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## Retinoic-acid-concentration-dependent acquisition of neural cell identity during in vitro differentiation of mouse embryonic stem cells

Yohei Okada<sup>a,b,c</sup>, Takuya Shimazaki<sup>a,c</sup>, Gen Sobue<sup>b</sup>, Hideyuki Okano<sup>a,c,\*</sup>

<sup>a</sup>Department of Physiology, Keio University School of Medicine, Shinjuku-ku, Tokyo 160-8582, Japan

<sup>b</sup>Department of Neurology, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya 466-8550, Japan

<sup>c</sup>Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan

Received for publication 11 February 2004, revised 19 July 2004, accepted 21 July 2004

Available online 8 September 2004

### Abstract

Retinoic acid (RA) is one of the most important morphogens, and its embryonic distribution correlates with neural differentiation and positional specification in the developing central nervous system. To investigate the concentration-dependent effects of RA on neural differentiation of mouse embryonic stem cells (ES cells), we investigated the precise expression profiles of neural and regional specific genes by ES cells aggregated into embryoid bodies (EBs) exposed to various concentrations of RA or the BMP antagonist Noggin. RA promoted both neural differentiation and caudalization in a concentration-dependent manner, and the concentration of RA was found to regulate dorso-ventral identity, i.e., higher concentrations of RA induced a dorsal phenotype, and lower concentrations of RA induced a more ventral phenotype. The induction of the more ventral phenotype was due to the higher expression level of the N-terminus of sonic hedgehog protein (Shh-N) when treated with low concentration RA, as it was abrogated by an inhibitor of Shh signaling, cyclopamine. These findings suggest that the concentration of RA strictly and simultaneously regulates the neuralization and positional specification during differentiation of mouse ES cells and that it may be possible to use it to establish a strategy for controlling the identity of ES-cell-derived neural cells.

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**Keywords:** Embryonic stem cells; Neural differentiation; Retinoic acid; Sonic hedgehog; N-terminus of Sonic hedgehog; Positional identity; Motor neuron; Morphogen

### Introduction

Embryonic stem cells (ES cells) are clonal cell lines derived from the inner cell mass (ICM) of developing blastocysts and under appropriate conditions are capable of proliferating extensively and generating various cell types derived from the three primary germ layers of the embryo in vitro. This pluripotency of ES cells provides a powerful in vitro model for investigating the mechanisms that control differentiation in early embryonic development. The basic strategy for in vitro differentiation usually adopted is to induce cell aggregation into so-called embryoid bodies

(EBs) through suspension culture in nonadhesive dishes or hanging drops. Different inducing conditions during EB formation can drastically affect the proportions of the various cell types that differentiate in EBs. For example, exposure to high-concentration RA strongly drives neural induction, whereas low-concentration RA induces cardiomyocyte differentiation (Rohwedel et al., 1999). Because neural cells represent only a small percentage of cells in EBs cultured in the presence of fetal bovine serum (FBS) and the absence of an exogenous inducer, efficient generation of neural cells requires an additional inductive stimulus or other differentiation method.

There are two major strategies for generating neural cells from mouse ES cells: EB formation and serum-free direct induction. The former includes treatment with high-concentration RA (Bain et al., 1995; Fraichard et al., 1995; Strubing et al., 1995), which has been shown to promote

\* Corresponding author. Department of Physiology, Keio University, School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Fax: +81 3 3357 5445.

E-mail address: [hidokano@sc.itc.keio.ac.jp](mailto:hidokano@sc.itc.keio.ac.jp) (H. Okano).

neural gene expression and repress mesodermal gene expression (Bain et al., 1996), and serum-free culture after EB formation, which enables selection of neural cells (Okabe et al., 1996). Direct induction methods consist of a co-culture system with stromal cell line PA6 as a neural inducer that has been found to have stromal-cell-derived inducing activity (SDIA) (Kawasaki et al., 2000), a low-cell-density neural stem cell (NSC) culture (neurosphere culture) with growth factors (Tropépe et al., 2001), and an adherent monolayer culture method (Ying et al., 2003).

Sequences of events leading to lineage commitment similar to those *in vivo* are often observed with all of these culture strategies, and, for example, exposure to Noggin or other manipulations that inhibit bone morphogenetic protein (BMPs) signaling, which blocks neural differentiation in the early development, facilitates neural differentiation of ES cells also *in vitro* (Finley et al., 1999; Gratsch and O'Shea, 2002; Kawasaki et al., 2000; Tropépe et al., 2001).

During the development of the mammalian central nervous system (CNS), the differentiation properties of neural stem cells (NSCs) vary depending on the stage at which they are generated (temporal identity) and their location (positional identity). These properties define the induction and sequential rounds of neurogenesis and gliogenesis, which seem to be regulated by both intrinsic and extrinsic factors, and limit their plasticity (Temple, 2001). Depending on their location, their differentiation is usually regulated by secreting signals that modulate the rostro-caudal or dorso-ventral axis of the body and by regional cues that define the borders of each CNS segment (Hitoshi et al., 2002; Temple, 2001). In view of these characteristics of NSCs, the temporal and positional identity of NSCs derived from ES cells may be controlled *in vitro* by the conditions under which they differentiate, the same as specification *in vivo*. Indeed, much interest has been focused on the generation of specific types of neurons or neural progenitors from ES cells by producing these identities with inductive signals, such as fibroblast growth factor (FGF) 8 and Shh, or with SDIA for dopaminergic neurons (Kawasaki et al., 2000, 2002; Kim et al., 2002; Lee et al., 2000; Ying et al., 2003), RA and Shh for motor neurons (Renoncourt et al., 1998; Wichterle et al., 2002), and a combination of SDIA and BMPs for dorsal and neural-crest-derived cells (Mizuseki et al., 2003).

RA is well known as the biologically active form of vitamin A and has been shown to play an important role during embryogenesis (Ross et al., 2000). RA influences neural development in the early stage of CNS development and is required to establish patterned territories of cell groups, which, for example, has been observed in rostro-caudal axis formation, according to the distribution of RA in experiments on *Xenopus* (Blumberg et al., 1997; Sive et al., 1990) and mice (Kessel, 1992; Kessel and Gruss, 1991; Marshall et al., 1992). For these reasons, RA has been thought to be one of the most important extrinsic inductive signals that can be used for neural differentiation of mouse

ES cells *in vitro*. However, its overall effects have yet to be clearly identified, and precise analysis of alterations of gene expression caused by RA treatment should be useful for establishing proper culture protocols for the differentiation of ES cells. In the present study, we demonstrated that RA promotes neural differentiation and caudalization in a concentration-dependent manner, and that the concentration of RA affects dorso-ventral positional identity, by determining the precise gene expression profiles during differentiation of ES cells.

## Materials and methods

### ES cell culture

Mouse ES cells (EB3) were maintained and used for induction. ES cells were grown on gelatin-coated (0.1%) tissue culture dishes in standard ES-cell culture medium containing GMEM (Sigma G6148) supplemented with 10% FBS, glutamine (2 mM), nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), 2-mercaptoethanol (2-ME) (0.1 mM), sodium bicarbonate (3 mM), HEPES (5 mM), and mLIF. EB3 is a subline derived from E14tg2a ES cells (Hooper et al., 1987) that was generated by targeted integration of Oct3/4-IRES-BSD-pA vector (Niwa et al., 2000) into the *Oct3/4* allele, and it was maintained in the medium containing 10 µg/ml blasticidin S to eliminate differentiated ES cells.

### Differentiation of ES cells

For embryoid body (EB) formation, ES cells were detached and dissociated into single cells with 0.25% trypsin–EDTA and then plated onto a bacteriological dish (Kord-Valmark™) in 10 ml of αMEM (Gibco 11900-024) supplemented with 10% FBS, sodium bicarbonate (3 mM), and 0.1 mM 2-ME (EB medium) at a density of  $5 \times 10^4$  cells/ml. On day 2, various concentrations of all-*trans*-retinoic acid (RA; Sigma R 2625) were added to the culture medium (2–/4+ protocol). RA was reconstituted with 100% ethanol to prepare a stock solution. It should be noted that the effective RA concentrations at which ES cells grow into EBs may be considerably higher than those indicated in the text, because FBS contains significant levels of RA. Furthermore, cells within EBs may produce endogenous RA, possibly as a secondary effect of the initially added RA. However, we used conditions in which FBS did not contain exogenous RA as a negative control (stated control in the figures), and evaluated the results in terms of relative concentrations of RA. Recombinant mouse Sonic Hedgehog (Shh) protein (amino-terminal peptide) (Shh-N; R&D Systems Inc., 461-SH) and cyclopamine (0.1 µM, 1 µM, Toronto Research Chemicals Inc., C988400) were also added on day 2 of the experiment. For Noggin treatment, 10% (v/v) culture supernatant of Cos7

cells transfected with *Xenopus* Noggin/MC BOS (a gift of Y. Takahashi) (Kohyama et al., 2001; Tonegawa and Takahashi, 1998) (xNoggin conditioned medium) was added.

EBs were collected at day 6 of culture and allowed to settle to the bottom of the tube for a few minutes. The cells were then washed once with PBS and incubated with 0.25% trypsin–EDTA for 5 min at 37°C. The enzymatic reaction was quenched by addition of an equal volume of EB medium, and the cells were dissociated with a transfer pipette by triturating 30 times. The cells were then washed twice with serum-free  $\alpha$ MEM and resuspended in Media hormone mix (MHM) medium, which contains DMEM/F-12 (1:1) (Gibco 12100-046, 21700-075), glucose (0.6%), glutamine (2 mM), sodium bicarbonate (3 mM), HEPES (5 mM), insulin (25  $\mu$ g/ml), transferrin (100  $\mu$ g/ml), progesterone (20 nM), sodium selenate (30 ng), and putrescine (60 nM) (all from Sigma except for DMEM/F-12) as described previously (Shimazaki et al., 2001). The dissociated EBs were plated on poly-L-ornithine/fibronectin-coated 10-mm cover glasses (Matsunami) at a cell density of  $1.6 \times 10^5$  cells/0.75 cm<sup>2</sup> on a 48-well culture plate (Coaster) and allowed to differentiate for 24 h.

To clarify the effects of RA added at different points in time or of exposure for different periods of culture, ES cells were differentiated into EBs based on 2–/2+/2–, 2–/2+/2+ and 4–/4+ protocols (Suppl. Fig. 1). In the 2–/2+/2– and 2–/2+/2+ protocol, various concentrations of RA were added on day 2, and on day 4 the culture medium was replaced with freshly prepared medium containing the same concentrations of RA (2–/2+/2+ protocol) or no RA (2–/2+/2– protocol). In the 4–/4+ protocol, various concentrations of RA were added to the culture medium on day 4. Total RNA was isolated at day 0, 2, 4, 6, and 8 and processed for RT-PCR analysis.

#### *Immunocytochemistry*

Dissociated EBs were cultured for 24 h and fixed with 4% paraformaldehyde for 20 min at room temperature. The cells were rinsed with PBS twice and pretreated with PBS containing 0.3% Triton X-100 for 5 min at room temperature. After blocking in TNB buffer (Provided by NEN™ Life Science Products, Inc.) for 1 h at room temperature, the cells were incubated at 4°C overnight with the following antibodies: anti-Nestin (Rat-401, mouse IgG, 1:200), anti-Islet-1/2 (40.2D6, mouse IgG, 1:500), anti-Lim3 (67.4E12, mouse IgG, 1:1000), anti-HB9 (81.5C10, mouse IgG, 1:100), anti-Otx1 (Otx-5F5, mouse IgG, 1:500000), anti-Nkx2.2 (74.5A5, mouse IgG, 1:5000), anti-Pax7 (mouse IgG, 1:5000) (Developmental Studies of Hybridoma Bank: DSHB), anti- $\beta$ III-tubulin (mouse IgG, 1:1000, Sigma T8660), anti-Olig2 (rabbit IgG, 1:30000) (Mizuguchi et al., 2001; Takebayashi et al., 2000), anti-Phox2b (rabbit IgG, 1:25000) (Pattyn et al., 1997), anti-Nkx6.1 (Ab174.3, rabbit IgG., 1:200000) (Jensen et al.,

1996), anti-Group B1 Sox [Sox1/(2)/3] (rabbit IgG, 1:10000) (Tanaka et al., unpublished). Anti-Group B1 Sox [Sox1/(2)/3] antibody is weakly reactive with Sox2, which is expressed not only by the neural primordium but by undifferentiated ES cells, and with Sox1 and Sox3 (with preference for Sox1 and Sox3 over Sox2). However, as all Group B1 Sox genes are expressed in neural primordium (Wood and Episkopou, 1999), we used this antibody to detect neural progenitors, by determining the immunostaining conditions under which undifferentiated ES cells, which were used as a negative control, did not stain (data not shown). Antigen retrieval was accomplished by incubating the samples in the boiled PBS for 10 min for anti-Islet-1/2 and anti-Lim3, in boiled Target Retrieval Solution (DAKO) for 10 min for anti-Nkx2.2, or in 1 N HCl at 30°C for 15 min for anti-Pax7. After washing with PBS three times, the cells were incubated for 1 h at room temperature with secondary antibodies conjugated with Alexa 488 or Alexa 568 (Molecular Probes). For anti-Islet1/2, anti-Lim3, anti-HB9, anti-Olig2, anti-Phox2b, anti-Otx1, anti-Nkx2.2, anti-Pax7, and anti-Nkx6.1 staining, we used biotinylated secondary antibodies (Jackson Immunoresearch Laboratory, Inc.) after exposure to 1% H<sub>2</sub>O<sub>2</sub> for 15 min at room temperature to inactivate endogenous peroxidase. The signals were then enhanced with streptavidin-HRP (SA-HRP), followed by TSA™ Fluorescein System (NEN™ Life Science Products, Inc.). After washing with PBS, the samples were mounted on slides and examined with a universal fluorescence microscope (Axiophot 2, Carl Zeiss) and a confocal laser scanning microscope (LSM510, Carl Zeiss). The nuclei of all samples were stained with hoechst33342 (1  $\mu$ g/ml, Sigma B2261). For statistical analysis, at least 200 cells per cover glass were examined, and the numbers of cells that had immunoreacted with each antibody were counted and expressed as a percentage of the total number of cells whose nuclei stained with hoechst33342. The *P* values for statistical significance (*t* test) are stated in the figure legends.

#### *Western blot analysis*

Western blot analysis was performed by the previously established method. A 20  $\mu$ g protein sample of a total cell extract was run on 7.5–15% SDS-PAGE, transferred to nitrocellulose, and probed with each antibody. The blot was probed with the following antibodies: anti-Nestin (Rat-401, mouse IgG, Developmental Studies of Hybridoma Bank: DSHB), anti- $\beta$ III-tubulin (mouse IgG, Sigma T8660), anti-Glial Fibrillary Acidic Protein (GFAP) (rabbit IgG, DAKO Z0334), anti-CNPase (mouse IgG, Sigma C5922), and anti-Shh N-terminal fragment (goat IgG, Santa Cruz sc-1194). Signals were detected with HRP-conjugated secondary antibodies (Jackson Immunoresearch Laboratory, Inc.) by using an ECL kit (Amersham Biosciences). Quantitative analysis was performed with Scion Image (Scion Corpo-

ration). The amounts of proteins loaded in each slot were normalized to those of  $\alpha$ -tubulin.

#### RNA isolation and RT-PCR

RT-PCR analysis of at least two independent cultures was performed in most of the experiments, and were similar results obtained. Total RNA was isolated with Trizol reagent (Invitrogen™ 15596-018) and DNase I treatment, or by the RNeasy Mini Kit (Qiagen). Total RNA (1–3  $\mu$ g) was used to synthesize cDNA with 500 ng oligo-d(T)<sub>12–18</sub> primers. The cDNA synthesis was performed at 42°C for 50 min in a final volume of 20  $\mu$ l according to the manufacturer's instructions for Superscript II RNase H<sup>-</sup> reverse transcriptase (Invitrogen™). To analyze relative expression of different mRNAs, the amount of cDNA was normalized based on the signals from ubiquitously expressed  $\beta$ -actin mRNA. The PCR was carried out by using a KOD Plus kit (Toyobo) according to the manufacturer's standard protocol in a final volume of 25  $\mu$ l. Primer sequences and PCR cycling conditions will be provided upon request. To provide negative controls and exclude contamination by genomic DNA, the reverse transcriptase was omitted in the cDNA synthesis step, and the samples were subjected to the PCR reaction in the same manner with primer sets for  $\beta$ -actin, and are indicated at the bottom of each figure as RT(-). PCR products were electrophoresed in agarose gel, and bands were visualized with ethidium bromide under UV light. The identity of the PCR products was confirmed by sequencing.

## Results

#### Differentiation potential of mouse ES cells regulated by RA

RA has been shown to be one of the most important extrinsic morphogens and precisely modulates the differentiation properties of ES cells into various cell types, including neural cells, skeletal muscle cells, adipocytes, cardiomyocytes, and vascular smooth muscle cells, in an incubation-time- and concentration-dependent manner (Rohwedel et al., 1999). To examine the concentration-dependent effects of RA on the differentiation of ES cells, we first differentiated ES cells by inducing the formation of EBs in the presence of various concentrations of RA. We also used Noggin, a secreted protein that plays a role in neural induction by inhibiting BMP-signaling (Finley et al., 1999; Gratsch and O'Shea, 2002; Kawasaki et al., 2000; Smith and Harland, 1992; Tropepe et al., 2001; Zimmerman et al., 1996), to investigate RA-independent neural differentiation. ES cells were plated onto bacteriological dishes and had been cultured for 6 days in medium containing various concentrations of RA (added on day 2) or xNoggin conditioned medium (Fig. 1), and they were analyzed by RT-PCR for markers of the three primary germ layers

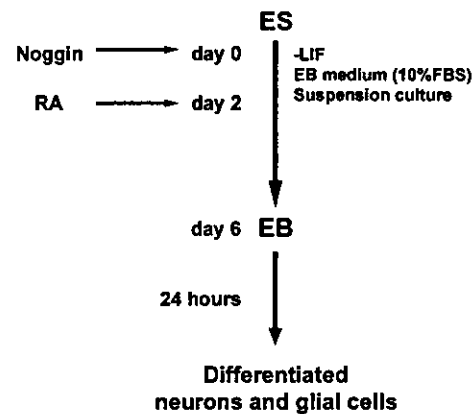


Fig. 1. Experimental protocol for differentiation of ES cells with retinoic acid (RA) or xNoggin conditioned medium. ES cells were cultured in the bacteriological dish for 6 days and formed embryoid bodies (EBs). Various concentrations of RA were added on day 2 of EB formation. Then, EBs were dissociated and differentiated on poly-L-ornithine/fibronectin-coated cover glasses.

(Fig. 2). On day 2, *oct3/4*, which is a marker for undifferentiated ES cells, was expressed by both control and Noggin-treated EBs. From day 4 of EB formation onward, *oct3/4* expression was gradually down-regulated by RA in a concentration-dependent manner and in a culture period-dependent manner, and it became undetectable on day 6 under all conditions, indicating that most of the ES cells had differentiated by 6 days of EB formation. On day 6, expression of *ck-17* (*cytokeratin 17*), a marker of epidermis (McGowan and Coulombe, 1998), and expression of *ngn2*, which is expressed in neuronal progenitors (Mizuguchi et al., 2001; Novitch et al., 2001; Ross et al., 2003), were enhanced by high-concentration RA treatment ( $>10^{-7}$  M; high-RA), and thus ectodermal differentiation was promoted by exposure to high-RA. Expression of *ck-17* mRNA in undifferentiated ES cells, which also expressed *oct3/4* (Fig. 2), was also demonstrated in a previous study (Tropepe et al., 2001). In the control, Noggin, and low-RA-treated EBs, its expression coincided with expression of *oct3/4* at day 4, and was then down-regulated by day 6 along with extinction of *oct3/4*. In the high-RA-treated EBs, on the other hand, expression of *ck-17* mRNA was detected at day 4 and day 6, without expression of *oct3/4*. The expression of *ck-17* in the absence of expression of *oct3/4* can be understood as indicating promotion of epidermal differentiation in EBs treated with high-RA. Mesodermal differentiation, represented by expression of *brachyury*, which is essential for the formation and organization of mesoderm (Herrmann et al., 1990; Wilkinson et al., 1990), and expression of homeobox gene *nkx2.5*, the earliest known marker of cardiac development (Komuro and Izumo, 1993; Lints et al., 1993), were facilitated by low-concentration RA treatment ( $10^{-9}$ – $10^{-8}$  M; low-RA). Endodermal markers, including *gata4*, expressed in primitive endoderm (Arceci et al., 1993), and *pdx1*, expressed in developing pancreas (Jonsson et al., 1994; Offield et al., 1996), were

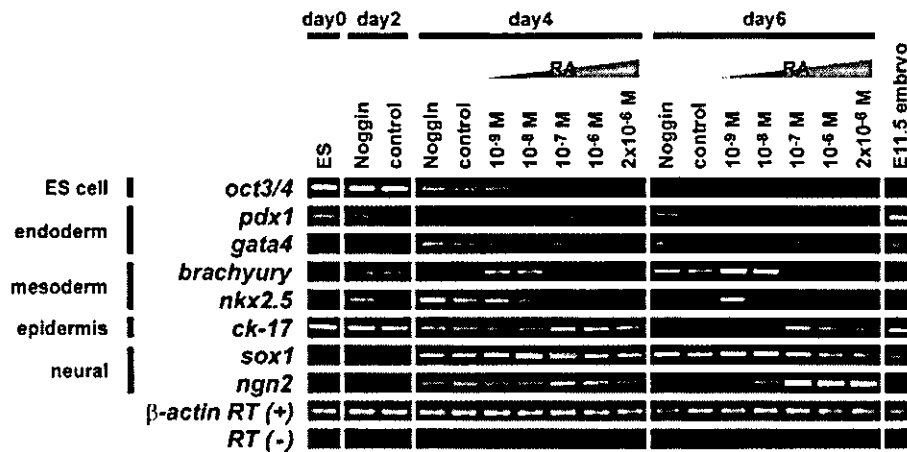


Fig. 2. Expression of markers of the three primary germ layers in EBs exposed to various concentrations of RA. RNA was isolated from ES cells (day 0) and EBs (days 2, 4, 6) and analyzed by RT-PCR for expression of markers of undifferentiated ES cells (*oct3/4*), endoderm differentiation (*pdx1* and *gata4*), mesoderm differentiation (*brachyury* and *nkx2.5*), epidermis differentiation (*ck-17*), and neural differentiation (*sox1* and *ngn2*). To normalize their expression to the amount of cDNA present in the sample, the cDNA for endogenous  $\beta$ -actin was amplified.

expressed unstably at low levels, and their levels correlated poorly with the concentrations of RA.

*Neural induction of mouse ES cells by RA*

It has been shown that neural differentiation of ES cells can be promoted by RA, especially by early exposure of EBs to relatively high RA concentrations (Bain et al., 1995, 1996; Fraichard et al., 1995; Gajovic et al., 1997; Renoncourt et al., 1998; Rohwedel et al., 1999; Strubing et al., 1995; Wichterle et al., 2002). However, as the effect of different RA concentrations had never been precisely described, we next investigated how RA promotes neural differentiation. EBs that had been cultured for 6 days were analyzed for differentiation markers of neural cells (progenitors, neurons, and glia) by Western blotting (Figs. 3A, B). Nestin, which is expressed in undifferentiated neural progenitors, was more strongly expressed in EBs treated with low-RA. Expression of  $\beta$ III-tubulin and GFAP, which are markers of differentiated neurons and astrocytes, respectively, increased in a concentration-dependent manner in EBs exposed to RA (Figs. 3A, B). By contrast, RT-PCR analysis on day 6 showed that expression of *sox1* (a marker of undifferentiated neural cells; Pevny et al., 1998; Wood and Episkopou, 1999) mRNA was higher in EBs treated with low-RA on day 6 (Fig. 2). Expression of CNPase, a marker of oligodendrocytes, was detected only slightly under all of the differentiating conditions, and its expression was not very strongly affected by the concentration of RA (Figs. 3A,B). To better understand the effects of RA on neural differentiation of EBs, we performed immunocytochemistry of markers of various neural lineages (Figs. 3C–E and 5A,B). EBs that had been cultured for 6 days were dissociated and differentiated on poly-L-ornithine/fibronectin-coated cover glasses for 24 h and then processed for immunocytochemistry of markers

of undifferentiated neural cells (Nestin, Group B1 Sox, Olig2) and postmitotic neurons ( $\beta$ III-tubulin). Olig2 is a basic-helix-loop-helix (bHLH) transcription factor that is expressed in most of the ventral neural progenitor cells around the period of neural tube closure (Takebayashi et al., 2000). Treatment of EBs with low-RA ( $10^{-8}$  M) induced a 1.6-, 3.0-, and 9.1-fold increase in Nestin-, Group B1 Sox, and Olig2-positive undifferentiated neural progenitors, respectively, over those treated with high-RA ( $2 \times 10^{-6}$  M) (Figs. 3C–E). Treatment of EBs with high-RA ( $2 \times 10^{-6}$  M) induced very few Nestin-, Group B1 Sox-, and Olig2-positive progenitor cells, and instead induced many  $\beta$ III-tubulin-positive postmitotic neurons [3.0-fold more than by treatment with low-RA ( $10^{-8}$  M)] (Figs. 3C–E and 5A). RT-PCR analysis showed that *olig2* was expressed in EBs treated with high-RA at day 4 and down-regulated by day 6, whereas it was expressed highly on day 6 in EBs treated with low-RA (Fig. 6A). These results indicate that higher concentrations of RA facilitate differentiation of neural progenitors into postmitotic neurons and glia, in contrast to lower concentrations of RA, which preferentially induce undifferentiated neural progenitor cells from ES cells; that is, that RA strongly promotes terminal differentiation of ES-cell-derived neural progenitors in a concentration- and culture-period-dependent manner in addition to its action that promotes neural induction of ES cells.

*ES-cell-derived neural cells acquire positional identity through EB formation*

To investigate how RA regulates the specification of rostral-caudal and dorso-ventral positional identity during EB formation, RT-PCR analysis of regionally specific markers was performed (Carpenter, 2002; Caspary and Anderson, 2003; Helms and Johnson, 2003; Hitoshi et al., 2002; Jessell, 2000; Marquardt and Pfaff, 2001;

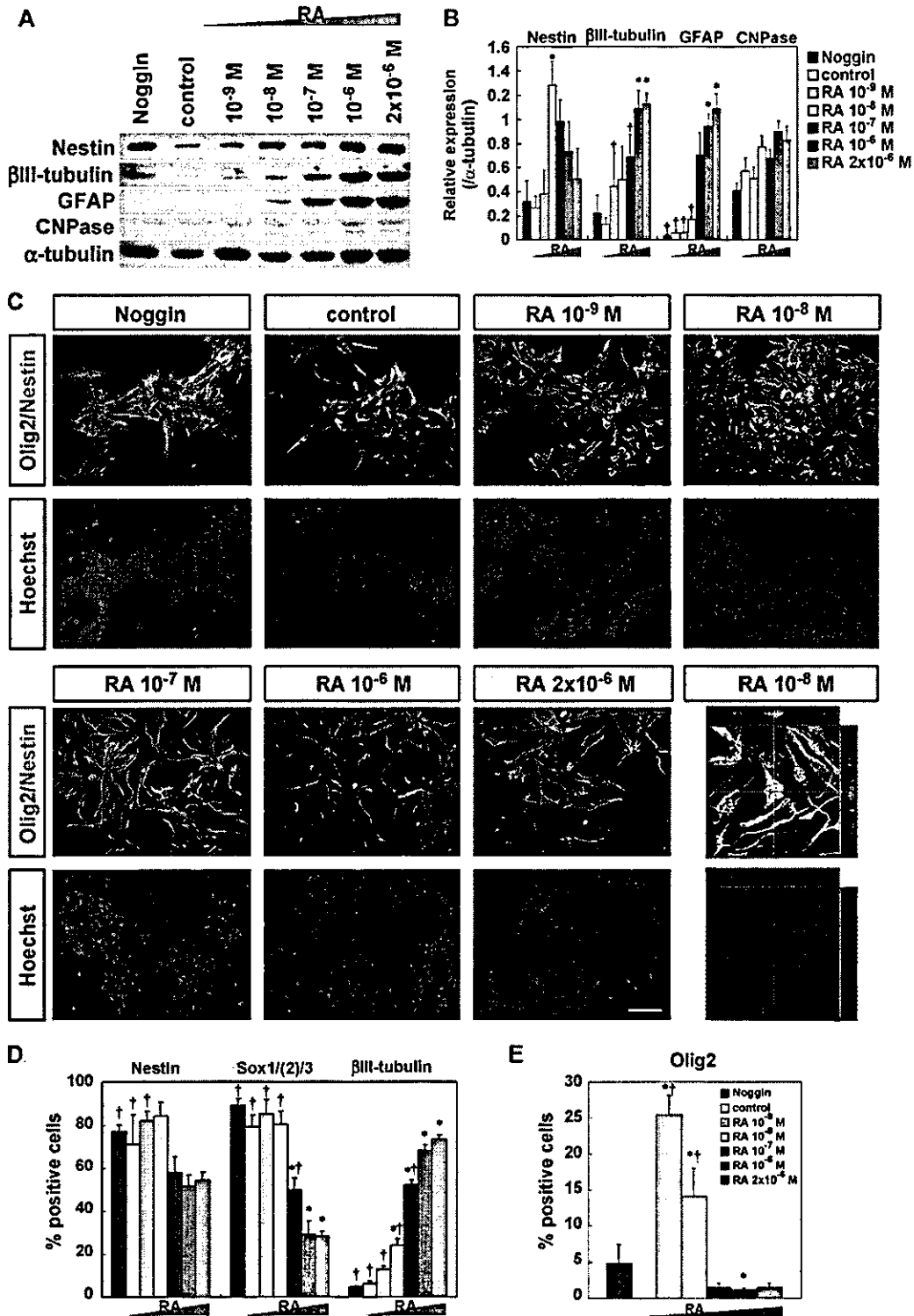


Fig. 3. RA promotes neural differentiation in a concentration-dependent manner and regulates the differentiation of ES-cell-derived neural progenitors. (A) Western blot analysis of markers for neural differentiation in EBs cultured for 6 days. (B) Quantitative analysis was performed with Scion Image. The amounts of proteins were normalized to those of  $\alpha$ -tubulin ( $n = 3$ , mean  $\pm$  SEM, \*,  $P < 0.05$  vs. control, †,  $P < 0.05$  vs. RA  $2 \times 10^{-6}$  M). (C) Immunocytochemistry of dissociated EBs for Olig2 and Nestin. Nuclear localization of Olig2 in Nestin immunoreactive cells was confirmed by three-dimensional reconstruction of confocal microscopic images (right end of lower panels). (D, E) The proportions of cells positive for Nestin, Group B1 Sox, Olig2, and  $\beta$ III-tubulin among the total number of cells in dissociated EBs were determined by immunocytochemically. Immunoreactive cells as a percentage of the total number of cells counted on the basis of nuclear staining with hoechst33342 are shown ( $n = 3$ , mean  $\pm$  SEM, \*,  $P < 0.05$  vs. control, †,  $P < 0.05$  vs. RA  $2 \times 10^{-6}$  M). The percentages of cells expressing Olig2, Group B1 Sox, and Nestin were higher in dissociates of EBs treated with low-RA ( $< 10^{-8}$  M) than in EBs treated with high-RA ( $> 10^{-7}$  M). Scale bar: 50  $\mu$ m.

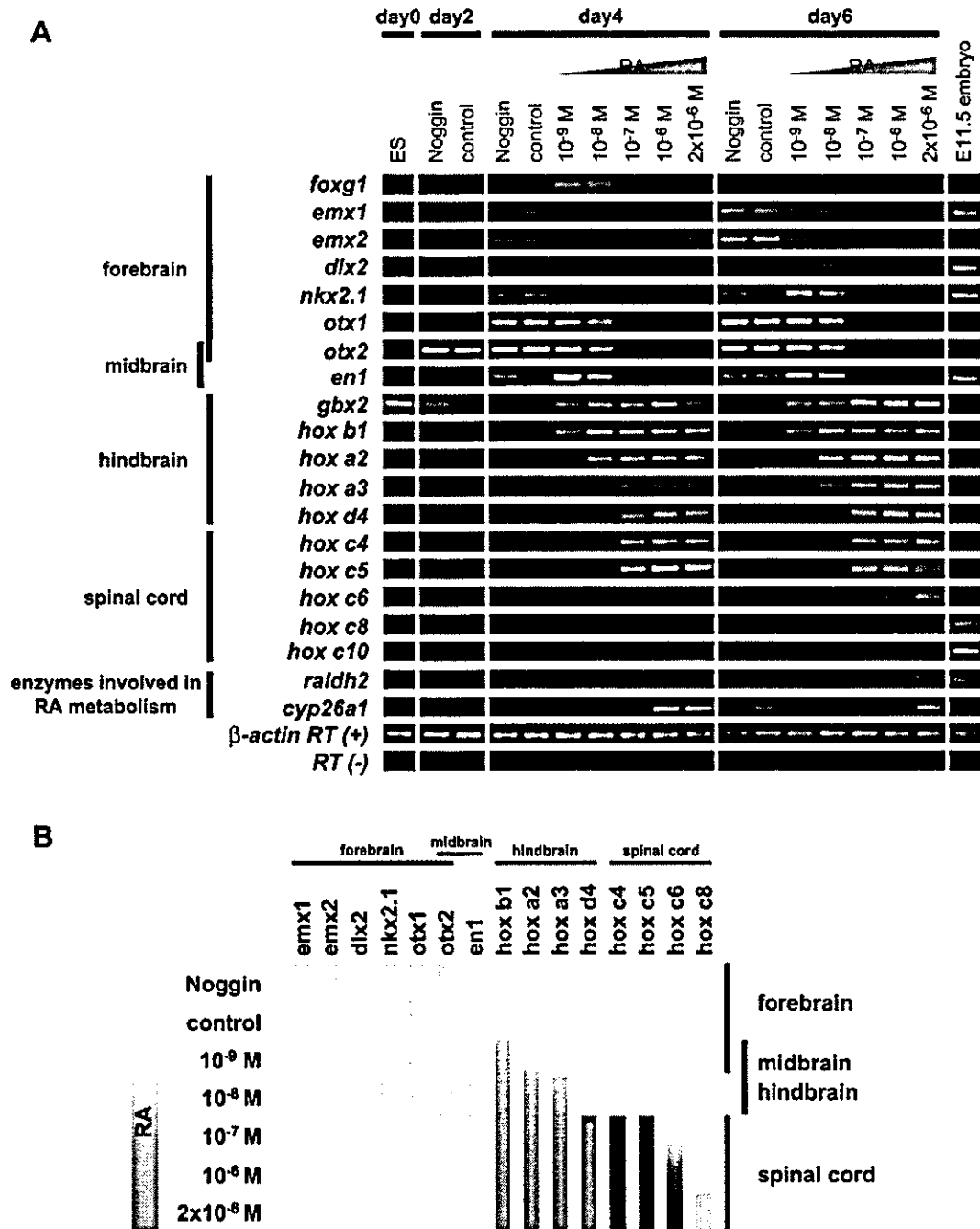
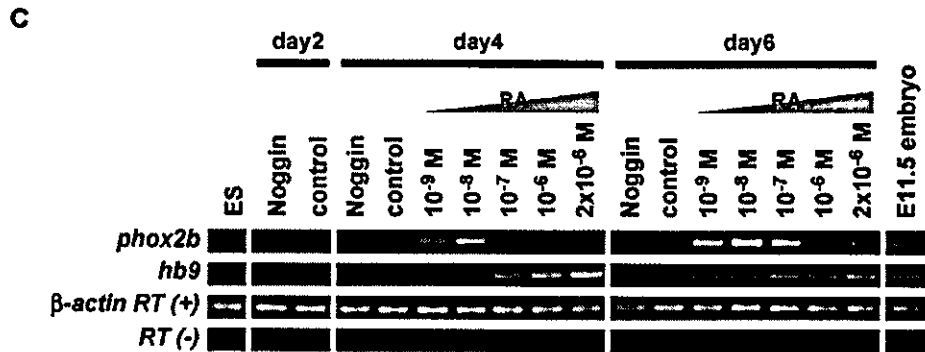
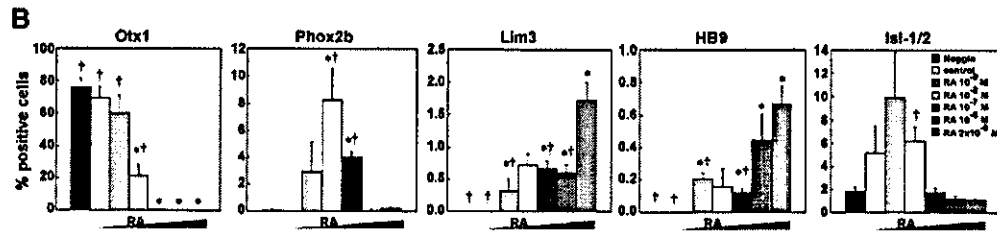
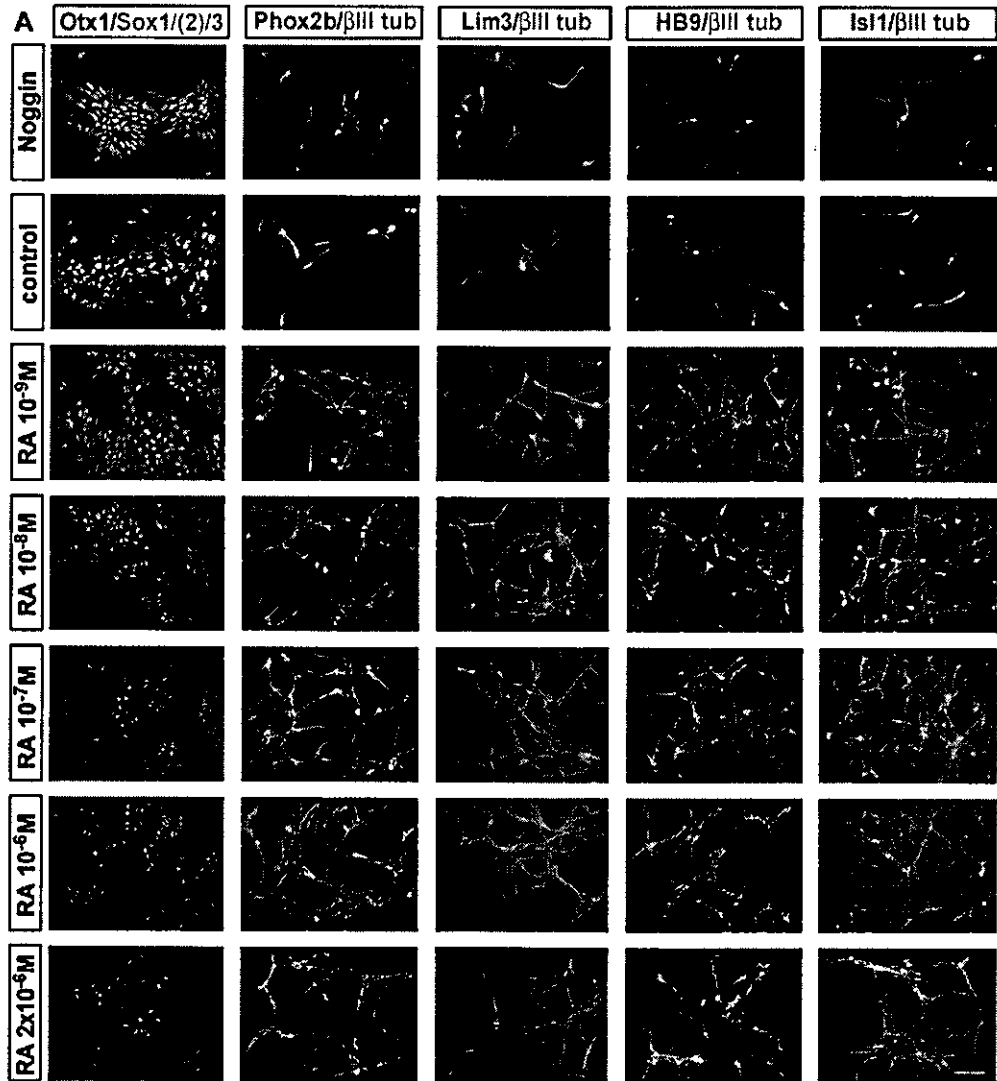


Fig. 4. Concentration-dependent effects of RA on the specification of rostral-caudal positional identity of ES-cell-derived neural progenitors. (A) Effect of RA on rostral-caudal axis formations was analyzed by RT-PCR on days 0, 2, 4, and 6 of differentiation. The expression patterns are summarized in (B). RA caudalized ES-cell-derived neural cells in a concentration-dependent manner. Control and Noggin-treated-EBs expressed forebrain-type markers, whereas EBs treated with low-RA and high-RA expressed midbrain–hindbrain-type markers and spinal-cord-type markers, respectively.

Fig. 5. RA caudalizes EB-derived neurons in a concentration-dependent manner. (A) Immunocytochemical analysis of neural progenitors and neurons differentiated from dissociated EBs with Otx1, which is expressed in developing forebrain and midbrain, and Phox2B, Lim3, HB9, and Isl-1/2, which are expressed in developing motor neurons and their progenitors. Immunoreactive cells as a percentage of the total number of cells counted on the basis of the nuclear staining with hoechst33342 are shown in B ( $n = 3$ , mean  $\pm$  SEM, \*,  $P < 0.05$  vs. control, †,  $P < 0.05$  vs. RA  $2 \times 10^{-6}$  M). (C) RT-PCR analysis of *phox2b* and *hb9*. Control and Noggin-treated EBs generated significant numbers of Otx1- and Group B1 Sox-positive anterior neural progenitors. Low-RA ( $10^{-9}$ – $10^{-8}$  M) induced many Phox2b-positive hindbrain brachial and visceral motor neurons and fewer Otx1/Group B1 Sox-positive anterior neural progenitors, whereas high-RA ( $>10^{-7}$  M) induced more HB9-positive hindbrain and spinal cord somatic motor neurons without any Otx1-positive cells. Scale bar: 50  $\mu$ m.





Niederreither et al., 2000; Schuurmans and Guillemot, 2002; Wurst and Bally-Cuif, 2001). As shown in Fig. 4, EBs were caudalized in a concentration-dependent manner during the first 2 days of RA exposure (days 2–4). After day 4, control and Noggin-exposed EBs expressed genes specific to forebrain (*emx1*, *emx2*, *nkx2.1*, *otx1*, *otx2*) and midbrain–hindbrain (*otx1*, *otx2*, *en1*), but no hindbrain or spinal cord markers. EBs treated with low-RA mainly expressed midbrain–hindbrain markers (*otx1*, *otx2*, *en1*, *gbx2*, *hoxb1*, *hoxa2*, *hoxa3*), and did not express spinal cord markers (*hoxc4*, *hoxc5*, *hoxc6*, *hoxc8*, *hoxc10*). Expression of telencephalic markers (*emx1*, *emx2*, *dlx2*) in EBs treated with low-RA was lower than in control and Noggin-exposed EBs. However, at day 4, expression of one of the telencephalic markers, *foxg1*, was somehow highest in the EBs exposed to low-RA. On the other hand, high-RA induced expression of hindbrain and rostral spinal cord markers (*hoxc4*, *hoxc5*, *hoxc6*) and reduced expression of forebrain and midbrain markers. These patterns of gene expression were detected at day 4 and were maintained thereafter. The expression levels of enzymes involved in RA metabolism, *raldh2* and *cyp26a1* (Fig. 4A), were higher in EBs treated with high-RA, a finding that was consistent with the EBs exposed to high-RA acquiring the identity of rostral spinal cord, where the concentration of RA and the expression level of its synthesizing enzyme Raldh2 are the highest in the developing CNS (Swindell et al., 1999). The RA catabolizing enzyme Cyp26a1 may have been induced by high-RA as part of a negative feedback mechanism. The total gene expression patterns indicating rostro-caudal specification in EBs differentiated under different conditions are summarized in Fig. 4B. The concentration-dependent caudalization of EBs by RA treatment shown by the result of the RT-PCR analysis was confirmed by immunocytochemistry of dissociated EBs with antibodies for markers expressed in developing forebrain and midbrain (Otx1) (Acampora et al., 1998), visceral or brachial motor neurons in the hindbrain (Phox2b) (Pattyn et al., 2000), and somatic spinal motor neurons (HB9, Lim3) (Arber et al., 1999) (Figs. 5A,B). Virtually all of the marker-positive cells were also positive for either a neural progenitor marker Group B1 Sox, or pan-neuronal marker  $\beta$ III-tubulin. A significant number of cells derived from EBs and grown under all conditions expressed Isl-1/2, a marker of postmitotic cholinergic neurons, including not only spinal motor neurons but those in ventral forebrain (Kohtz et al., 2001; Wang and Liu, 2001). Somatic motor neurons of the hindbrain and spinal cord expressing Lim3 and HB9 were found more frequently when treated with high-RA, whereas hindbrain visceral or brachial motor neurons expressing Phox2b were found more frequently when treated with low-RA. By contrast, an enormous number of neural progenitors that were positive for both Otx1 and Group B1 Sox and acquired anterior positional

identity were induced from control and Noggin-treated EBs, and less frequently from low-RA treated EBs, whereas no such cells were induced from high-RA-treated EBs. Taken together, these findings indicate that RA induced both caudalization of EBs based on the expression patterns of regionally specific genes during neural induction and neuronal differentiation in a concentration-dependent manner, resulting in significant generation of forebrain and midbrain (control and Noggin), hindbrain (low-RA), and spinal cord (high-RA) types of neural progenitors or neurons, respectively.

#### RA controls dorso-ventral axis formation

To determine the effect of RA on dorso-ventral axis specification of EB-derived cells, we investigated the expression of class I genes (*pax7*, *dbx1*, *dbx2*, *irx3*, *pax6*, whose expression is repressed by Shh in early CNS development) and class II genes (*nkx6.2*, *nkx6.1*, *olig2*, *nkx2.2*, whose expression is activated by Shh). These genes are differentially expressed along the dorso-ventral axis in the progenitor domains of developing hindbrain and spinal cord (Jessell, 2000). As shown in Fig. 6, EBs treated with low-RA expressed both class I and class II genes, indicating that they were composed of various populations that had acquired their identities throughout the dorsal to ventral neural tube. Interestingly, on the other hand, treatment with high-RA raised the expression levels of class I genes and significantly reduced those of class II genes except *olig2* at day 4, in comparison to treatment with low-RA. Thus, high-RA caused dorsalization of neural progenitor cells in EBs. To investigate the mechanism underlying the action of RA in specifying dorso-ventral identity, we investigated its effects on expression of the N-terminus of Shh protein (Shh-N) and *sonic hedgehog* (*shh*) mRNA. Mouse Shh is produced as a 49-kDa secreted protein that post-translationally cleaves to yield two mature proteins: an approximately 19-kDa N-terminal fragment that contains the signaling portion of the molecule and an approximately 27-kDa C-terminal fragment, which has auto-processing activity (Marti et al., 1995; Porter et al., 1995, 1996; Roelink et al., 1995). We found that expression of both the Shh-N protein and *shh* mRNA was significantly up-regulated by exposure to low-RA in day 4–6 EBs (Figs. 7A–C), but that further increasing the RA concentration ( $>10^{-7}$  M) induced their down-regulation instead. More specifically, the RA-responsive increase in Shh-N expression appeared to be concentration-dependent up to  $10^{-8}$  M, but was completely abrogated at  $10^{-7}$  M and higher concentrations. On the other hand, the peak level of full-length Shh protein expressed in response to exposure to  $10^{-8}$  M of RA was maintained even in EBs exposed to higher concentrations of RA. These results suggested that the ventralization of neural progenitors in EBs exposed to low-RA might be caused by an enhanced expression of Shh-N. However, we

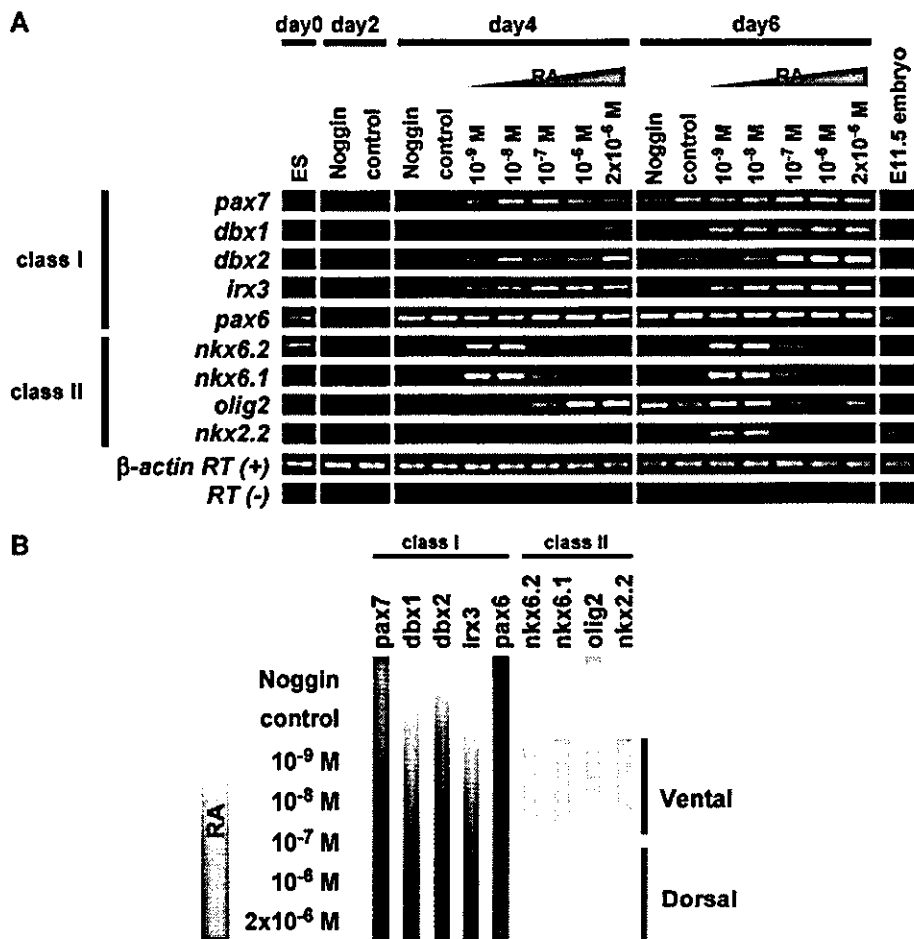


Fig. 6. Specification of the dorso-ventral identity of ES-cell-derived neural progenitors is regulated by RA. (A) RT-PCR analysis of class I and class II genes, which define dorso-ventral positional identity. The expression patterns are summarized in (B). EBs treated with low-RA ( $10^{-9}$ – $10^{-8}$  M) expressed both class I and II genes (class II > class I), which indicated that both ventral and dorsal neural progenitors had been induced, whereas EBs treated with high-RA ( $>10^{-6}$  M) expressed only class I gene, indicating dorsal neural progenitors had been induced.

could not rule out the possibility of the opposite causal relationship; that is, that low-RA induced enhanced expression of Shh-N protein and the expression of *shh* mRNA resulted from the ventralization of EB-derived cells that had been induced by low-RA treatment through an unknown mechanism. To address this issue, we treated EBs exposed to RA with recombinant Shh-N protein and cyclopamine, an inhibitor of Shh signaling (Chen et al., 2002a,b; Incardona et al., 1998). In the absence of cyclopamine treatment, EBs exposed to low-RA expressed both class I (*pax7*, *dbx1*, *dbx2*, *irx3*, and *pax6*) and class II genes (*nkx6.2*, *nkx6.1*, *olig2*, and *nkx2.2*), thereby indicating both dorsal and ventral phenotype. Treatment with 1  $\mu$ M cyclopamine strongly down-regulated the ventral class II genes (*nkx6.2*, *nkx6.1*, *olig2*, and *nkx2.2*) and some of the class I genes (*dbx1* and *dbx2*), indicating a dorsalized phenotype (Figs. 7D,E). In addition, exposure to 50 nM of recombinant Shh-N protein enhanced expression of class II genes (*nkx6.2*, *nkx6.1*, *olig2*, and *nkx2.2*) but reduced *pax7* expression. These effects were abrogated by treatment with 1  $\mu$ M cyclopamine (Figs.

7D,E). EBs treated with high-RA expressed higher levels of class I genes (*pax7*, *dbx1*, and *dbx2*, *irx3*, *pax6*), but lower levels of class II genes (*nkx6.2*, *nkx6.1*, *olig2*, and *nkx2.2*), thereby indicating a more dorsal phenotype than after low-RA treatment. However, high-RA treated EBs were ventralized by treatment with exogenous Shh-N, as shown by the up-regulation of class II genes (*nkx6.2*, *nkx6.1*, *olig2*, and *nkx2.2*) and down-regulation of *pax7*, and these changes were also abrogated by 1  $\mu$ M cyclopamine treatment (Figs. 7D,E).

This alteration of dorso-ventral identity by RA, Shh-N, and cyclopamine was confirmed by the immunostaining of dissociated EBs with antibodies against Pax7, Nkx6.1, and Nkx2.2 (Fig. 8). Virtually all the marker-positive cells also stained with the antibodies against Group B1 Sox or Nestin, indicating they are neural progenitor cells. It was noteworthy that Shh-N treatment could induce only Nkx6.1-positive but not Nkx2.2-positive neural progenitors in EBs treated with high-RA, indicating that the ventralmost neural progenitors could not be efficiently derived under such conditions, but that they were capable of increasing the

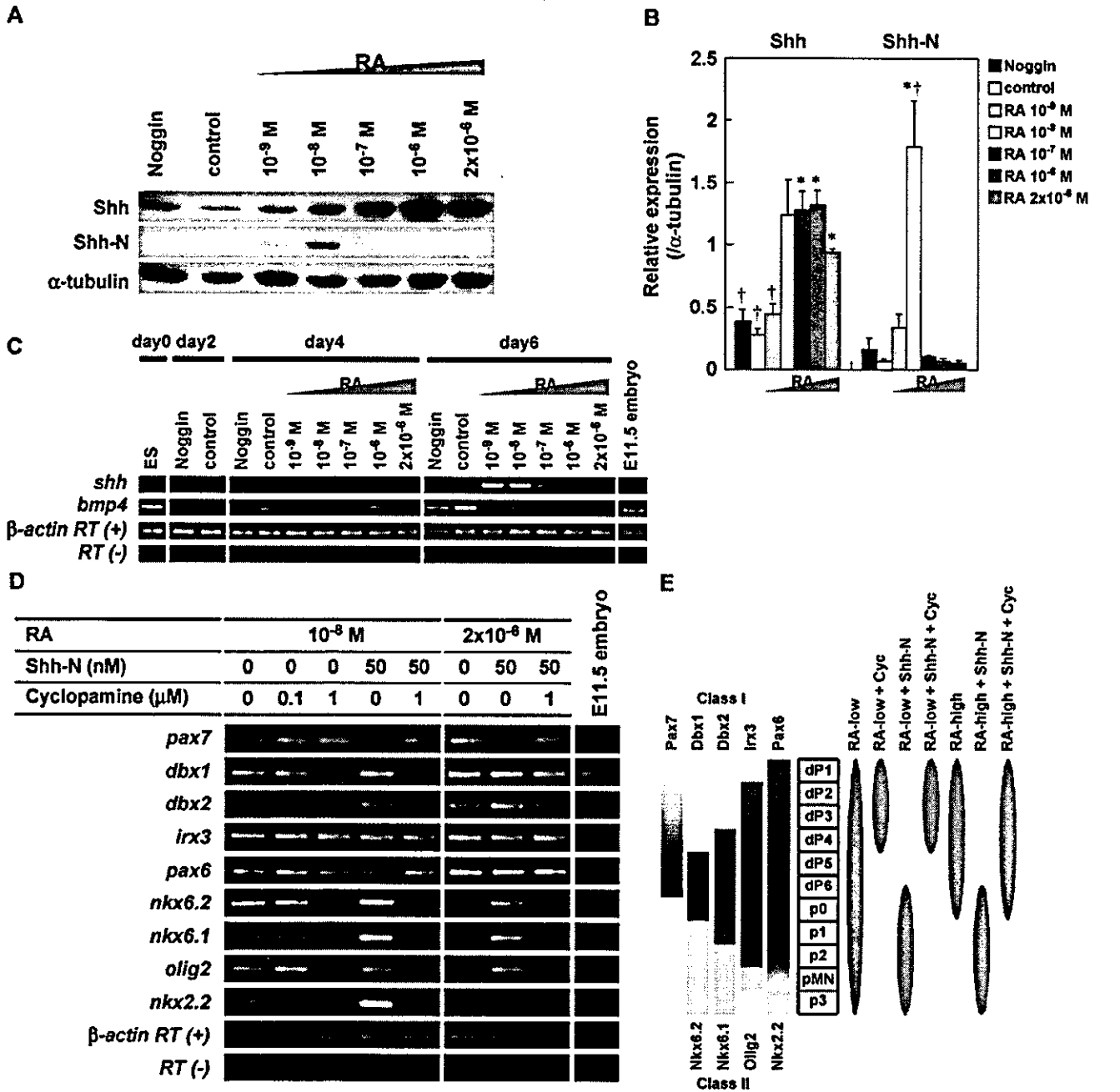
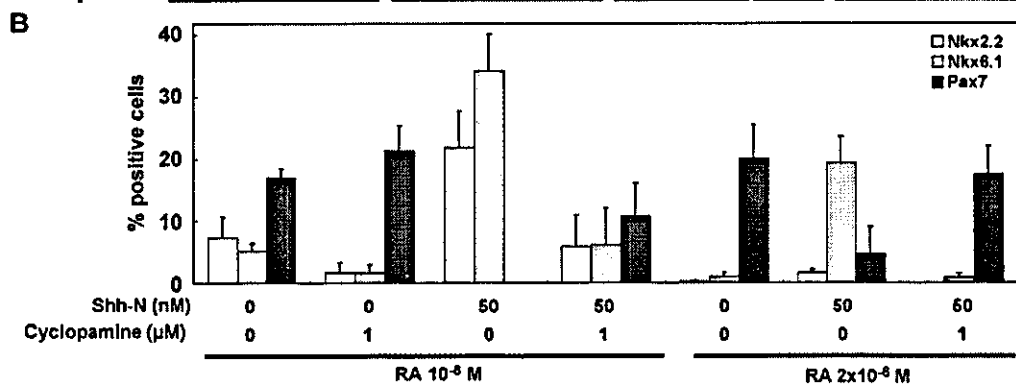
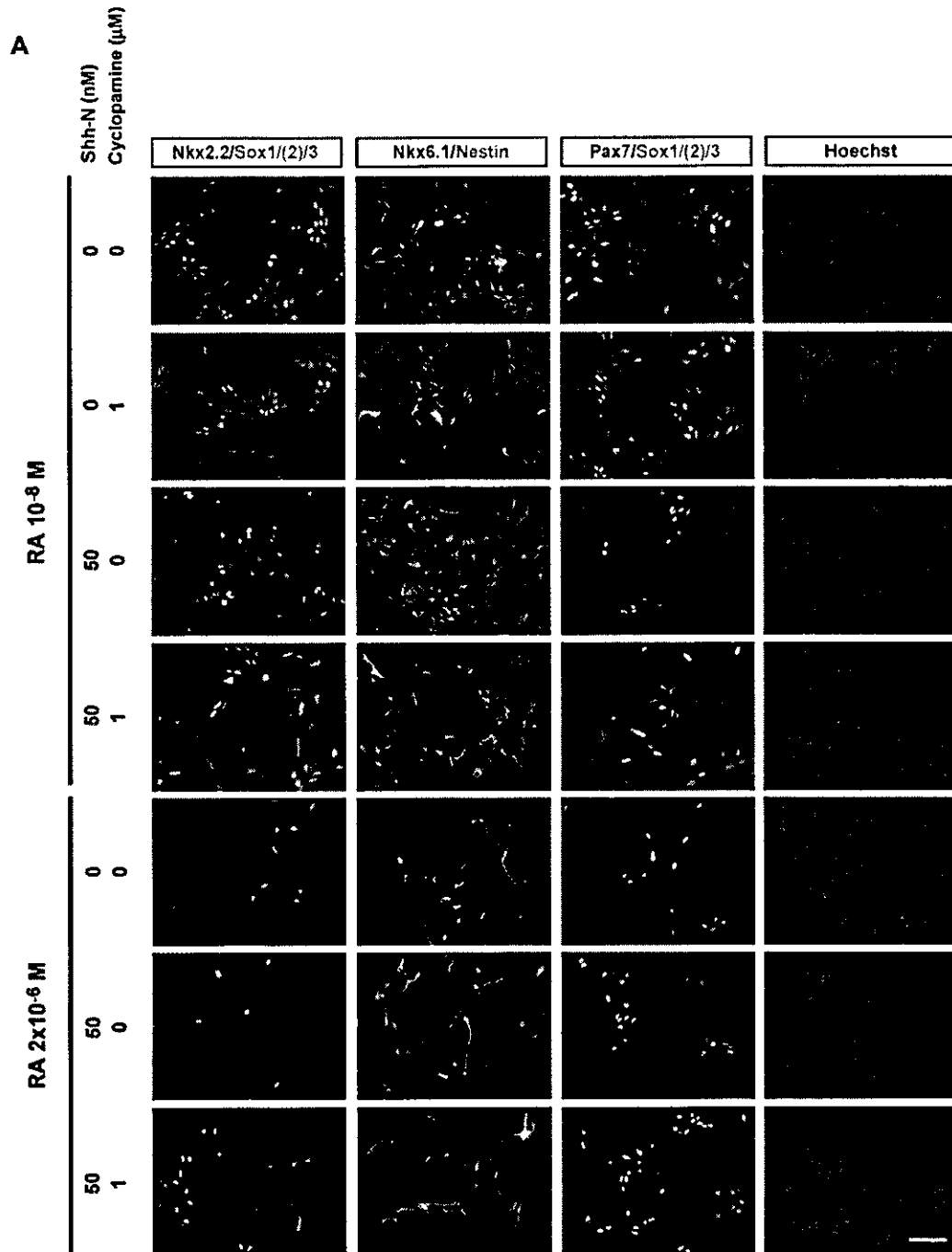


Fig. 7. Shh-N mediates RA-dependent dorso-ventral specification of ES-cell-derived neural progenitors. (A) Expression of Shh and its active N-terminal truncated form, Shh-N, in EBs cultured for 6 days, was analyzed by Western blotting. EBs were exposed to various concentrations of RA. Quantitative analysis was performed with Scion Image. The amounts of proteins were normalized to those of  $\alpha$ -tubulin (B) ( $n = 3$ , mean  $\pm$  SEM, \*,  $P < 0.05$  vs. control. †,  $P < 0.05$  vs. RA  $2 \times 10^{-6}$  M). (C) RT-PCR analysis of *shh* and *bmp4*. Shh-N was more highly expressed in EBs treated with low-RA ( $10^{-9}$  M– $10^{-8}$  M). (D) RT-PCR analysis of RA-exposed EBs treated with Shh-N and its inhibitor cyclopamine. Shh-N and cyclopamine were added together with RA on day 2. (E) Summary of expression patterns in vitro corresponding to in vivo. Cells from low-RA-treated EBs were a mixed population of dorsal-to-ventral neural progenitors and were capable of being dorsalized by inhibiting Shh signaling with cyclopamine. By contrast, exogenous Shh-N induced ventral neural progenitors were capable of being dorsalized by cyclopamine. Cells from high-RA-treated EBs showed dorsal positional identities. However, addition of Shh-N increased the number of ventral neural progenitors, and they were also capable of being dorsalized by cyclopamine treatment.

Fig. 8. Dorso-ventral specification of RA treated EBs is altered by Shh-N and cyclopamine. The alteration of dorso-ventral identity by RA, Shh-N, and cyclopamine was confirmed by the immunostaining of dissociated EBs with antibodies against Pax7 as a marker for dorsal neural progenitors, against Nkx6.1 as a marker for ventral neural progenitors, and against Nkx2.2 as a marker for ventralmost neural progenitors in combination with Group B1 Sox or Nestin as a marker for neural progenitors. Immunoreactive cells as a percentage of the total number of cells counted on the basis of the nuclear staining with hoechst33342 are shown in B ( $n = 3$ , mean  $\pm$  SEM). Scale bar: 50  $\mu$ m.



number of Nkx6.1- or Nkx2.2-positive ventral neural progenitors in EBs treated with low-RA. The observation in EBs treated with high-RA is consistent with the previous report (Wichterle et al., 2002).

One of the major dorsalizing molecules in the CNS, *bmp4* (Casparly and Anderson, 2003; Jessell, 2000; Knecht and Bronner-Fraser, 2002), was not very strongly affected by RA, Shh-N, or cyclopamine, suggesting a lesser contribution of the BMP signal to this RA-mediated dorso-ventral specification (Fig. 7C, data not shown). These results indicate that differentiating EBs were ventralized by low-RA through induction of endogenous Shh-N protein, and that the effect was abrogated by cyclopamine and enhanced by the addition of exogenous Shh signal (Figs. 7D,E and 8).

#### *Positional identity regulated by the RA concentration is mainly determined during the first 2 days of exposure to RA*

According to the RT-PCR analysis of EBs cultured according to the 2–/4+ protocol, the expression patterns of most of the regionally specific markers were determined by day 4 and maintained unchanged thereafter. This observation raised two possibilities. One possibility is that the first 2 days of exposure to RA are critical to the determination of positional identity, and the second is that the effect of the RA concentration was altered during the later culture period by degradation of RA. To determine which of these possibilities was true, we performed a RT-PCR analysis of EBs cultured according to other protocols in which the times when RA is added or the duration of exposure to RA (2–/2+/2–, 2–/2+/2+, and 4–/4+ protocols) is different from the 2–/4+ protocol (Suppl. Fig. 1). Expression of *oct3/4* had been maintained before the addition of RA, but no expression of other markers except *otx2*, whose mRNA was expressed even in undifferentiated ES cells, had been detected in any of the culture protocols, including the 4–/4+ protocol. The expression patterns of most of regionally specific markers were determined by day 4 of the 2–/2+/2– and 2–/2+/2+ or by day 6 of the 4–/4+ protocol, and were virtually the same in all protocols as observed in the 2–/4+ protocol, and they were maintained thereafter as well (Suppl. Fig. 2). Overall, positional identity is determined during the first 2 days of exposure to RA and is maintained thereafter regardless of the presence or absence of RA in later culture periods.

## Discussion

The pluripotent embryonic stem cell is a valuable in vitro model for studying the effects of various factors on cell lineage decisions in very early embryonic stages of mammalian development, and the effect of RA signaling on the differentiation of ES cells and neural induction, in particular, has been extensively studied. In addition to previous reports showing that RA promotes neural differ-

entiation of ES cells and caudalization of the positional identity of their progeny (Bain et al., 1995, 1996; Fraichard et al., 1995; Gajovic et al., 1997; Renoncourt et al., 1998; Strubing et al., 1995; Wichterle et al., 2002), the results of the present study demonstrate the novel and precise actions of RA on neural differentiation and acquisition of positional identity by ES-cell-derived neural cells.

#### *Effect of the concentration of RA on ES cell differentiation*

It is well known that exposure of growing EBs to high-RA markedly increases the rate of neural differentiation, whereas low-RA induces more mesodermal cells (Rohwedel et al., 1999). Higher concentrations of RA also promote faster differentiation of ES cells, as indicated by the pattern of *oct3/4* expression, which was down-regulated more rapidly in EBs exposed to higher concentrations of RA, and down-regulated more at day 6 than at day 4 (Fig. 2). The result of this study showed that RA also concentration-dependently facilitates terminal differentiation of neural cells derived from ES cells. The expression levels of markers of differentiated neurons and glia, i.e., of  $\beta$ III-tubulin and GFAP, respectively, was higher in EBs treated with higher concentrations of RA, whereas the expression levels of markers of undifferentiated neural cells, i.e., of Nestin, Group B1 Sox, Olig2, and *sox1* mRNA, was inversely correlated with the concentration of RA (Figs. 2 and 3). The findings are consistent with a high-RA enhancing differentiation of neural progenitor cells, as described previously (Bain et al., 1995, 1996; Fraichard et al., 1995; Gajovic et al., 1997; Renoncourt et al., 1998; Strubing et al., 1995; Wichterle et al., 2002).

Down-regulation of Wnt signaling has been shown to be one of the mechanisms involved in RA-induced neural differentiation of mouse ES cells (Aubert et al., 2002). Interestingly,  $\beta$ -catenin, which is a key molecule in Wnt signaling, has been shown to interact directly with retinoid receptor RAR, but not with RXR, in a retinoid-dependent manner, and as a result retinoids decrease  $\beta$ -catenin-Lef/Tcf-mediated transactivation in cultured cells in a dose-dependent manner (Easwaran et al., 1999). Wnt3a signaling through Lef/Tcf1 has also been implicated in suppression of neural differentiation and induction of mesodermal differentiation in the mouse embryo (Galceran et al., 1999; Yamaguchi et al., 1999; Yoshikawa et al., 1997). These findings raise the possibility that the one of the effects of RA in EBs is to inhibit the Wnt- $\beta$ -catenin anti-neural pathway by up-regulation of Secreted frizzled-related protein 2 (Sfrp2) (Aubert et al., 2002) and/or sequestration of  $\beta$ -catenin in a concentration-dependent manner, thereby resulting in the promotion of neural differentiation, and inversely in the suppression of mesodermal differentiation.

FGFs are another molecules that may be involved in the neurogenesis related to RA signaling. RA has been shown to promote neuronal differentiation by repressing FGF signalings from the posterior neural plate. Caudal FGF signalings

have the opposite effects and repress *Raldh2* (RA synthesis) in the presomatic mesoderm and generic neuronal differentiation in chick early neural tube (Diez del Corral et al., 2003; Novitch et al., 2003). These observations raise the possibility that RA inhibits the action of endogenously generated FGFs in a concentration-dependent manner during the culture of EBs. Further study of the associations between these signals is required to clarify the mechanism underlying the RA-promoted neural differentiation of ES cells.

#### *Acquisition of rostro-caudal identity depends on the concentration of RA*

A previous study on chick embryos showed that the default identity of early neural tissue is a rostral location and that neural cells can be caudalized by exogenous factors, such as the caudalizing activity of paraxial mesoderm, FGFs, and retinoid from the mesoderm, which induce midbrain, hindbrain, and spinal cord characters, when applied during the appropriate period of development (Muhur et al., 1999).

RA is one of the factors, that has been shown to be involved in hindbrain patterning and the caudalization of neural tissues in the early embryonic CNS *in vivo* (Maden, 2002). The distribution of endogenous RA has been examined in mouse and chick embryos, by various methods, including HPLC (high-performance liquid chromatography) (Horton and Maden, 1995; Maden et al., 1998), the use of *LacZ* reporter cells (Maden et al., 1998; Wagner et al., 1992), and the use of *RAR $\beta_2$ -LacZ* transgenic mice (Reynolds et al., 1991; Zimmer, 1992). This distribution of endogenous RA is correlated with the opposing action of the two main enzymes involved in RA-metabolism, RA-synthesizing enzyme, *Raldh2*, which is most strongly expressed in the paraxial mesoderm adjacent to the rostral spinal cord with the rostral boundary of the presumptive first somite (Berggren et al., 1999), and the catabolizing enzyme, *Cyp26a1*, which is expressed in anterior neuroepithelium. These spatially distributed enzymes create a rostro-caudal RA concentration gradient *in vivo* (Abu-Abed et al., 2001; Fujii et al., 1997; Maden et al., 1998; Sakai et al., 2001; Swindell et al., 1999), with the peak RA concentration occurring at the hindbrain/spinal cord boundary, with levels gradually decreasing anterior and posterior to it. Furthermore, it has been suggested that the patterning of the rhombomere is influenced over time by the constant supply of RA from the paraxial mesoderm, where the neuroepithelium grows and moves away from this source of RA. These findings imply that the more posterior rhombomeres that develop later than the more anterior rhombomeres may have been exposed to higher concentration of RA, leading to the expression of more posterior genes (such as posterior *hox* genes), which require a higher concentration of RA for activation *in vivo* (Maden, 2002). Our findings are consistent with the above-described putative regulatory mechanism of hindbrain/rostral spinal cord positional

specification correlated with the RA concentration gradient *in vivo* in the following manner. The default positional identity of ES-cell-derived neural cells is specified as anteriormost forebrain, which was acquired in the control and Noggin-exposed EBs. EBs treated with low-RA were specified as midbrain to hindbrain, which is generated earlier and require lower concentrations of RA *in vivo*, whereas EBs treated with high-RA were specified as posterior hindbrain to rostral spinal cord, which is generated later and requires higher concentrations of RA *in vivo*. In addition, the fact that even the EBs treated with high-RA expressed genes specific to rostral (*hoxc4* to *hoxc6*), but not to caudal spinal cord (*hoxc8* to *hoxc10*) is consistent with the putative gradient of endogenous RA *in vivo* with a higher concentration in the rostral spinal cord, and the proposed role of RA in rostral spinal cord determination (Liu et al., 2001). Moreover, other factors may be involved in the activation of RA-responsive genes and the specification of positional identity, such as RA binding proteins, including cellular retinoic acid binding protein (CRABP) 1, which limits the access of RA to the nuclear retinoid receptors. The spatiotemporal pattern of expression of CRABP1 suggests that the fine regional control of availability of RA to the nuclear receptors may also play an important role in the organization of the central nervous system and the differentiation of its progenitors *in vivo* (Leonard et al., 1995; Maden, 2001; Maden et al., 1992; Ruberte et al., 1993). The role of these RA binding proteins in the regulation of *in vitro* differentiation of ES-cell-derived neural cells should be investigated further in the future.

#### *RA also affects dorso-ventral positional identity*

In contrast to the acquisition of rostro-caudal identity, dorso-ventral identity was analyzed in terms of expression of the transcriptional control of the homeodomain (HD) and basic helix-loop-helix (bHLH) proteins. Previous studies have emphasized the role of *Shh* signaling in establishing the pattern of expression of ventral spinal cord patterning genes (Jessell, 2000). RA has also been reported to contribute to the ventral patterning of the spinal cord; that is, to the induction of ventral interneurons (V0 and V1) by inducing class I genes, including *Dbx1*, *Dbx2*, *Evx1*, *Evx2*, and *En* (Pierani et al., 1999), and to the specification of limb level motor neuron subtypes by the expression of *Raldh2* in LMC (Sockanathan and Jessell, 1998). Furthermore, recent studies have revealed involvement of RA from the paraxial mesoderm in the timing of neurogenesis and the patterning of the ventral spinal cord regulating the expression of class I and class II genes via inhibition of FGF signals and in combination with *Shh* signals (Diez del Corral et al., 2003; Novitch et al., 2003). However, the results of our study showed that the concentration of RA to which EBs were exposed was critical for acquisition of dorso-ventral identity by differentiating ES cells, and the concentration dependency showed a bell-

shaped pattern. This was shown by the pattern of the expression of class I and class II genes (Figs. 6A,B), which determines the dorso-ventral progenitor domains of developing hindbrain and spinal cord. EBs exposed to high-RA exhibit mainly dorsal phenotypes, whereas EBs exposed to low-RA exhibit more ventral phenotypes (Figs. 6–8). The expression pattern of *olig2*, higher at day 4 in EBs treated with high-RA and at day 6 in those treated with low-RA, seems to conflict with this finding; however, there are several possible explanations. One is that this alteration of the expression pattern of *olig2* mimics that in vivo according to the stage of development, with expression in most of the undifferentiated neural/glia progenitor cells in the ventral half of the spinal cord occurring around the period of neural tube closure and later being restricted to the motor neuron domain (pMN domain) of the ventral ventricular region, where the progenitors of motor neurons and oligodendrocytes arise sequentially (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2001). Thus, both the cells collected at day 4 from EBs exposed to high-RA and those collected at day 6 from EBs exposed to low-RA may consist of multipotent neural progenitors expressing *Olig2*. Furthermore, the role of RA in motor neuron development, such as its effect on the expression of bHLH and HD transcription factors, including *Olig2*, varies with the stage of development, according to a previous study that analyzed chick spinal cord development (Novitsch et al., 2003). Similar alteration of the effects of RA may occur in our culture system and be another possible explanation for the sequential expression pattern of *olig2* in EBs exposed to high-RA.

The ventralization of EBs treated with low-RA can be explained by the finding that the active form of Shh-N, which is secreted by the notochord and floor plate of the developing CNS and ventralizes gene expression of neural progenitors in a concentration-dependent manner in vivo (Jessell, 2000), is more highly expressed on day 6 in EBs treated with low-RA (Figs. 7A–C). The hypothesis that the concentration-dependent activity of RA that defines dorso-ventral identity is mediated by Shh-N was confirmed by the result of treatment with the inhibitor of Shh signaling, cyclopamine (Figs. 7D,E and 8) (Chen et al., 2002a,b; Incardona et al., 1998). Cyclopamine abrogated the ventralization activity of low-RA treatment, and addition of exogenous Shh-N more efficiently ventralized differentiating EB-derived cells that had been exposed to both low-RA and high-RA, in a cyclopamine-sensitive manner. The expression level of *bmp4*, which dorsalizes neural progenitor cells (Caspary and Anderson, 2003; Jessell, 2000; Knecht and Bronner-Fraser, 2002), was not very strongly affected by Shh-N or cyclopamine in EBs exposed to either low-RA or high-RA (Fig. 7C, data not shown), indicating that BMP signaling is not the major contributor to RA-mediated dorso-ventral specification. Taken together, these findings suggest that Shh-N expressed in EBs exposed to low-RA may be one of the major signals that ventralize neural progenitors and induce

expression of class II genes in addition to class I genes, and that the lack of the shh signal in EBs exposed to high-RA may result in expression of only class I genes and a more dorsalized phenotype, which can be ventralized by exogenous Shh-N protein. The results of this study are consistent with a previous report that RA enhances expression of class I genes, but not of class II genes, in developing chick spinal cord (Diez del Corral et al., 2003; Novitsch et al., 2003), and that exogenous Shh-N is required in addition to high-RA for efficient generation of motor neurons during EB formation in vitro (Renoncourt et al., 1998; Wichterle et al., 2002). Because EBs exposed to low-RA are mixed populations and contain many mesodermal cells (Fig. 2) (Rohwedel et al., 1999), they may secrete larger amounts of Shh-N than EBs treated with high-RA, which contain smaller proportions of mesodermal cells.

The discrepancy in response to RA between full-length Shh expression and Shh-N expressions detected by Western blotting (Figs. 7A,B) may be another important finding in this study. In contrast to the expression of full-length Shh being observed in EBs treated with RA at concentration  $10^{-8}$  M and above, generation of Shh-N was detected only in EBs exposed to lower concentrations of RA (Figs. 7A,B), indicating the possible existence of RA-dependent machinery controlling Shh-N production by modulating an auto-processing mechanism by the C-terminus of Shh, which processes full-length Shh into the N-terminus active form, or by altering degradation activity of Shh-N.

Use of mutant ES cells for *indian hedgehog* (*ihh*) and *smoothed* (*smo*) has shown that hedgehog signaling is also required for neural differentiation of mouse ES cells by RA (Maye et al., 2004). In our study, however, expression of Group B1 *Sox* and *sox1* mRNA in EBs treated with low- or high-RA and their dissociates were not down-regulated by cyclopamine (Fig. 8, data not shown), indicating that neural differentiation was not inhibited under our culture conditions even in the presence of cyclopamine. There are two possible explanations for this discrepancy. In our experiments, cyclopamine was added on day 2 after the start of ES cells differentiation, whereas in the mutant ES cells in which hedgehog signaling was disrupted it was disrupted at the start of differentiation, raising the possibility that hedgehog signaling may be one of the factors that is required for the initial commitment of neuroectodermal differentiation. The other possibility is that the concentration of cyclopamine used in our study may not have been adequate to completely block hedgehog signaling, and the residual signaling activity may have been sufficient for the transition of ES-cell-derived ectoderm into neuroectoderm, but not for the ventralization of neural cells.

The mechanism underlying these roles of hedgehog signals in differentiation and specification of ES-cell-derived neural cells needs to be elucidated in the future.



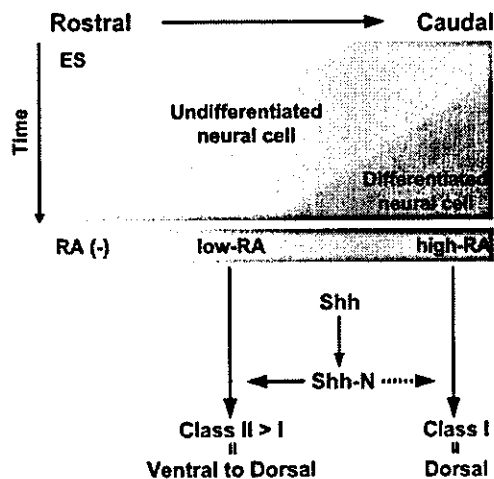


Fig 9. Schematic presentation of the concentration-dependent effects of RA on neural differentiation by mouse ES cells. RA simultaneously promotes both neural differentiation and caudalization in concentration-dependent manner. Low-RA induces a higher level of Shh-N, which endows ES-cell-derived neural progenitors with ventral identity, whereas high-RA poorly induces Shh-N, and they acquire dorsal neural identity instead.

RA is one of the most important inductive signals in vertebrate ontogeny and can be used to induce neural differentiation of mouse ES cells *in vitro*. However, its actions are complicated and difficult to deal with at will, because RA has the ability to induce various types of cells depending on its concentration, and it simultaneously affects both the timing of differentiation and the acquisition of positional identity, including rostro-caudal and dorso-ventral identity during neural differentiation (Fig. 9). Separation of these two phenomena is desirable to investigate the underlying mechanisms, and separation may have been accomplished, in part, by using SDIA, which is a culture protocol that induces neural cells without RA treatment. Thus, previous studies have shown involvement of RA at a single concentration in the caudalization of ES-cell-derived neural cells (Mizuseki et al., 2003; Wichterle et al., 2002). However, it is still not easy to separate these two phenomena completely during the neural induction of ES cells, because they are simultaneously affected by RA *in vivo* in combination with other signals, such as FGF and Shh signals, as shown by previous studies (Appel and Eisen, 2003; Diez del Corral et al., 2003; Novitch et al., 2003).

The present study identified detailed gene expression profiles and clarified the effects of the concentration of RA on ES cell differentiation, neuralization, and positional specification, though it may be impossible to map the patterns of expressions of the regional specific markers observed in ES-cell-derived neural cells directly to parallel expression of the markers *in vivo*. In combination with the RA-independent neural induction method using Noggin, this information will enable us to establish a strategy that will allow control of both the differentiation and the positional identity of neural cells

derived from mouse ES cells through EB formation *in vitro*, and it may be applicable to human ES cells, raising the possibility of application to the treatment of neurological diseases.

## Acknowledgments

We are grateful to Dr. H. Niwa for kindly providing ES cell line EB3, Dr. M. Nakafuku for the anti-Olig2 antibody, Dr. J.-F. Brunet for the anti-Phox2b antibody, Dr. O.D. Madsen and Dr. H. Duus for the anti-Nkx6.1 antibody, Dr. H. Kondoh for the anti-GroupB1 Sox antibody, Dr. Y. Takahashi for the xNoggin/BOS plasmid, J. Kohyama, S. Yuasa, and M. Yano for their thoughtful advice, and S. Nakamura for technical assistance. This work was supported by grants from CREST, Japan Society for the Promotion of Science to H.O.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.07.038.

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