Original Article

Differentiation of Mouse Embryonic Stem Cells into Dorsal Interneurons of the Spinal Cord Using BMP4 and Activin A

Farzaneh Najafi, M.Sc.[1,2], Narges-Zare Mehrjardi, M.Sc.[1], Maryam Hatami, M.Sc.[1], Hossein Baharvand, Ph.D.[1,3*]

1. Stem Cells and Developmental Biology Department, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
2. Biotechnology Department, Tehran University, Tehran, Iran.
3. Developmental Biology Department, University of Science and Culture, ACECR, Tehran, Iran

* Corresponding Address: P.O.Box: 19395-4644, Stem Cells and Developmental Biology Department, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
Email: Baharvand@royaninstitute.org

Abstract

Objective: In vertebrates, bone morphogenetic proteins (BMPs) and activin signals play multiple roles in dorso-ventral patterning and development of the spinal cord. Here the inductions of BMP 4 and activin A on embryonic stem cells (ESCs) into dorsal interneurons have been studied.

Materials and Methods: Four different treatments have been used for mESC derived neural precursors; they include BMP4 (1ng/ml and 10ng/ml), activin A (100 ng/ml), and activin A+BMP4 (100ng/ml, 10ng/ml). Induction's effect on expression of specific dorsal interneuron markers in mature neurons have been evaluated by the use of immunocytochemistry and RT-PCR.

Results: Treatment of ESC-derived neural precursors with BMP4, activin A, or both showed an increased generation of both dI1 and dI3 interneurons (Lhx2 and Isl-1-positive cells) compared to the control group. However, the synergistic effect in generation of dI3 was not observed when both factors were used. Moreover, RT-PCR analysis of differentiated cells showed the expression of Lhx9, Lhx1, and Isl1, the transcription factors that are markers of dI1, dI2, and dI3 interneurons respectively.

Conclusion: Our results showed that specific combination of developmental signaling molecules can direct the differentiation of ESCs into dorsal interneurons. Furthermore, qualitative and quantitative differences in signaling by different members of the TGF-β superfamily may play a role in the specification of different types of dorsal interneurons.

Keywords: Embryonic Stem Cells, Dorsal Patterning, BMP, Activin, Spinal Cord Interneurons

Introduction

The vertebrate central nervous system (CNS) is derived from a columnar epithelium, the neural plate which then folds to form the neural tube. Diverse cell types of the CNS are differentiated along the anteroposterior and dorsoventral axes of the neural tube. The most anterior region of the neural tube forms the brain structures while more posterior regions give rise to the spinal cord. The medial region of the neural plate develops into cells of the ventral half of the neural tube including floor plate cells, motor neurons, and ventral interneurons. The lateral region of the neural plate differentiates into the dorsal cells of the neural tube - neural crest cells, roof plate cells and dorsal sensory relay interneurons (1).

Through a variety of assays, the inductive signals that control the identity and pattern of these cell types have been revealed. The generation of ventral cell types is induced by sonic hedgehog (Shh) secreted from the notochord. In contrast, dorsal cell types are generated in response to signals derived from the epidermal ectoderm, probably from member(s) of the TGF-β family such as BMP4 (2). Roof plate cells are also generated in response to a BMP mediated signal from the epidermal ec-

toderm (3, 4). Roof plate is the direct source of BMP signals, notably BMP4 and BMP7, which specify D1 (dI1) and D2 (dI3) classes of dorsal interneurons. It is believed that a temporal change in the response of neural cells to TGF-β-signaling controls generation of distinct classes of dorsal interneurons; i.e. at the early stages when they acquire the capacity to generate D1 and D2 interneurons (3). In addition, it is believed that qualitative and quantitative differences in signaling by TGF-β molecules are important in identification of distinct classes of dorsal interneurons; for example, activin A and low concentrations of BMP4 induced dI3 (D2) interneurons while GDF7, a BMP family member, and higher levels of BMP4 induced dI1 (D1) interneurons (3, 4). Inhibition of BMP signaling via the expression of a secreted BMP inhibitor such as noggin, or a conditional inactivation of BMPR1A and BMPR1B resulted in the loss of dI1 and dI2 cells, indicating that BMP signaling is necessary for their production (5-7). TGFβ signaling also acts to block the formation of more medial interneurons (dI4-dI6) in the dorsal part of the neural tube, possibly via the repression of Lbx1 protein (8-10). Based on the development of the neural tube dorsal interneurons and previous studies on this issue, we examined the generation of these cell types in the mouse embryonic stem cells (ESCs). Embryonic stem cells are derived from a preimplantation embryo which has the potential for differentiation to derivatives of embryonic germ layers (11-16). Because ESCs can be easily maintained, propagated, and genetically manipulated they can be a better source of specialized cells for therapeutic purposes. However, one of the major challenges in cell therapy is to develop a reliable protocol for pre-differentiation of ESCs into specific neuronal phenotypes. While differentiation of ESCs into motor neurons may be successfully achieved by treatments with RA and Shh, the mechanism of differentiation of dorsal horn interneurons is far from clear. Also little is known about the signals that induce initial dorsal character (17, 18). Clues to potential factors involved in induction of neuronal phenotype can be unveiled by studying neural development. Therefore, to identify the signals that induce initial dorsal characteristics , and to differentiate dorsal interneurons of the spinal cord for future spinal cord cell therapy, we have first induced generation of neuroepithelial precursor cells (nestin-positive cells); then, to induce specification of dorsal interneu-

rons, the neural precursors were treated with retinoic acid and the dorsalizing factors BMP4 and activin A.

To generate different types of dorsal interneurons, different concentrations of BMP4 and two distinct members of TGF-β superfamily were applied, which resulted in the greatest number of specified dI1 and dI3 neurons following treatment with activin A and higher concentrations of BMP4.

Materials and Methods
Culture of undifferentiated mouse ESCs
ES cells of line Royan B 1 (19) which were derived from the C57BL/6 mouse strain were grown on a feeder layer of primary mouse embryonic fibroblasts (MEF) in tissue culture flasks (stage 0). Cells were maintained in the presence of 1000 IU/ml leukemia inhibitory factor (LIF, ESGRO, Chemicon, ESGl107) in the ESC medium (stage 0) consisting of Knockout ™DMEM (Gibco,10829-018), 15% ESC qualified fetal bovine serum (FCS, Gibco,16141-079), 0.1 mM β-mercaptoethanol (Sigma, M7522), 2 mM L-glutamine (Gibco,25030-024), 0.l mM non-essential amino acids (Gibco, 11140-035) and penicillin and streptomycin (Gibco, 15070-063).

Induction of differentiation
The protocol of differentiation was summarized (Fig 1) (20,21). To induce differentiation (stage1), ESCs were dissociated into a single cell suspension by using Trypsin-EDTA in HBSS (Gibco,25300-054). 650000 cells were then transferred to bacteriological Petri dishes and cultivated for 4 days in the medium described above without β-mercaptoethanol and LIF in order to make ESC aggregates or embryoid bodies (EBs). At day 4 (4d), about sixty EBs were plated onto 6-well plates and coated with Poly-L-ornithine (Sigma, P3685)/ laminin (Sigma, L2020) containing the same medium. One day after the attachment of EBs, selection of nestin-positive cells (stage 2) was carried out by replacing the ESC medium with serum-free DMEM/F12 medium (Gibco, 12456018) supplemented with 5 μg/ml insulin (Sigma,16634), 30 nM sodium selenate (Sigma, S5261), 50 μg/ml transferrin (Sigma,T5391), and 5 μg/ml fibronectin (Invitrogen, 12173-091). The culture medium was replaced after 2 days. After 4 days of selection, differentiation of neural precursors (stage 3) was initiated; the cells were cultured in medium containing 25

µg/ml insulin instead of 5 µg/ml insulin, and supplemented with 1 µg/ml laminin, 10^{-6} M retinoic acid (RA), as well as different concentrations and combinations of BMP4 and activin A in 5 groups. neither BMP4 nor Aactivin A was considered as a control group, the other four groups were treated with 1 ng/ml BMP4, 10 ng/ml BMP4, 100 ng/ml activin A, and 10 ng/ml BMP4 plus 100 ng/ml activin A. The five groups were incubated for 4 days, with their mediums being replaced after 2 days. They were then dissociated with trypsin-EDTA (Gibco, 25300-054), and replated onto poly-L-ornithine/laminin-coated 24-well and 6-well plates containg neurobasal medium (Gibco, 211030-049) supplemented with 10% FCS, L-glutamine, nonessential amino acids, antibiotics, 2% B27 (Gibco, 17504-044), and 100 µM ascorbic acid (stage 4). To prevent glial cell proliferation, 10 µM of cytosine arabinoside (Sigma, C1768) was added as well. Half of the medium was changed every 2 days and the cells were allowed to differentiate under a maturation condition for 6 days.

Immunocytochemistry analysis
The cells were immunostained with nestin after stage 2, and with MAP-2, Lhx2, and Isl1 antibodies following stage 4.
After the cells were fixed with ice-cold 4% paraformaldehyde for 20 minutes and rinsed three times with PBS, they were incubated for 30 minutes in 5% goat serum, 0.3% bovine serum albumin, and 0.25% Triton X-100 in Tris-buffered saline (TBS). This blockade of nonspecific binding and permeabilization was followed by an overnight incubation with primary antibodies in optimal concentrations at 4°C. After five PBS rinses, the samples were incubated with the primary antibodies for 60 minutes in darkness at room temperature. The primary antibodies were: microtubule-associated protein 2 (MAP2) mouse monoclonal antibody (Sigma M2320, 1:250), nestin mouse monoclonal antibody (Chemicon MAB353, 1:200), Isl1 rabbit polyclonal antibody (Sigma, I5783, 1:500), and Lhx2 goat polyclonal antibody (Santa Cruz, sc-19344, 1:250). After washing, the cells were incubated with the following secondary antibodies: FITC conjugated goat anti-mouse (Chemicon, AP124F, 1:100), FITC conjugated goat anti-rabbit IgG (H+L) (Jackson Immunoresearch, 111-095-003, 1:200) and FITC conjugated mouse anti-goat IgG (H+L) (Sigma, F7367, 1:200). The cells were then counterstained with 1µg/ml propid-

ium iodide (PI, Sigma, P4864) or DAPI (Sigma) for 3 minutes at room temperature. Omission of primary antibodies was used as a control for all markers. Labeled cells were examined with a fluorescence microscope (Olympus, BX51, Japan) and images were acquired with an Olympus D70 camera. For cell counting, 10 random images prepared from fluorescent stained cells from different sites on each slide were counted in each image to determine the number of positive cells per total number of cells (stained with propidium iodide).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis
Total RNA was isolated as described using the RNeasy Mini Kit (Qiagen, 74104). Prior to reverse transcription (RT), a sample of the isolated RNA was treated with 1 U/µl of RNase-free DNaseI (EN0521, Fermentas) per 1 µg of RNA (in order to eliminate residual DNA) in the presence of 40 U/µl of ribonuclease inhibitor (E00311, Fermentas) and 1x reaction buffer with MgCl2 for 30 minutes at 37 ºC. Standard reverse-transcription reactions were performed with 2 µg total RNA using Oligo (dT)18 as a primer and Superscript II RNase H- reverse transcriptase kit (Invitrogen™, 11917-010), according to manufacturer's instructions. For every reaction set, one RNA sample without reverse transcriptase (RT- reaction) was to provide a negative control in the subsequent PCR. The primer sequences were: Lhx9 were: F: 5'-gacggtagcatttactgcaag -3' and R: 5'- gtgatgcttgaaagaagttc -3', Lhx1; F: 5'- caaggagcgaaggatgaaaca g -3' and R: 5'-cagatgatggcacaaagggta g -3', Isl1; F: 5'- gactttgagcaaggggttacg -3' and R: 5'- acatgaaaagtggcaagtctcc -3' and β-tubulin, forward: 5'-tcactgtgcctgaacttacc-3' and reverse: 5'-ggaacatagccgtaaactgc-3'. Amplification conditions consisted of initial denaturation at 94°C for 5 minutes followed by 30 cycles, denaturation at 94°C for 45 seconds, annealing for 45 sesonds, extension at 72°C for 30 seconds, and a final polymerization at 72 °C for 10 minutes on a Mastercycler gradient machine (Eppendorf, Germany). Each PCR was performed under linear conditions with beta-tubulin used as an internal standard. Products were electrophoresed on 1.7 % agarose gel. The gel was stained with ethidium bromide (0.5 µg/ml) and photographed on a UV transilluminator (Uvi-doc, UK). Gel images were analyzed using the UVI bandmap program (Uvitec, Cambridge, UK).

Statistical analysis

The data were expressed as Mean ± SD. One-way ANOVA followed by Tukey's post hoc test for multiple group comparison was used to analyze group differences of the data collected from image analysis. A difference between groups was considered as statistically reliable if $p < 0.05$.
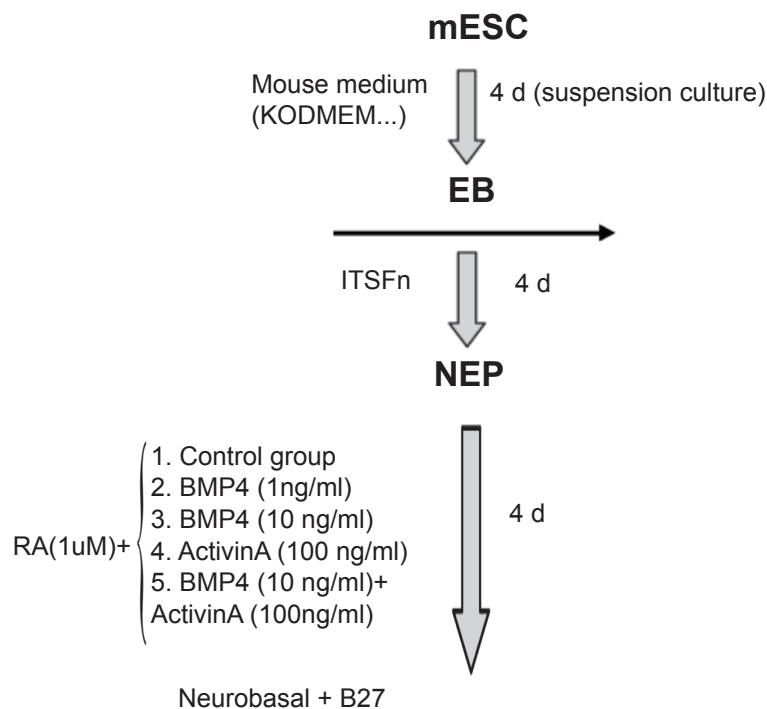
Results

To determine whether mESCs can differentiate into distinct classes of dorsal interneurons of the spinal cord, nestin positive cells were first induced (Fig 2A); they were then treated with RA and BMP4 and/or activin A to induce neurons (MAP 2 positive cell, Fig 2B) and dorsal interneurons of the spinal cord. Some of the experiments were just treated with RA, and were considered as control groups.
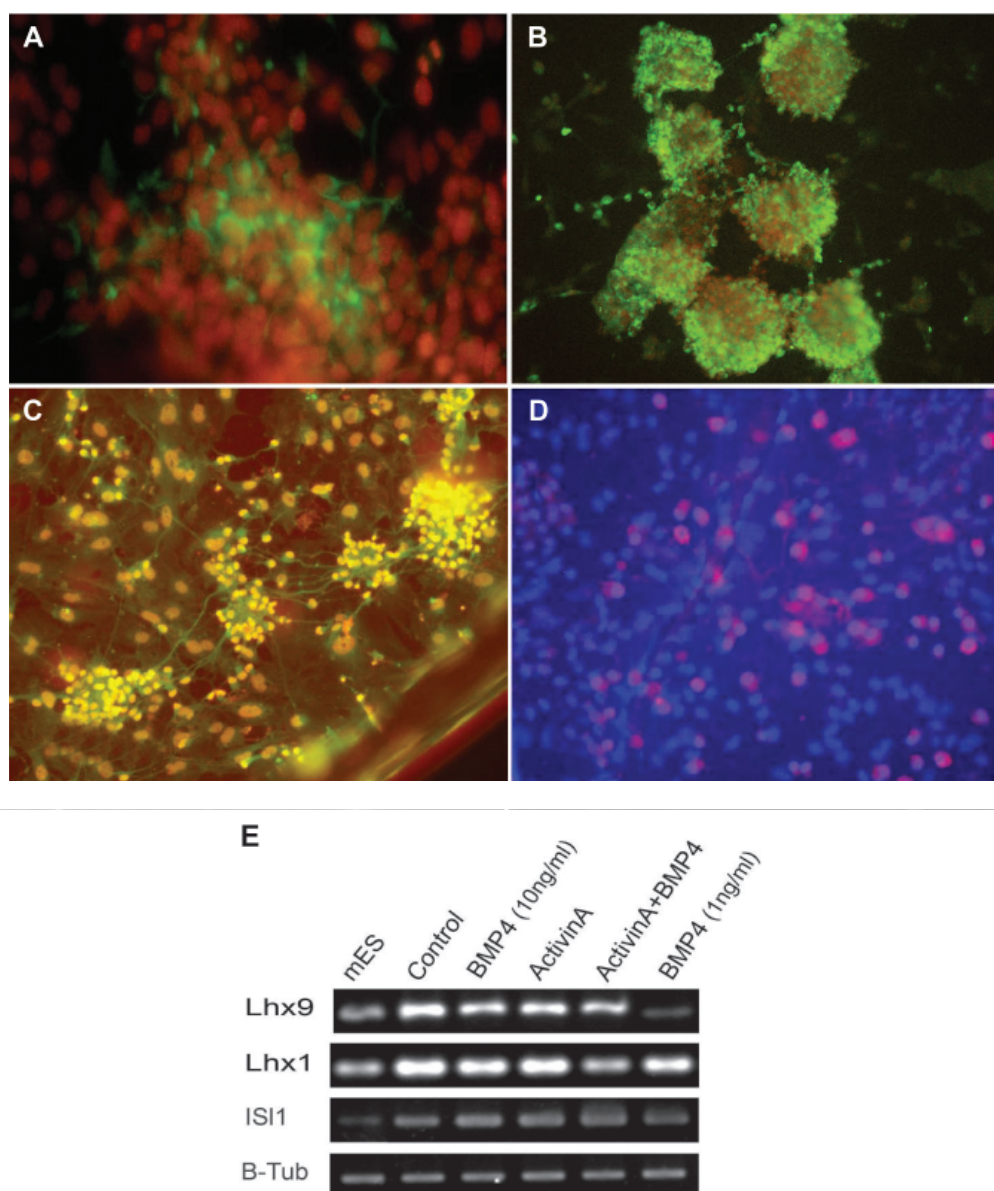
Since most spinal cord dorsal interneurons (dI1) are exposed to higher levels of roof-plate-derived TGFβ-mediated dorsalizing signals, it is possible that applying different concentrations of BMP4 can result in generation of distinct classes of dorsal interneurons (6, 22). To address this, cells were treated with 1 ng/ml BMP4 or 10 ng/ml BMP4. To examine the number of dI1 and dI3 interneurons differentiated under various treatments, stage 4 cells were immunostained with Lhx2 (Fig 2C) and Isl1 (Fig 2D) antibodies which are the markers of dI1 and dI3 interneurons respectively (3, 4, 8, 9, 23). We found that cells treated with 10 ng/ml BMP4 responded with the greater generation of dI1 and dI3 interneurons compared to the control group (Fig 3, $p < 0.0001$, $p < 0.003$, respectively). Furthermore, exposure of the cells to 1 ng/ml BMP4 did not induce a significant increase in the number of dI1 or dI3 interneurons compared to the control group (data not shown). These findings indicate that probably a concentration threshold of the dorsalizing signal BMP4 is required for the generation of distinct classes of dorsal sensory interneurons.

The same experiment was performed with activin A to test whether distinct members of the TGF-β superfamily can induce distinct types of dorsal interneurons. Based on the experiments done on explants of chick embryos, activin A can preferentially induce generation of dI3 (D2) neurons (3).

Our results indicate that cells subjected to activin A differentiated into more dI1 and dI3 neurons in comparison to the control group (Fig. 3, $p < 0.001$ and $p < 0.001$, respectively). Moreover, compared to the 1ng/ml BMP4 group, activin A-treated cells generated more dI3 (Isl1-positive) interneurons, but not dI1 neurons, (Fig 3, $p < 0.003$); these results provide evidence that treatment with activin A can induce more dI3 interneurons than treatment with lower concentrations of BMP4.

mESC

Mouse medium (KODMEM...) → 4 d (suspension culture)

EB

ITSFn → 4 d

NEP

RA(1uM)+
1. Control group
2. BMP4 (1ng/ml)
3. BMP4 (10 ng/ml)
4. ActivinA (100 ng/ml)
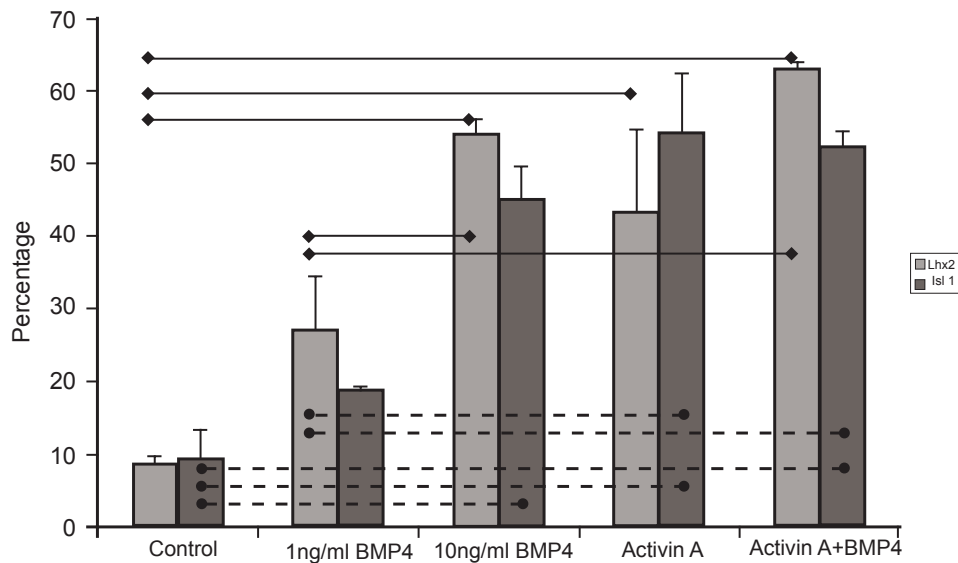5. BMP4 (10 ng/ml)+ ActivinA (100ng/ml)

4 d

Neurobasal + B27

Fig 1: Illustration of the differentiation induction protocol of mouse embryonic stem cells (mESCs) into dorsal interneurons of the spinal cord. NEP: neural precursors, EB: Embryoid body

Fig 2: *Characterization of mouse ESC-derived dorsal interneurons. Immunofluorescence staining of neural progenitor cells and neurons with ani-nestin at stage 2 (A), anti MAP2, Lhx2 (C), and Isl1 (D) at stage 4. Red and blue colors in A, B, C, and D represent nuclei counterstained with propidium iodide and DAPI, respectively. (E) RT-PCR analysis of differentiated neurons at step 4.*

In another experiment, dorsalizing factors were used together to test whether they have an additive effect on the generation of dorsal interneurons. Although a considerable increase in the number of Lhx2-positive and Isl1-positive neurons (dI1 and dI3 interneurons respectively) was observed in compared to the control group (Fig 3, $p<0.0001$ and $p<0.001$, respectively), there was no significant increase in the number of interneurons in this group compared to the 10 ng/ml BMP4-treated group and the activin A-treated group.

However, the group treated with both activin A and BMP4 resulted in generation of more dI3 neurons, but not dI1 neurons, in comparison to the 1 ng/ml BMP4-treated group ($p<0.004$), again confirming the previous result regarding preferential induction of dI3 neurons through treatment with the dorsalizing factor activin A (Fig 3). Moreover, RT-PCR analysis of differentiated cells showed the expression of Lhx9, Lhx1 and Isl1, the transcription factors that are markers of dI1, dI2, and dI3 interneurons respectively (Fig 2E).

Fig 3: Percentage of dI1 (Lhx2 positive) and dI3 (Isl1 positive). Solid line: significant difference (p<0.05) between different groups immunostained for Lhx2. Dashed line: significant difference (p<0.05) between different groups stained for Lhx2.

Discussion

Here, we examined the effects of activin and BMP4 on the differentiation of embryonic stem cells into interneurons *in vitro*. We found that the treatment of ESC-derived neural precursors with BMP4, activin A, or both increased the generation of both dI1 and dI3 interneurons (Lhx2 and Isl1 positive cells) compared to the control group. Timmer et al. previously suggested that activin signaling may promote the formation of the dI3 precursor cells within a region circumscribed by BMP signals, and that this function is not dependent upon BMP signaling.

However, the synergistic effect in generation of dI3 was not observed when both factors were used. *In vitro* data suggested that BMP and activin signaling has overlapping but distinct roles in patterning the dorsal neural tube (3, 4, 24).

Treatment of neural explants of chicken embryo with activin ligands indicated that activin signaling could repress Pax6 expression while promoting the expression of LHX2/9 and Islet1 (10). Although this is a subset of the activities ascribed to BMP signaling, there were significant differences in how these genes responded to different levels of BMP and activin signaling. Timmer et al. have described that activin pathway signaling elicits different downstream effects than BMP signaling *in vivo*.

Moreover, RT-PCR analysis of differentiated cells showed the expression of Lhx9, Lhx1 and Isl1, the transcription factors that are markers of dI1, dI2, and dI3 interneurons, respectively. Therefore, our results show that BMP4/activin A signals are required for the differentiation of specific dorsal interneurons of the spinal cord. Previous studies on neural explants of chick embryos have shown that BMP4 and activin A mimic the ability of epidermal ectoderm to induce neural crest cells and roof plate cells (3, 4). The present study extends this issue to the mouse ESCs as well. Furthermore, apparently a qualitative difference in the signaling of the TGF-β members may generate distinct types of dorsal interneurons, i.e. activin A may preferentially induce specification of dI3 interneurons versus dI1 neurons. Moreover, Murashov et al. (18) demonstrated that orderly application of developmental signaling molecules including RA, Shh, BMP2, and Wnt3A directs differentiation of ESCs into dorsal interneurons possessing appropriate neuronal markers, synaptic proteins, and neurotransmitter machineries.

Our results show that distinct classes of dorsal interneurons of the spinal cord require a specified threshold of BMP4 signaling. ES cells exposed to low BMP4 concentrations (1 ng/ml) generated a considerably smaller number of dorsal interneurons dI1 and dI3 than those treated with higher levels of BMP4 (10 ng/ml).

Conclusion

Mouse ESCs have the ability of differentiating to enormous cell types including dorsal cells of the neural tube. Increasing evidences places TGF-β signaling as an early event in the specification of dorsal identity in the neural tube which apparently implicates quantitative and qualitative differences in TGF-β signaling. Future studies aimed at dissecting molecular context that allows TGF-β signaling to regulate ESC differentiation into dorsal interneurons should yield important insights into mechanisms that control the development of dorsal horn neuronal population. The identification of these mechanisms may provide the means to generate specific classes of interneurons from ESCs for therapeutic applications.

Acknowledgments

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