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Sox6 overexpression causes cellular aggregation and the neuronal differentiation of P19 embryonic carcinoma cells in the absence of retinoic acid

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Abstract The Sox6 gene is a member of the Sox gene family that encodes transcription factors. Previous studies have suggested that Sox6 plays an important role in the development of the central nervous system. Aggregation of embryonic carcinoma P19 cells with retinoic acid (RA) results in the development of neurons, glia and fibroblast-like cells. In this report, we have shown that Sox6 mRNA increased rapidly in P19 cells during RA induction and then decreased during the differentiation of P19 into neuronal cells. To explore the possible roles of Sox6 during this process, stably Sox6-overexpressing P19 cell lines (P19[Sox6]) were established. These P19[Sox6] had acquired both characteristics of the wild-type P19 induced by RA. First, P19[Sox6] cells showed a marked cellular aggregation in the absence of RA. Second, P19[Sox6] could differentiate into microtubule-associated protein 2 (MAP2)-expressing neuronal cells in the absence of RA. Sox6 expression could cause the activation of endogenous genes including the neuronal transcription factor Mash-1, the neuronal development-related gene Wnt-1, the neuron-specific cell adhesion molecule N-cadherin, and the neuron-specific protein MAP2, resulting in neurogenesis. Moreover, E-cadherin, a major cell adhesion molecule of wild-type P19, was strongly induced by Sox6, resulting in cellular aggregation without RA. Thus Sox6 may play a critical role in cellular aggregation and neuronal differentiation of P19 cells.

Key words: Sox6; Neuronal differentiation; Embryonal carcinoma

1. Introduction

The cloning of the mammalian sex-determining gene, SRY, has led to the identification of a large number of related genes [1–6]. These called Sox (Sry-related HMG box) genes are characterized by the presence of a DNA-binding domain, the HMG box (high mobility group) [1,2,7–9]. The HMG box is an 80 amino acid motif, which mediates sequence-specific DNA binding. Sox genes encode a group of proteins implicated in transcriptional regulation. Sox genes are expressed in various phases of embryonic development and cell differentiation in a cell-specific manner. Sox proteins have been found throughout the animal kingdom and are involved in the regulation of developmental processes such as sex determination, sex differentiation, neurogenesis, chondrogenesis, and thymocyte differentiation [2,5,10–12]. Some Sox proteins, including Sox1, Sox2, Sox3, Sox6, Sox9 and Sox11, are expressed in the central nervous system (CNS) during embryogenesis.

Sox2 and 3 are mainly expressed in the developing CNS [7]. In the adult mouse, spermatogenesis is associated with the expression of Sox5 and Sox6, suggesting an overlapping function of these genes in the testis [13,14]. Sox6 was estimated to play a role in the developing nervous system because it is specifically expressed in the nervous system at the initial stage (9.5–12.5 days post-coitum) in the embryos. Sox6 was initially isolated from an adult mouse testis cDNA library. We isolated a cDNA encoding a rat homologue of the previously characterized mouse Sox6 [15]. Recently, a Sox6 null mutant has been identified in the mouse [16]. The result of study with the Sox6 null mutant showed that the Sox6 protein is likely to be involved in maintaining the normal physiological function of muscle tissue, including the heart [16]. However, the role of Sox6 is still unknown in the development of the CNS.

In this study, we investigated the possible role of Sox6 in neuronal cell differentiation of murine P19 embryonic carcinoma (EC) cells [17]. For the induction of neuronal differentiation, P19 cells were allowed to aggregate for 4 days in the presence of retinoic acid (RA) and were replated for 5–7 days without RA. P19 cells can also differentiate into mesodermal and endodermal cells with dimethylsulfoxide (DMSO) stimulation and without any inducer, respectively [18]. Here, we demonstrate that RA-induced P19 cells express Sox6, and constitutive overexpression of Sox6 in P19 cells directs the cells to differentiate into neuronal cells and undergo cellular aggregation without RA.

2. Materials and methods

2.1. Tissue culture

The P19 EC cell line was purchased from American Type Culture Collection. The cells were maintained in α-modified minimum essential medium supplemented with 2.5% fetal bovine serum and 7.5% calf serum. For the induction of neuronal cells, aggregate formation was

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Abbreviations: CNS, central nervous system; RA, retinoic acid; MAP2, microtubule-associated protein 2; HMG, high mobility group

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performed in the presence of 5×10^{-7} M RA and initiation of the neuronal differentiation was carried out by replating into a monolayer culture on polyornithine-coated dishes in the absence of RA. Aggregation of the P19 cells was done by culturing the cells on bacterial grade dishes (Iwaki) for 4 days (P19/RA). Subsequently, the aggregants were replated on polyornithine-coated tissue culture dishes for 5–7 days (P19/NEU).

2.2. Plasmid DNAs and transfection

Sox6 cDNA was kindly provided by Dr. Shinya Yamashita (Nippon Suisan Kaisha, Tokyo, Japan). To generate an expression construct for Sox6, a Sox6 *Eco*RI/*Bsa*AI fragment was inserted in pcDNA3.1 myc/his B vector (Invitrogen, San Diego, CA, USA). Stable transfectants were obtained using the electroporation method, followed by selection with 500 mg/l G418 (Nakalai, Kyoto, Japan) for 14 days. The colonies were selected and clones were established by limiting dilution.

2.3. Reverse transcription polymerase chain reaction (RT-PCR) analysis

RT-PCR was performed using MulV RNA polymerase (Perkin Elmer) and Taq polymerase (Perkin Elmer) as described in the manufacturer's manual. The first strand cDNA was synthesized using oligo(dT) primer. PCR amplification was performed using the synthesized cDNA as a template as previously described [19]. For the internal control, the synthesized cDNA was amplified with glyceraldehyde-3-phosphate dehydrogenase (G3PDH)-specific primers. The oligonucleotides used were as follows: Sox6, 5′-TACAGCAGCACAAGATTA-3′ annealed to 5′-CGTGTTCCTTCTCAGT-3′; G3PDH, TGAAGGTCGGTGTGAACGGATTTGGC-3′ annealed to 5′-CATGTAGGCCATGAGGTCCACCAC-3′.

2.4. Cell counting

The cells (10^6) were cultured for 2 or 4 days after replating. P19 cells, Sox6 transfectants and LacZ transfectants were cultured with usual media without RA on culture grade dishes. P19 cells were cultured with 5×10^{-7} M RA on bacterial grade dishes (P19/RA). P19 cells during neuronal differentiation (P19/NEU) were cultured with usual media on 0.1% polyornithine-coated dishes after preculture with RA for 4 days on bacterial grade dishes. After the cells were dispersed using 0.025% trypsin and 1 mM EDTA, the cells were counted.

2.5. Immunocytochemistry

The cells were grown on 0.1% polyornithine-coated coverslips and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). Subsequently, the cells were dehydrated with 5% acetic acid in methanol, washed three times with Tris-buffered saline containing 0.05% Tween 20 (TTBS), permeabilized with Triton X-100/Tris-buffered saline. The cells were incubated for 1 h at 37°C with monoclonal microtubule-associated-protein 2 (MAP2) antibodies (1:500) (Amersham) or normal mouse serum, washed three times with TTBS and incubated with biotinylated sheep anti-mouse IgG (Amersham) as the second antibody (1:1000) for 1 h at room temperature. Following an additional rinse, the samples were incubated for 30 min at room temperature with streptavidin POD (1:1000) (Boehringer Mannheim), washed three times with TTBS, and mounted in color developer (Lipshow).

The cells were cultured for 3 days after replating, dispersed using 0.025% trypsin and 1 mM EDTA, and fixed with 4% paraformaldehyde in PBS. The cells were stained with anti-MAP2 antibody and the stained cells counted.

2.6. Immunofluorescence

The cells were grown for 1 day on 0.1% polyornithine-coated coverslips and fixed with 4% paraformaldehyde in PBS. Subsequently, the cells were washed with PBS, permeabilized with 0.2% Triton X-100 in PBS for 5 min, washed again in PBS and incubated with 0.1% sodium borohydride in PBS. After washing in PBS containing 1% bovine serum albumin twice, the cells were incubated with anti-myc antibody (1:200) (Invitrogen) or normal mouse serum and then cells were washed three times with TTBS and incubated with Alexa 546-conjugated goat anti-mouse IgG (1:1000) (Molecular Probes).

2.7. Flow cytometry

The cells were dissociated with 0.025% trypsin and 1 mM EDTA in PBS, 70% ethanol fixed at −20°C for 4 h, cytoplasmic membrane removed by 0.1% Triton X-100 in PBS for 10 min on ice and stained with 100 mg/l of 4′,6-diamino-2-phenylindole dihydrochloride/100 mg/l of RNase A in PBS for 30 min at room temperature. Samples were analyzed for DNA content on a Cell Counter Analyzer CCA (Partec).

3. Results and discussion

3.1. The change in Sox6 mRNA during neuronal differentiation of P19 cells

P19 cells are known to differentiate into neuronal cells by the two elements of RA stimulation and cellular aggregation [17,18]. The expression of Sox6 was determined during the neuronal differentiation of P19 cells as shown in Fig. 1. The

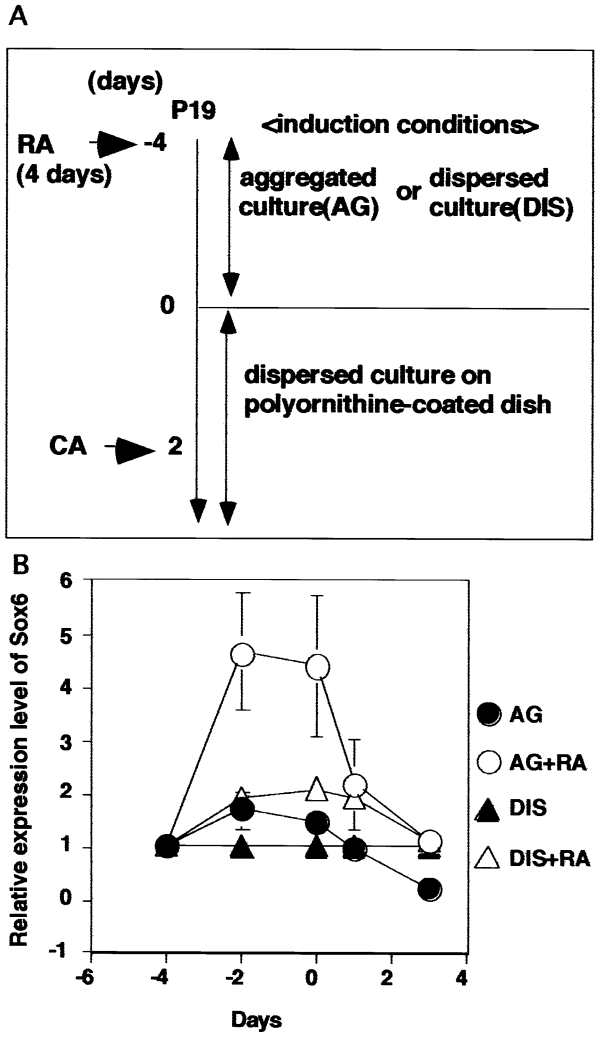

Fig. 1. Change in Sox6 mRNA in the differentiation of P19 cells into neuronal cells in response to RA with or without cellular aggregation. A: Culture conditions for P19 cells. P19 cells were cultured in the presence or absence of RA either on bacterial grade dishes as an aggregated culture or on tissue culture dishes as a dispersed culture. B: Sox6 mRNA levels in P19 cells. The Sox6 mRNA level in cultured P19 cells was determined by RT-PCR. To normalize for sample loading, the ratio of the quantitative detection of each Sox6 band to the corresponding G3PDH band was taken. Each bar represents the mean ± S.E.M. of three experiments in each group. ○: aggregated with RA; ●: aggregated without RA; △: dispersed with RA; ▲: dispersed without RA (non-induction).

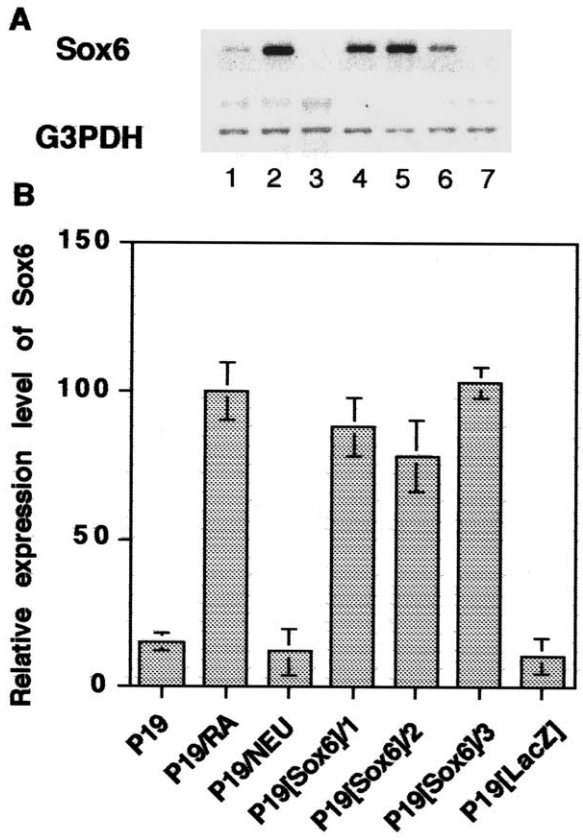

Fig. 2. The expression of Sox6 mRNA in P19 cells during RA-induced neuronal differentiation and Sox6-transfected P19 cells. Aggregated P19 cells were cultured with RA for 4 days (P19/RA), replated and cultured on polyornithine-coated dishes for 3 days (P19/NEU). Sox6- or LacZ-transfected P19 cells were harvested on culture grade dishes for 3 days after replating for RT-PCR. A: Gene expression of Sox6 was determined by RT-PCR in RA-induced P19 cells, three clones of Sox6-transfected P19 cells, and LacZ-transfected P19 cells. Lane 1, wild-type P19 cells; lane 2, P19/RA; lane 3, P19/NEU;, lanes 4–6, P19[Sox6]; lane 7, P19[LacZ]. B: Relative levels of Sox6 mRNA expression using NIH image.

cells were cultured for 4 days under four conditions with or without RA on either bacterial grade or tissue culture dishes. Subsequently, the cells were cultured under the same conditions for differentiation into neuronal cells. The Sox6 mRNA expression increased markedly during cellular aggregation with RA, and then decreased during the neuronal differentiation, while it increased slightly without cellular aggregation and with RA. Regardless of the aggregation of the cells, the expression of Sox6 was almost unchanged when RA was not present.

P19 cells could not differentiate into neuronal cells in the absence of RA, and although P19 cells differentiated into neuronal cells when dispersed with RA, the frequency of the differentiation into neuronal cells was about a third of that when aggregated with RA (data not shown).

3.2. *Effect of ectopic Sox6 expression on the neuronal differentiation of P19 cells*

To examine whether Sox6 expression is necessary for the neuronal differentiation of P19 cells, we isolated the clone of P19 cells that stably expressed the Sox6 gene. A cytomegalovirus promoter-driven expression vector for Sox6 was constructed by insertion of Sox6 open reading frame into the mammalian expression vector pcDNA3.1/myc/his. After G418 selection for 2 weeks, 86 clones were isolated by the limiting dilution method. The level of Sox6 mRNA expression in three clones with medium expression level of the gene in the isolated clones is shown in Fig. 2. The transfectants expressed 5–10-fold Sox6 mRNA in comparison with wild-type P19 and the P19 cells transfected with LacZ (P19[LacZ]). The level of Sox6 mRNA expression in three clones was the same as that in the P19 cells induced with RA (P19/RA). To detect the recombinant Sox6 protein, the cells were immunofluorescently stained with anti-myc antibody. Almost all the cells were stained strongly with anti-myc antibody (data not shown). The results of the immunofluorescent study suggest that recombinant Sox6 protein was produced in P19[Sox6].

3.3. *Sox6 overexpression causes cellular aggregation and neuronal differentiation in P19 cells*

P19 cells differentiate into neuroectodermal lineages when aggregated with RA, but not when aggregated with DMSO or in the absence of RA. To examine whether Sox6 can induce neurogenesis in the absence of RA when overexpressed in P19 cells, Sox6 transfectants were morphologically observed.

Sox6 transfectants began cellular aggregation on culture grade dishes 2 days after replating (Fig. 3B). Furthermore, they began extension of neurite-like structures at 3 or 4 days after replating without RA (Fig. 3C) and then the number and length of neurite-like structures increased and they

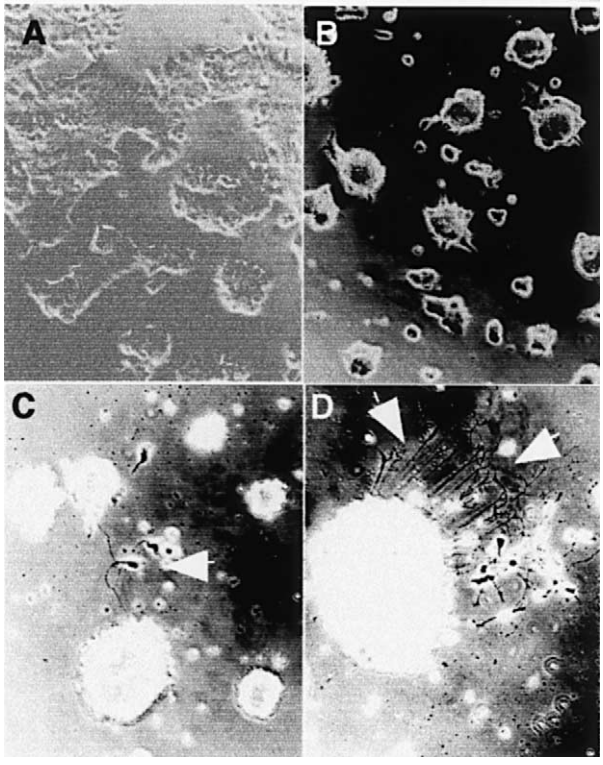

Fig. 3. Morphology of Sox6-overexpressed P19 cells during neuronal differentiation. P19[LacZ] cells were cultured for 2 days on polyornithine-coated coverslips without RA (A). P19[Sox6] cells were cultured for 2 days (B), 4 days (C), and 6 days (D) on polyornithine-coated coverslips without RA and observed morphologically with a phase contrast microscope. Arrows indicated the neurite-like structures.

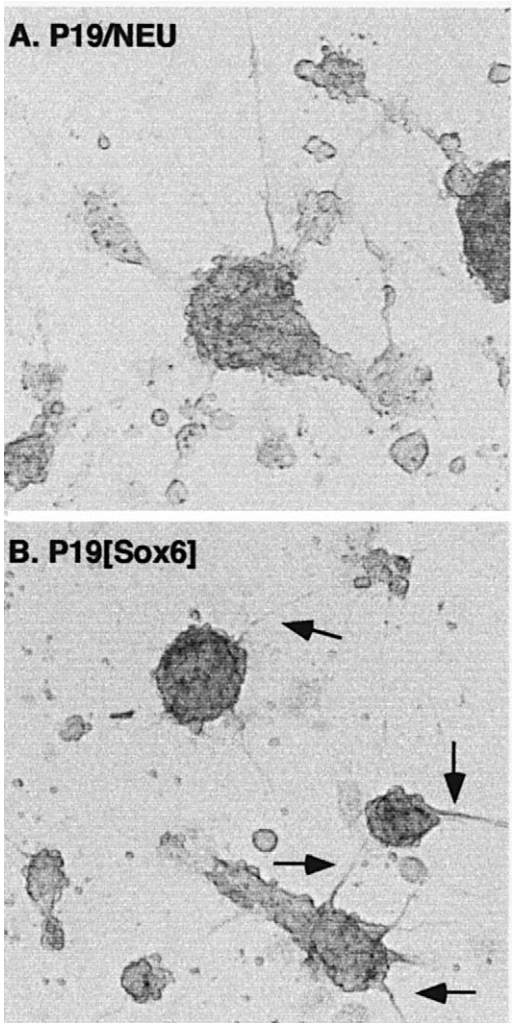

Fig. 4. Immunostaining of Sox6-transfected P19 cells with anti-MAP2 antibody. P19 cells were cultured for 4 days as aggregates with RA and then cultured for 5 days on polyornithine-coated coverslips without RA, and then the cells were stained with anti-MAP2 antibody (A) as described in Section 2. P19[Sox6] cells were cultured for 5 days on polyornithine-coated coverslips without RA, and then P19[Sox6] cells were fixed for immunostaining with anti-MAP2 antibody (B). Arrows indicated staining neurites.

connected to each other at 6 days after replating (Fig. 3D), while wild-type P19 cells and LacZ transfectants were adherent on the dish, and they maintained epidermal-like morphology (Fig. 3A).

To examine whether the neurite-like structures of Sox6 transfectants have the same characteristics as the neurites, Sox6 transfectants were stained using MAP2 antibody specific to the neurite. The neurite-like structures of P19[Sox6] cells were stained with MAP2 antibody to the same extent as those of P19/NEU cells (Fig. 4).

About 60% of P19[Sox6] cells were MAP2-positive at 3 days after replating (Fig. 5). Most P19/RA cells and P19[LacZ] cells were MAP2-negative, like the P19 cells, and about 90% of P19/NEU cells were MAP2-positive cells at 3 days after replating. The proportion of MAP2-positive cells increased dramatically by the Sox6 overexpression.

The morphological observation and the immunofluorescence study with anti-glial fibrillary acidic protein (GFAP, a

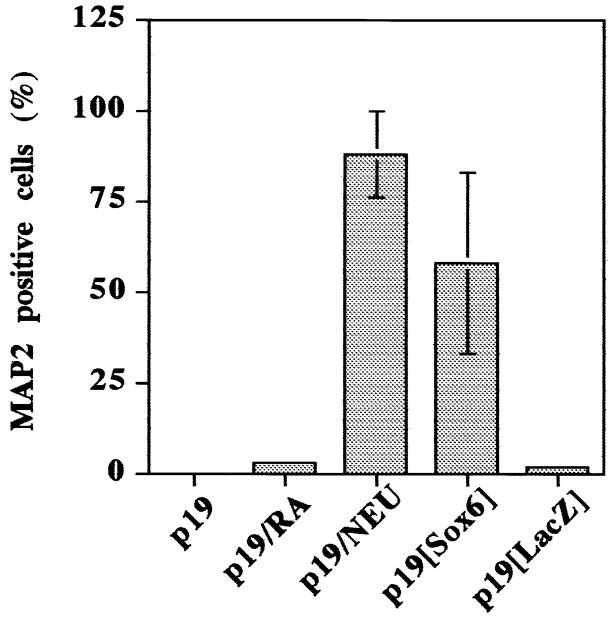

Fig. 5. The MAP2-positive cells in Sox6-overexpressing P19 cells. P19 cells were cultured for 4 days as aggregates with RA (P19/RA) and then cultured for 3 days on polyornithine-coated dishes without RA (P19/NEU). Untreated P19 cells (P19), P19[Sox6] cells and P19[LacZ] cells were cultured for 3 days on culture dishes without RA. Cells were dissociated by trypsinization, fixed by paraformaldehyde, and stained with anti-MAP2 antibody.

glial cell marker) antibody showed that there were almost no glial cells in P19[Sox6] until 11 days after replating (data not shown). In contrast, GFAP-positive cells started to appear in the culture of P19/RA cells 5 days after replating [15]. On the other hand, it is also known that P19 cells differentiate into myocytes under these conditions. But the myocyte-like cells and the expression of myocyte-markers had not been observed in P19[Sox6] cells (data not shown).

These results showed that the overexpression of Sox6 induced specifically neuronal differentiation of P19 cells without RA. Thus, these findings may suggest that Sox6 play roles in the neuronal differentiation of P19 cells by the RA signaling pathway.

The cell population of the wild-type P19 cells increased about 15-fold for 4 days after replating into a monolayer culture on culture dishes in the absence of RA (Table 1). P19[Sox6] and P19[LacZ] cells cultured with RA in culture dishes proliferated about 10-fold for 4 days as well as the P19/RA cells cultured with RA in bacterial grade dishes. However, P19/NEU cells did not always proliferate. The cell cycle profiles of these cells are shown in Table 2. The ratio of the S- and G2/M-phase cells in P19[Sox6] cells was similar to that of P19/RA cells. This result may suggest that P19[Sox6]

Table 1
Effect of ectopic Sox6 expression for cell growth

Day	Wild-type P19			P19[Sox6]	P19[LacZ]
	Untreated	P19/RA	P19/NEU		
0	10	10	10	10	10
2	54 ± 8	38 ± 15	7 ± 2	44 ± 14	48 ± 7
4	148 ± 10	103 ± 18	15 ± 4	110 ± 16	115 ± 4

Cell number is shown $\times 10^5$.

Table 2
The cell cycle profile of P19 cells

	Wild-type P19		P19[Sox6]	P19[LacZ]
	Untreated	P19/RA		
G1	20.0 ± 5.2	33.5 ± 7.5	28.5 z± 8.8	18.6 ± 3.9%
S	60.4 ± 6.2	49.7 ± 5.2	57.2 ± 6.7	56.8 ± 5.2
G2/M	19.6 ± 3.8	16.8 ± 5.4	14.3 ± 2.2	24.6 ± 4.1

P19 cells were cultured for 4 days as aggregates with RA on bacterial grade dishes (P19/RA). Untreated P19 cells, P19[Sox6] cells and P19[LacZ] cells were cultured for 3 days on culture dishes without RA. Cells were dissociated by trypsinization, fixed using ethanol for flow cytometric analysis.

cells are in the early stages of neuronal differentiation, and are also maintaining the ability to proliferate.

Thus, these findings may suggest that Sox6 play roles in the neuronal differentiation of P19 cells by the RA signaling pathway.

3.4. *Expression profiles of development-related genes during the neuronal differentiation of Sox6-transfected P19 cells*

The overexpression of Sox6 triggered neuronal differentiation of P19 cells in the absence of RA. To examine whether Sox6 can activate the expression of endogenous genes in the neuronal pathway, the expression patterns of development-related genes, such as nestin [20], Oct-3/4, Wnt-1 [21] and Mash-1 [22], were determined using RT-PCR during neuronal differentiation of P19[Sox6] cells and P19/RA cells (Fig. 6).

Nestin, an intermediate filament protein of neuroepithelial stem cells known to decrease during neurogenesis and maturation, has been used as a marker of neuronal stem cells. In P19/NEU cells at 3 days after replating and P19[Sox6] cells, the nestin mRNA level was equivalent or slightly lower than that in undifferentiated P19 cells (Fig. 6A). The expression of nestin mRNA in the P19/NEU cells decreased slightly 5 days after replating, and then decreased after 7 days to half the level observed 3 days after replating (data not shown). Nestin mRNA has been previously reported to decrease during the maturation of neurons in P19 cells. However, Gao et al. [19] reported that the nestin mRNA was also highly expressed in P19[N-cad] cells, which overexpressed the N-cadherin protein and could differentiate into neurons in the absence of RA. The reasons for the high expression of nestin in P19[Sox6] cells despite the ability of these cells to differentiate into neurons in the absence of RA remain unclear at present.

Oct-3/4 is known as another stem cell marker. Oct-3/4 is a transcriptional factor that expresses in many stem cells with the ability of self-reproduction, and the expression disappears at the initiation of differentiation. The expression level in P19[Sox6] cells was equivalent or a little less than in P19 cells (Fig. 6B). The Sox6-overexpressing cells retained the proliferation ability still, and they were also almost equal to P19[LacZ] cells in growth rate. These results may suggest that P19[Sox6] cells partially retain the stem cell-like properties from their expression of Oct3/4 and proliferation capability.

Mash-1, one of the neuron-specific helix-loop-helix type transcriptional factors, is an essential factor in the development of autonomic and sensory neurons in mice. In P19 cells, Mash-1 was thought to be one of the master genes determining the cellular fate of neurons. Mash-1 mRNA was not detected in undifferentiated P19 and P19[LacZ] cells, but it began to increase by RA induction (P19/RA) and then reached a peak at 3 days when P19 cells differentiated into neurons (P19/NEU) as shown in Fig. 6C. The Mash-1 mRNA level in P19[Sox6] was almost the same as that in P19/RA.

Wnt-1 is known as a midbrain morphogenesis-related protein [23]. Furthermore, Wnt-1 is known to be also induced by RA in P19 cells [24]. The Wnt-1 expression level was low in undifferentiated P19 cells, but it increased rapidly when aggregated with RA (P19/RA), and then disappeared at 3 days when these cells differentiated into neurons (P19/NEU), as shown in Fig. 6D. Wnt-1 mRNA in P19[Sox6] was expressed at about five-fold that in undifferentiated P19 cells. In comparison with P19/RA, the expression of mRNA in P19[Sox6] was 50–70%.

Smolich and Papkoff [24] reported that the overexpression of Wnt-1 in P19 cells could activate the expression of other Wnt genes, but these cells could not differentiate into neuron-like cells without RA. However, Tang et al. [26] reported that Wnt-1-overexpressing P19 cells could differentiate only into neurons in the absence of RA, and the Mash-1 gene was up-regulated during the neuronal differentiation state in these cells. The discrepancy between these two studies is unclear at present.

These results may suggest that the ectopic expression of Sox6 could initiate neuronal differentiation of P19 cells, which is comparable to that induced by RA in terms of the expression profiles of neuronal development-related genes. Overexpression of Sox6 caused an increase in both Wnt-1 and Mash-1 expression, suggesting that Sox6 is involved upstream in the Wnt-1 signaling pathway in the early stage of neuronal differentiation. This may be supported by the fact that Sox6 and Wnt-1 are co-expressed in the developing brain [13,23].

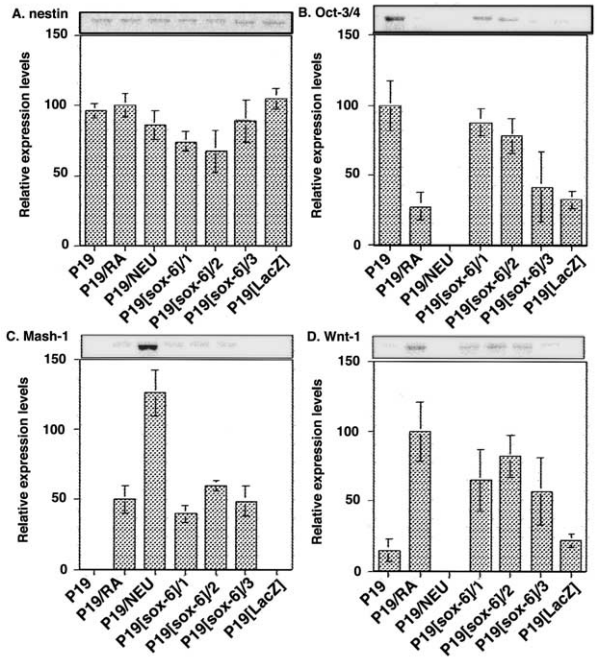

Fig. 6. Expression profiles of development-related genes during the neuronal differentiation in P19[Sox6] cells and P19/RA cells. RT-PCR analysis of nestin, Oct-3/4, Mash-1, and Wnt-1 mRNA expression during neuronal differentiation of P19[Sox6] cells, P19/RA cells and P19/NEU cells as described in Fig. 2. Samples were prepared from cell lysates of P19[Sox6] cells, P19/RA cells and P19/NEU. Taking the value of P19/RA cells as 100%, the relative level of expression is shown.

3.5. Sox6 up-regulates cell adhesion molecules such as E-cadherin and N-cadherin

On culture grade dishes, Sox6 overexpression caused marked cellular aggregation in the absence of RA. Wild-type P19 cells usually differentiate into neurons through the two sequential stages of RA stimulation and cellular aggregation. In wild-type P19 cells, RA is an essential factor in neuronal differentiation, while cellular aggregation promotes the process. It is known that wild-type P19 cells express E-cadherin during undifferentiation, but E-cadherin is substituted by N-cadherin, a neuron-specific cell adhesion molecule, during the neuronal differentiation by RA [19].

We examined whether Sox6 overexpression causes the substitution of cell adhesion molecules. The overexpression of Sox6 induced higher E-cadherin expression in comparison with wild-type P19 cells, P19[LacZ], and even RA-induced P19 cells (Fig. 7A). The overexpression of Sox6 also increased N-cadherin expression in comparison with wild-type P19 cells and P19[LacZ], but the level of N-cadherin expression was half of that in P19/RA cells (Fig. 7B). These results may suggest that the marked cellular aggregation in P19[Sox6] cells was caused by the higher expression of E-cadherin only or both E-cadherin and N-cadherin.

Sox6 overexpression could cause the activation of endogenous genes including Wnt-1, Mash-1, N-cadherin, and MAP2, resulting in neurogenesis. Moreover, E-cadherin was also strongly induced by Sox6, resulting in marked cellular aggregation.

Thus, these results may suggest that Sox6 induces neuronal differentiation of P19 cells by the two elements of RA stimulation and cellular aggregation. In the first, Sox6 induced development-related genes, such as Wnt-1 and Mash-1, downstream of the RA signaling pathway leading to neurogenesis. In the second, Sox6 induced cell adhesion molecules, such as E-cadherin or N-cadherin, promoting neuronal differentiation by stimulating cellular aggregation or the cell to cell interaction.

P19[Sox6] cells had the property that was similar to P19/RA cells in the expression level of Mash-1, Wnt-1 and E-cadherin genes. However, levels of expression of the Oct-3/4 gene in P19[Sox6] cells were almost equal to the untreated wild-type P19 cells. The largest difference between the phenotypes of P19[Sox6] cells and P19/RA cells was observed in the number of MAP2-positive cells (Figs. 4 and 5) and the morphological features of these cells (Fig. 3). About 60% of P19[Sox6] cells were MAP2-positive, but most of P19/RA cells were MAP2-negative, like untreated P19 cells. The expression profiles of these genes, cell growth (Table 1) and the cell cycle profiles (Table 2) may suggest that P19[Sox6] cells most resemble neuronal progenitor-like cells, and the properties of neuronal stem cell and immature neuron have also been inherited a little.

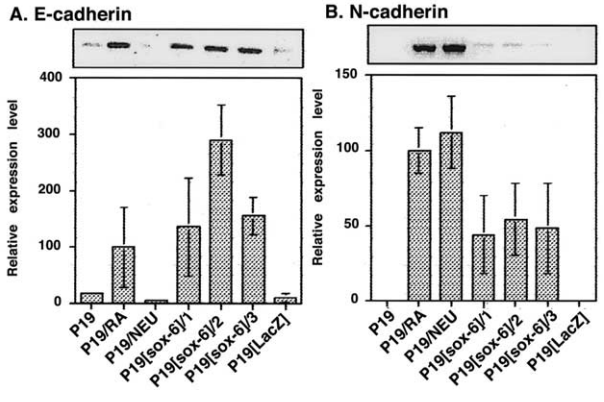

Fig. 7. Expression profiles of cell adhesion molecule genes during the neuronal differentiation in P19[Sox6] cells and P19/RA cells. RT-PCR analysis of E-cadherin and N-cadherin mRNA expression during neuronal differentiation of P19[Sox6] cells, P19/RA cells and P19/NEU cells as described in Fig. 2. Samples were prepared from cell lysates of P19[Sox6] cells, P19/RA cells and P19/NEU. Taking the value of P19/RA cells as 100%, the relative level of expression is shown.

It has previously been reported that the overexpression of N-cadherin [19] or MEF 2C [25], a myocyte enhancer factor, in P19 cells was sufficient to trigger neuronal differentiation of P19 cells in the absence of RA. Two contradictory results on the Wnt-1-overexpression in P19 cells were reported. In one report, the overexpression of Wnt-1 [24] in P19 cells was still dependent on RA for neuronal differentiation, though the overexpression suppressed SSEA-1, an early embryonic ectodermal cell marker, and activated other Wnt genes such as Wnt-4 or -6, both early neuronal development-related genes. In another report, Wnt-1-overexpressing P19 cells could only differentiate into neurons without RA and the overexpression increased Mash-1 and Ngn-1, both basic helix-loop-helix genes. However, cellular aggregation was still necessary for these N-cadherin-, MEF 2C-, and Wnt-1-overexpressing P19 cells to undergo neuronal differentiation. The overexpression of N-cadherin, MEF 2C and Wnt-1 could substitute for the role of RA, but not the role of cellular aggregation in the initial stage of neuronal differentiation. In this study, we showed that the overexpression of Sox6 in P19 cells could substitute for the role of both RA and cellular aggregation.

Gao et al. [19] suggested that N-cadherin might initiate the neuronal differentiation of P19 cells through the Wnt-1 signaling pathway, because the Wnt-1 gene was up-regulated in N-cadherin-overexpressing P19 cells, but could not be detected in the wild-type P19 cells. In the P19[Sox6] cells, N-cadherin, Wnt-1 and Mash-1 were up-regulated, suggesting the possibility that Sox6 induces neuronal differentiation of P19 cells through the N-cadherin signaling pathway.

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