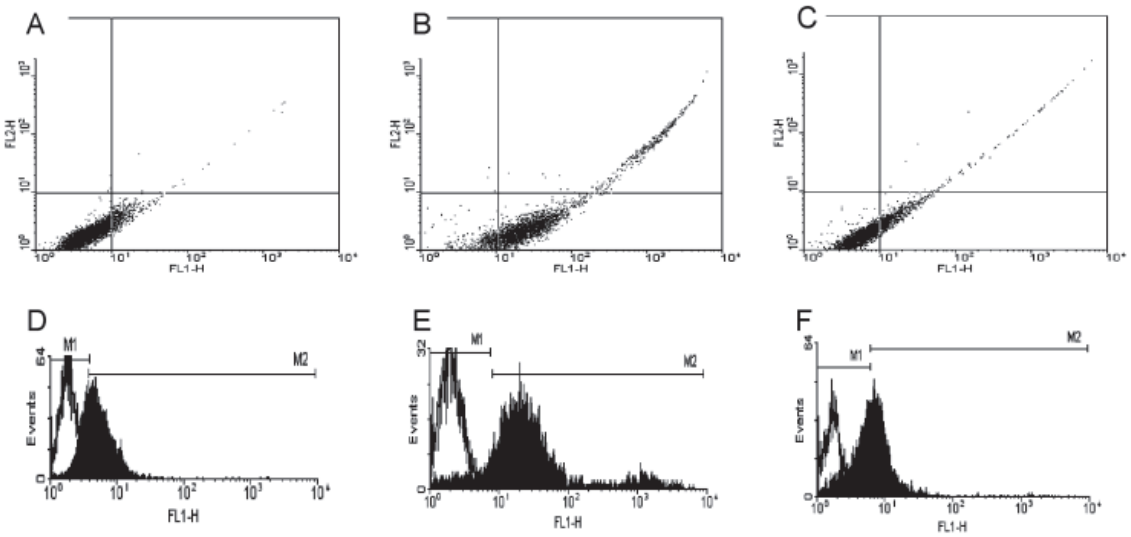

Fig 2: Flowcytometry analysis of mouse MSCs for Nestin positivity under different treatments. Dot blot (A-C) and histogram analyses (D-F) of MSCs (A and D), 7 days after induction with bFGF and EGF (B and E), 7 days after exposure to bFGF and EGF and BMP4 (200 ng/ml, 4 days) (C and F).

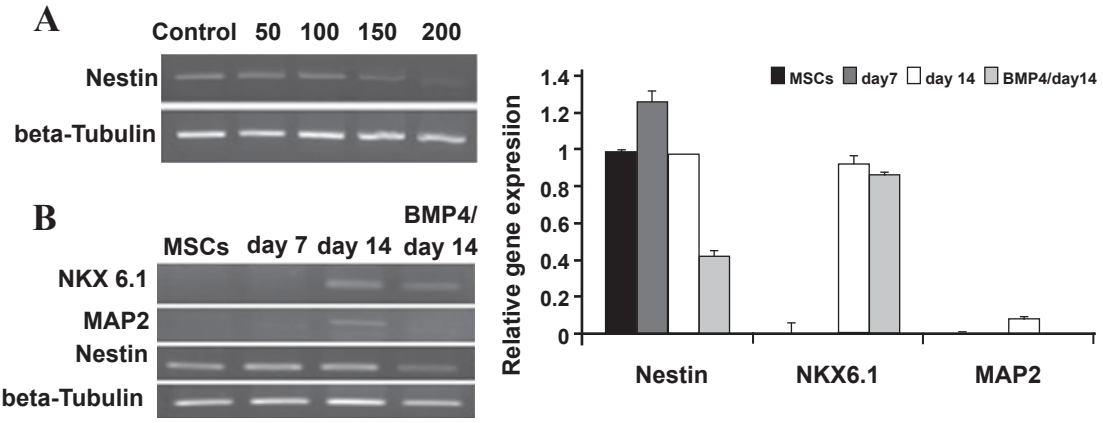

Fig 3: RT-PCR analysis of neural markers in treated mouse MSCs. (A) Effect of different concentrations (ng/ml) of BMP4 during the first 4 days on expression of Nestin, 7 days after exposure to bFGF and EGF. Expression of neural markers (B) and their relative gene expression (C) 7 and 12 (N.B. Here and in the text it is 7 and 12 days, but in Fig 3 below it is 7 and 14 days) days post neural induction and following exposure to BMP4 (200ng/ml) during the first 4 days. The results reveal Nestin expression only before neural induction, while NKX6.1, MAP2 are also expressed after neural induction. The neural expression of these neural markers decreases upon treatment with BMP4 (n=2).

In order to measure the effect of BMP4 on the neural fate of MSCs by evaluating Nestin expression, the cells were exposed to different concentrations of BMP4 (0, 50, 100, 150, 200 ng/ml) for the first 4 days. RT-PCR analysis of Nestin expression at day 12 showed a significant decrease at 200 ng/ml of BMP4 (Fig 3A). Therefore, the experiments were continued using this concentration. Flow cytometry analysis at day 12 showed a 50% reduction in Nestin expression with 200 ng/ml BMP4 (Fig 2C and 2F). This finding was further supported with semi-quantitative RT-PCR analysis of NKX6.1, Nestin, and MAP2 which showed significant reduction in expression of the neural markers in the presence of 200 ng/ml of BMP4 (Fig 3B and 3C).

Discussion

Several recent studies suggest that MSCs might be able to break the barriers of germ layer commitment and differentiate in vitro and/or *in vivo* into cells of different tissues. In addition, these cells were reported to differentiate in vitro and in vivo into cells expressing neuronal and glial markers (6-8, 15, 16). This plasticity of MSCs has provided a new area in which it is possible to use these cells for cell-replacement therapy. By far the most prominent advantage of using MSCs over other cell types in cell-replacement therapy

is their autologous character. Hence, in this study, MSCs were directly exposed to bFGF and EGF for up to 12 days to induce neural differentiation. Gradually, MSC-derived neurons displayed distinct neuronal morphologies, ranging from simple bipolar to large, extensively branched multipolar cells. RT-PCR and immunofluorescence analyses of these cells showed that they expressed significant amount of Nestin, Map2, and β-tubulin III, postneural induction. Flowcytometry analysis also confirmed our results and showed that more than 62% of MSCs could express the neural precursor marker (Nestin) after 7 days, in comparison to the control group which was 11.00 ± 0.04% Nestin-positive.

It has been shown that BMP signaling has an inhibitory effect on neural induction in vertebrate embryos (17). So, the inhibition of BMP would induce ectodermal cells toward neurogenesis by default (2). On the other hand, several possibilities have been proposed for bFGF-mediated neural differentiation of MSCs (18). Thus, during this study we aimed to evaluate whether BMP4 functions as a neural differentiation inhibitor in MSCs and could suppress the bFGF and EGF neurogenic effect on MSCs. To address this question MSCs were exposed to different concentrations of BMP4 ranging from 50 to 200 ng/ml in the first week of MSCs neural induction with bFGF and EGF. RT-PCR analysis shows that exposure to 200 ng/ml BMP4 downregulated the expression of Nestin. Flowcytometry analysis also showed that the percentage of Nestin-positive MSCs decreased from 70% in the bFGF and EGF group to 20% in the bFGF, EGF and BMP4 group. This indicates that BMP4 could affect neural differentiation of MSCs with bFGF and EGF. Existing literature on the effect of BMP4 on the neural differentiation of embryonic stem cells (ESCs) reveals that BMP4 in a restricted window inhibited neural induction, and incubation with BMP4 for the first 8 days of aggregation resulted in a strong inhibition of neuronal differentiation that did not reverse when the cells were subsequently cultured (12). Although we did not carry out a time course study on the effect of BMP4 on neural induction, the results presented in this study are in concordance with the observation obtained in ESCs. Even though the MSCs were exposed to BMP4 in the presence of bFGF and EGF in the first 4 days of culture, the expression of the neural markers was also reduced 12 days post induction. This suggests that, like ESCs, the effects of BMP4 on MSCs are not reversible and inhibit neural differentiation. In ESCs a lower concentration of BMP4 (8 ng/ml) is required to inhibit neural differentiation (12), while in this study neural inhibition was observed with only 200 ng/ml of BMP4. This difference might be due to epigenetic modification which has been induced in these cells and high signaling intensity might be required to reverse this modification.

Conclusion

The results of this study indicate that MSCs can differentiate into neural-like cells. *In vivo* these cells may only differentiate into a mesoderm lineage and not an ectodermal linage, possibly due to the secretion of BMP4 in their niche, the bone marrow. Therefore, if this inhibitory signal is removed and inducers of neural lineage are provided, these cells may gain neural specification and provide a suitable cell source for cell therapy in neurodegenerative diseases.

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