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Suppression of Sox6 in P19 cells leads to failure of neuronal differentiation by retinoic acid and induces retinoic acid-dependent apoptosis ☆

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Abstract The Sox6 gene is a member of the Sox gene family, which encodes transcription factors, and previous studies have suggested that it 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. Sox6 mRNA increases rapidly in P19 cells during RA induction and then decreases during differentiation into neuronal cells. To investigate whether Sox6 expression is essential for neuronal differentiation, we established Sox6-suppressed P19 (P19[anti-Sox6]) cells by transfection of antisense-Sox6 cDNA. Most of the P19[anti-Sox6] cells showed no neurites and were not stained by the anti-MAP 2 antibody, while the suppression of Sox6 expression nearly totally blocked neuronal differentiation in P19 cells. Further, Sox6 suppression caused RA-dependent apoptosis by P19[anti-Sox6] cells: RA-treated P19[anti-Sox6] cells showed chromatin condensation, DNA fragmentation, and an increase in caspase-3-like activity. Thus, Sox6 is considered essential for neuronal differentiation and may play an important role in the early stages of neuronal differentiation or apoptosis.

Keywords: Sox6; Neuronal differentiation; Embryonal carcinoma P19; Apoptosis; Retinoic acid

1. Introduction

Sox proteins are important factors in neurogenesis [1], among which Sox2 and 3 are mainly expressed in the developing central nervous system, while Sox6 plays a role in the early stage of the developing nervous system, as it is specifically expressed during the initial stage (9.5–12.5 days post-coitus) of mouse embryogenesis [2]. Sox6 was initially isolated from an adult mouse testis cDNA library [2,3], after which we isolated cDNA encoding a rat homolog of the previously characterized mouse Sox6 [4]. Recently, a Sox6 null mutant was produced in mice and results of that study showed that Sox6 protein is involved in maintaining the normal physiological function of muscle tissues, such as of the heart [5]. However, the exact role of Sox6 during development of the central nervous system remains to be elucidated.

Mouse embryonal carcinoma cells (P19 cells) have been extensively used as a model system for in vitro differentiation of neuronal cells, as well as skeletal and cardiac muscle cells [6]. Following treatment with retinoic acid (RA) and cellular aggregation, P19 cells differentiate into neurons and glia, though they form skeletal or cardiac muscle, endoderm, and other cell types under different conditions [6]. In studies of neurogenesis, gene-transfected P19 cells have been found useful [7,8]. Since numerous genes are activated in P19 cells in response to RA, we previously investigated whether Sox6 can directly induce neuronal cells from P19 cells. In that study, we produced P19[Sox6] cells that overexpressed P19, which caused remarkable cellular aggregation and differentiation into neuronal cells in the absence of RA [9].

In the present study, we used an antisense Sox6 suppression approach to determine whether Sox6 expression is essential for neuronal differentiation by P19 cells. We found that the expression of antisense Sox6 RNA induced RA-dependent apoptosis and blocked the differentiation of P19 cells into neuronal cells by RA.

2. Materials and methods

2.1. Tissue culture

A P19 EC cell line was purchased from American Type Culture Collection, then maintained in α-modified minimum essential medium supplemented with 2.5% fetal bovine serum (FBS) and 7.5% calf serum (CS). For the induction of neuronal cells, P19 cells were cultured in bacterial grade dishes (IWAKI) in the presence of 500 nM of RA for 4 days. Cells were floated on the dishes and formed large aggregates, which were trypsinized for dispersion and re-plated in poly-ornithine-coated tissue culture dishes or transferred to coverslips without RA for 5–7 days.

2.2. Plasmid DNA and transfection

Sox6 cDNA was kindly provided by Dr. Shinya Yamashita (Nippon Suisan Kaisya Ltd, Tokyo, Japan). To generate an antisense Sox6 construct, Sox6 fragments were amplified by PCR using the restriction site tagging primers 5′-caaagcttcctctccatccgc-3′ and 5′-gatctagattacagatgtgcgtctgg-3′. The *Hin*dIII/*Xba*I fragment of the Sox6 PCR products was reverse inserted into the *Xba*I/*Hin*dIII site of a

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pcDNA3.1 myc/his B vector (Invitrogen, San Diego, CA). Antisense Sox6 expression vectors were transfected into P19 cells and stable transfectants were obtained following selection with 500 μg/ml of G418 (Nakarai, Kyoto, Japan) for 14 days. A pcDNA3.1 LacZ vector (Invitrogen, San Diego, CA) was transfected into P19 cells using the same procedure as for the antisense Sox6 expression vectors.

2.3. Immunocytochemistry

An immunocytochemical study was carried out as previously described [9]. Briefly, cells were fixed with 4% paraformaldehyde in PBS, dehydrated with 5% acetic acid in methanol, and permeabilized with Triton X-100/Tris-buffered saline. They were stained with monoclonal microtubule-associated-protein 2 (MAP2) antibodies (1:500) (Amersham) or normal mouse serum.

2.4. Cell count

The cells were then cultured in tissue culture grade dishes in the presence of RA for 24 or 48 h, treated with trypsin for collection, resuspended in Tripton solution, and counted using a Coulter counter.

2.5. Nuclear staining

Cells were cultured in 24-well plates as floating cultures in the presence of RA for 24 or 48 h. They were then exposed to 1 ng/ml of Hoechst 33342, to stain nuclei of living cells, and 2 ng/ml of propidium iodide (PI), to stain nuclei of dead cells, for 30 min at room temperature.

2.6. Isolation of fragmented DNA

To examine DNA fragmentation, approximately 10^6 cells were cultured in bacterial grade dishes in the presence of RA for 48 h. The cells were then harvested, washed twice with PBS, and used for DNA isolation. Next, they were added to 100 μl of lysis buffer (10 mM Tris–HCl buffer, pH 7.4, containing 10 mM EDTA and 0.5% Triton X-100), incubated for 10 min at 4 °C, and centrifuged at 16 000 rpm for 20 min at 4 °C. The supernatant was treated with 200 μg/ml of RNase A for 1 h at 37 °C and then with 200 μg/ml of proteinase K for 1 h at 37 °C. Twenty microliters of 5 M NaCl and 120 μl of isopropanol were added to 200 μl of supernatant, and then incubated overnight at −20 °C and centrifuged at 16 000 rpm for 20 min at 4 °C. The pellets were dried and dissolved in 20 μl of TE buffer (10 mM Tris–HCl buffer, pH 7.4 and containing 1 mM EDTA). DNA ladders were visualized by running 20 μl on a 2% agarose gel, with a 123-bp ladder used as the standard.

2.7. Caspase-3-like activity

Approximately 10^6 cells were cultured in bacterial grade dishes in the presence or absence of RA for 48 h. After incubation, the cells were washed twice with PBS and lysed in PBS containing 0.2% Triton X-100 on ice for 10 min. After centrifugation at $10\,000 \times g$ for 20 min, cell extracts were measured for Ac-DEVD-pNA cleavage activity using a Caspase-3 Colorimetric Activity Assay Kit (Chemicon), according to the manufacturer's instructions.

2.8. RT-PCR analysis

The expression of Oct-3/4, Wnt-1, Mash-1, N-cadherin and Sox6 was measured by semiquantitative RT-PCR as previously described [9]. 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. For the internal control, the synthesized cDNA was amplified with glyceraldehydes-3-phosphate dehydrogenase (G3PDH)-specific primers. The oligonucleotides used were as follows: 5′-tacagcagcacaagatta-3′ and 5′-cgtgttccttctcagt-3′ for Sox6; 5′-tttgtgttagcctgcgagttac-3′ and 5′-ttagacagttgcgagagacaccat-3′ for anti-Sox6; 5′-tgaaggtcggtcggtgtgaacggatttggc-3′ and 5′-catgtaggccatgaggtccaccac-3′ for G3PDH.

3. Results

3.1. Antisense-Sox6 (anti-Sox6) suppressed RA-induced endogenous Sox6 expression

To suppress endogenous Sox6 expression, we constructed an anti-Sox6 expression vector (Fig. 1A) and transfected it into P19 cells. A cytomegalovirus (CMV) promoter-driven expression vector for anti-Sox6 was constructed by inserting a 1-kb Sox6 fragment in reverse orientation into the mammalian expression vector pcDNA3.1/myc/his. After G418 selection for 2 weeks, 10 clones were isolated by limiting dilution. Anti-Sox6 mRNA expression in two isolated clones with high expression levels of the exogenous gene is shown in Fig. 1B. The expression of anti-Sox6 was detected in clones 2–5 and 2–8, whereas

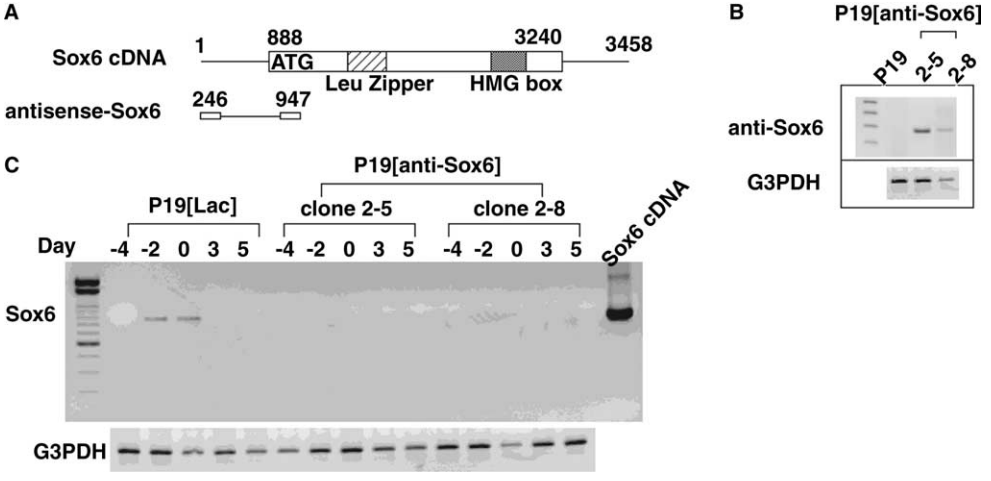

Fig. 1. Suppression of Sox6 mRNA expression by anti-Sox6. (A) Schematic diagram of Sox6 cDNA structure and an anti-Sox6 fragment. Anti-Sox6 fragments were amplified by PCR using restriction site tagging primers shown in the open frame of antisense-Sox6. (B) Expression of anti-Sox6 in transfected P19 cells. RNA was isolated from cells cultured in tissue culture grade dishes in the absence of RA and subjected to RT-PCR analysis using anti-Sox6 primers. (C) Suppression of Sox6 mRNA in P19[anti-Sox6] cells treated with RA. Cells were cultured in the presence of RA in bacterial grade dishes for 4 days (Day −4 to Day 0) and then re-plated in poly-ornithine-coated dishes for neuronal differentiation at Day 0. RNA was isolated from the cells and then subjected to RT-PCR analysis using Sox6 primers. Sox6 cDNA used as a PCR control and G3PDH was a positive control for reverse transcription.

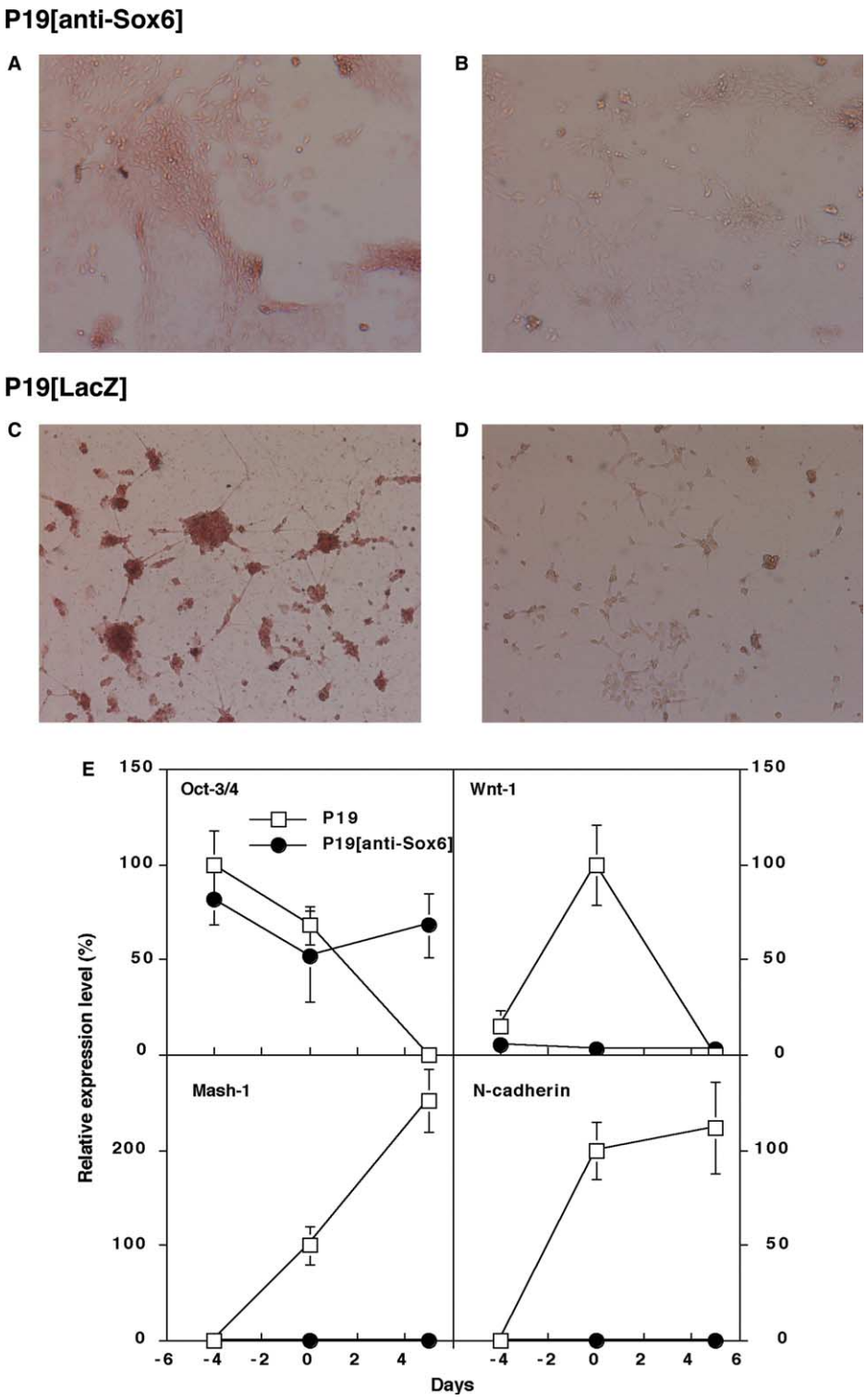

Fig. 2. Failure of neuronal differentiation in P19[anti-Sox6] cells. (A–D) Cells were cultured in bacterial grade dishes in the presence of RA for 4 days and transferred to poly-ornithine-coated coverslips for 5 days. The cells were fixed for immuno-staining with an anti-MAP2 antibody. (A) Clones 2–5 from P19[anti-Sox6] cells, (B) clones 2–8 from P19[anti-Sox6] cells, (C) P19[LacZ] cells stained with anti-MAP2 antibody as a positive control, (D) P19[LacZ] cells stained with normal mouse serum as a negative control. (E) RT-PCR analysis of Oct-3/4, Wnt-1, Mash-1, and N-cadherin during neuronal differentiation of P19 and P19[anti-Sox6] cells. Cells were cultured in bacterial grade dishes in the presence of RA for 4 days (Day −4 to Day 0), then transferred to poly-ornithine-coated coverslips for 5 days (Day 5). Taking the value for P19 at Day 0 as 100%, the relative level of expression is shown.

there was no expression in the P19 cells. To examine whether the expressed anti-Sox6 suppressed endogenous Sox6 mRNA, the clones were treated with RA to induce Sox6 mRNA (Fig. 1C). In P19[LacZ] cells, endogenous Sox6 was highly induced by RA and cellular aggregation occurred from day −2 to day 0. In contrast, no endogenous Sox6 was expressed in the two P19[anti-Sox6] clones. These results suggest that anti-Sox6 suppressed the transcription or enhanced the degradation of endogenous Sox6 mRNA.

3.2. Sox6, an essential factor for neuronal differentiation of P19 cells

P19 cells are known to differentiate into neuronal cells by both RA stimulation and cellular aggregation [8,9]. To determine whether Sox6 expression is essential for neuronal differentiation, P19[anti-Sox6] cells were cultured in accordance with standard procedures required for neuronal differentiation to occur. Briefly, the cells were cultured in bacterial grade dishes for cellular aggregation in the presence of RA for 4 days, re-plated on poly-ornithine-coated coverslips in the absence of RA for 5 days, and then fixed for immuno-staining (Fig. 2). P19[LacZ] cells showed extended neurites and differentiated into MAP2-positive neurons (Fig. 2C). As for P19[anti-Sox6] cells, most on the coverslips had no neurites and were not stained by the anti-MAP 2 antibody (Fig. 2A and B), while morphological observations showed them to be similar to untreated P19 cells. Further controls are in Supplementary Figure. Oct-3/4, a molecular marker of undifferentiated cells, remained expressed in P19[anti-Sox6] cells during the RA-induction and in monolayer cultures, after being re-plated in the absence of RA (Fig. 2E). The neuronal cell markers, Wnt-1, Mash-1, and N-cadherin, were not detected during the culture of P19[anti-Sox6] cells by RT-PCR (Fig. 2E). These results suggest that anti-Sox6 nearly totally blocked the neuronal differentiation of P19 cells.

3.3. Sox6 suppression causes a decrease in cellular aggregation and RA-dependent cell death

Sox6 mRNA was rapidly increased in P19 cells during RA stimulation, as we previously described. To examine the effects of Sox6 suppression, P19[anti-Sox6] cells were observed morphologically during RA stimulation. P19[LacZ] cells formed many large aggregates in bacterial grade dishes with RA stimulation (Fig. 3B), whereas P19[anti-Sox6] cells formed few and small aggregates, with many cellular fragments caused by the process of cell death (Fig. 3D).

To determine the number of living cells, P19[anti-Sox6] cells were cultured for 4 days in culture grade dishes in the presence of RA. Cells that adhered to the dishes were collected after washing away dead cells and counted using a Coulter counter. In the absence of RA, two P19[anti-Sox6] cell lines proliferated in a manner equivalent to P19[LacZ] cells, while in its presence, P19[anti-Sox6] clones 2–5 proliferated slightly and clones 2–8 did not increase, while the growth of P19[LacZ] cells was half of that without RA (Fig. 4). Similar results were obtained using Trypan blue staining for counting after the cells were cultured for 4 days in bacterial grade dishes in the presence of RA, though we could not precisely count the number of living cells, because of the interference of many small cell fragments (data not shown). As for P19 [anti-Sox6] cells, cell fragments caused by RA-dependent cell death were seen, whereas such fragments were not found in the dishes used for P19[LacZ] cells.

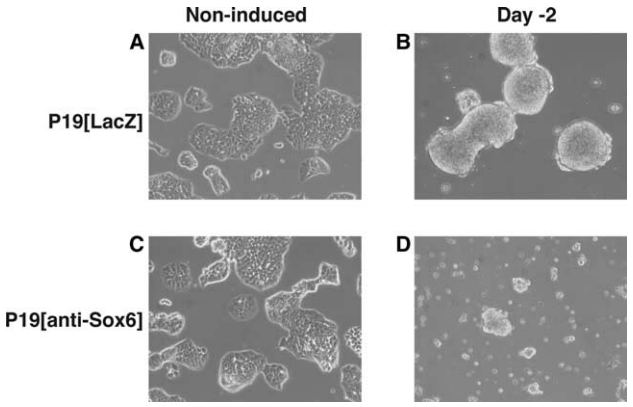

Fig. 3. Sox6 suppression decreased cellular aggregation and increased cell death in the presence of RA. Cells were cultured in the presence of RA in bacterial grade dishes for 4 days and observed using phase-contrast microscopy. Upper panels: P19[LacZ] cells; lower panels: clones 2–8 from P19[anti-Sox6] cells. Left panels: Cells without RA treatment in tissue culture dishes; right panels: 2 days following RA treatment in bacterial grade dishes.

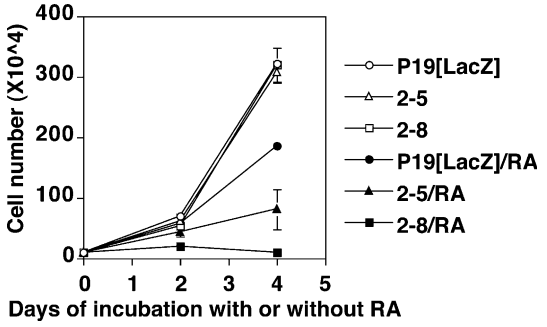

Fig. 4. Growth curves of P19[anti-Sox6] cells cultured with or without RA. Cells were cultured in tissue culture grade dishes in the presence or absence of RA for 2 or 4 days, after which they were treated with trypsin for collection, re-suspended in Tripton solution, and counted using a Coulter counter. Without RA: open symbols; open circle; P19[LacZ] cells, open triangle; clones 2–5 from P19[anti-Sox6] cells, open square; clones 2–8 from P19[anti-Sox6] cells. With RA: closed symbols; closed circle; P19[LacZ] cells, closed triangle; clones 2–5 from P19[anti-Sox6] cells, closed square; clones 2–8 from P19[anti-Sox6] cells.

These results suggest that the low increase in cell number for P19[anti-Sox6] cells was caused by cell death rather than a decrease in the rate of cellular proliferation in the presence of RA.

3.4. Sox6 suppression causes RA-dependent apoptosis

To examine whether RA-induced apoptosis in P19[anti-Sox6] cells, chromatin condensation was used as an index for apoptotic cells. Cells were cultured in a floating state and stained without fixation using two types of fluorescent reagents, Hoechst 33342 for the nuclei of living cells and PI for those of dead cells. In the absence of RA, P19[LacZ] and P19[anti-Sox6] cells formed a number of aggregates (Fig. 5A and D), and contained large and regularly shaped nuclei with distinct nucleoli that were diffusely stained by Hoechst 33342 (Fig. 5B and E). In contrast, in the presence of RA,

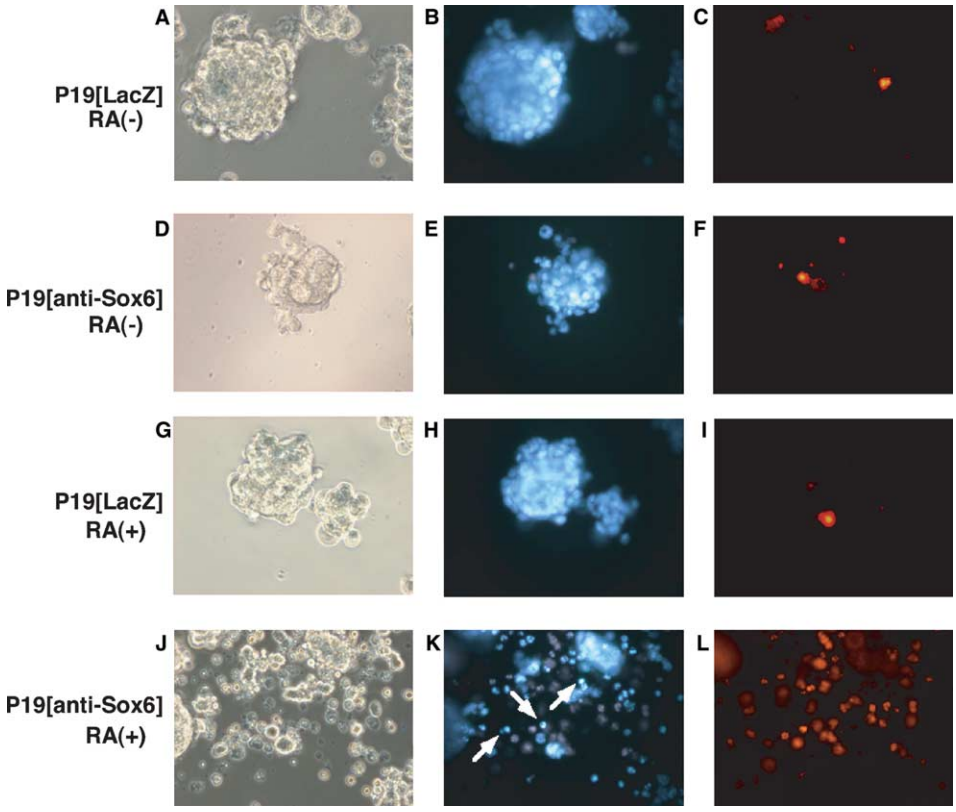

Fig. 5. RA-dependent cell death of P19[anti-Sox6] cells. Cells were cultured in 24-well plates as floating cultures in the presence of RA for 48 h, then stained with Hoechst 33342 or PI without fixation. Left panels show observations using phase-contrast microscopy. Middle and right panels show observations after Hoechst 33342 and PI staining, respectively, using fluorescence microscopy. (A–C) P19[LacZ] cells in the absence of RA, (D–F) clones 2–8 from P19[anti-Sox6] cells in the absence of RA, (G–I) P19[LacZ] cells in the presence of RA, (J–L) clones 2–8 from P19[anti-Sox6] cells in the presence of RA. Arrows in K show apoptotic nuclei with chromatin condensation. Micrographs show a continuous subset of cells in the same fields, which moved slightly because the cells were floating.

P19[anti-Sox6] cells contained nuclei that were smaller and brightly stained, as well as irregularly shaped nuclei, which is characteristic of chromatin condensation associated with apoptosis (Fig. 5K). Almost none of the P19[LacZ] cells contained such nuclei (Fig. 5H). This remarkable increase in dead nuclei was also observed following PI staining of P19[anti-Sox6] cells treated with RA (Fig. 5L), in contrast to P19[LacZ] cells, which had only a small number of dead nuclei (Fig. 5I).

To examine cellular viability, cells were cultured in a floating state for 4 days in the presence of RA, then collected, and dispersed by pipetting. The cells were stained with Hoechst 33342 and PI and counted without fixation. The viability rates of two clones, 2–5 and 2–8, from P19[anti-Sox6] cells and the P19[LacZ] cells were $22.0 \pm 7\%$, $13.2 \pm 4.1\%$, and $86.6 \pm 5.0\%$, respectively, at a low concentration of RA (500 nM). Further, nearly all P19[anti-Sox6] cells died with a high concentration of RA (1.5 μM), whereas the survival rate of P19[LacZ] cells was about 70%. In P19[LacZ] cells only slight DNA fragmentation (another index of apoptosis) was detected 48 h after the addition of RA (Fig. 6A), whereas it strengthened over time in P19[anti-Sox6] cells. DNA fragmentation in P19[anti-Sox6] cells was also confirmed in experiments that used 33342 or PI for nuclear staining with Hoechst (Fig. 5K and L).

Caspase-3-like activity is known to increase during RA-induced neuronal differentiation and apoptosis of wild-type P19 cells. In the present study, caspase-3-like activity remarkably increased in RA-treated P19[anti-Sox6] cells, but not in RA-treated P19[LacZ] cells (Fig. 6B). Overall, chromatin condensation, DNA fragmentation, and caspase-3-like activity increased in RA-treated P19[anti-Sox6] cells, showing that the RA-dependent cell death of P19[anti-Sox6] cells is caused by apoptosis.

4. Discussion

Various factors, including transcriptional factors such as Mash-1, Ngn-1, and Sox6, are induced by the RA signaling pathway during neuronal differentiation of P19 cells [7,9–11]. An important role of the Wnt family, which encodes secretion proteins associated with morphogenesis in the central nervous system, has been suggested for P19 cells during RA-induced neuronal differentiation [12]. Some transcriptional factors have been transfected into P19 cells, in which the overexpression of Wnt-1, Mash-1, and N-cadherin induced neuronal differentiation, even when RA was not present [7–10,13]. However, none of these factors were able to play a role equal to RA when transfected into P19 cells and cellular aggregation was still required for neuronal differentiation.

We previously reported that the overexpression of Sox6 caused cellular aggregation and neuronal differentiation in the

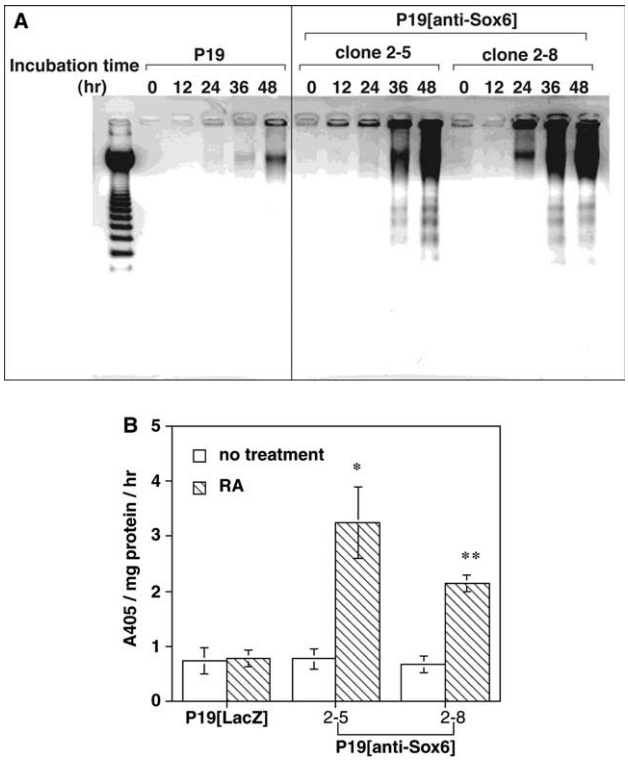

Fig. 6. RA-dependent apoptosis in P19[anti-Sox6] cells. (A) RA-dependent DNA fragmentation in P19[anti-Sox6] cells. Cells were cultured in bacterial grade dishes in the presence of RA for the indicated times and DNA fragments were collected, as described in Section 2. DNA ladders were visualized by running 20 μl on a 2% agarose gel, with a 123-bp ladder used as the standard. (B) Caspase-3-like activity increased in P19[anti-Sox6] cells with RA-treatment. Cells were cultured in bacterial grade dishes in the presence or absence of RA for 48 h, after which cell extracts were collected, as described in Section 2. Caspase-3-like activity was measured for 1 h using Ac-DEVD-pNA as the substrate. Data presented represent means ± S.D. of five replicate experiments. Differences were considered significant at $*P < 0.01$ and $**P < 0.05$ (Student's unpaired t test).

absence of RA [9]. In that study, Sox6 induced an increased expression of neuronal development-related genes such as Wnt-1 and Mash-1, and also increased the expression of cell adhesion molecules such as E-cadherin and N-cadherin. These results suggest that Sox6 is committed upstream of these factors in RA-signaling pathways.

In the present study, to examine whether Sox6 expression is essential for neuronal differentiation, the anti-Sox6 gene was transfected into P19 cells to specifically suppress the Sox6 gene. Our results showed that P19[anti-Sox6] cells had the same morphological features and growth rate as RA-untreated P19 cells, and could not differentiate into MAP2 positive neurons (Fig. 2). Thus, the suppression of Sox6 blocked the neuronal differentiation of P19 cells by RA. Oct-3/4, a molecular marker of undifferentiated cells, remained expressed in P19[anti-Sox6] cells during all culture stages. Wnt-1, Mash-1, and N-cadherin, neuronal cells markers, were not detected during the culture of P19[anti-Sox6] cells with RA. Though the Wnt-1 has already and remarkably increased by RA in the neuronal induction stage of P19 cells, its gene is not completely induced in this stage for P19[anti-Sox6] cells. These results may suggest that the suppression of Sox6 inhibits neuronal differentiation at the stage of neural induction and allows P19 cells remain undifferentiated. In P19[anti-Sox6] cells, aggregates decreased in number and size, possibly due to a decrease in E-cadherin expression. Although Sox6 overexpression caused an increase in N- and E-cadherin expression in P19 cells, the level of E-cadherin decreased and N-cadherin was not detected in P19[anti-Sox6] cells in the presence of RA (Fig. 2E and data not shown). Gao et. al. [10] previously reported that N-cadherin was increased in RA-induced P19 cells and that its overexpression caused neuronal differentiation with cellular aggregation in the absence of RA. We speculated that a decrease in N- or E-cadherin expression is one of the causes of the failure of neuronal differentiation by P19[anti-Sox6] cells.

We did not anticipate that Sox6 suppression would cause RA-dependent cell death in P19[anti-Sox6] cells. RA partially induces apoptosis in wild-type P19 cells during neuronal differentiation. However, most of the P19[anti-Sox6] cells died after the addition of RA in the present experiments. We considered that cell death progressed by apoptosis, as it was accompanied by chromatin condensation and DNA fragmentation.

The susceptibility of wild-type P19 cells to RA-induced apoptosis has been reported to be altered by additional endogenous or exogenous substances [14], of which BMP-2 and 4 are cooperative factors [15–17]. Fujita et al. [16] noted that many apoptotic cells were observed in wild-type P19 cells treated with both RA and BMP-4. However, they were not found among those treated with RA or BMP alone. Further, Tang et al. [13] examined the expression profile of BMP-4 in wild-type P19 cells and showed that it was not expressed in P19 cells untreated with RA, while it was increased during cellular aggregation in the presence of RA and decreased with the neuronal differentiation of P19 cells. In that study, remarkable cell death was not observed even with a higher expression of BMP-4. In the present results obtained during cellular aggregation with RA, we also did not observe cell death in wild-type P19 cells (Fig. 2), but RA-dependent cell death was seen in P19[anti-Sox6] cells when the expression of Sox6 was suppressed. Therefore, we considered it important to examine the expression of BMP-4 in P19[anti-Sox6] cells. In preliminary experiments, BMP-4 was increased in P19[anti-Sox6] cells treated with RA. Further, Sox6 may be a gene and a key for solving this contradiction. Thus, Sox6 may play an important role in the turning-point at which P19 cells stimulated with RA proceed to cell death or differentiate into neuronal cells.

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References

[1] Scotting, P.J. and Rex, M. (1996) Neuropathol. Appl. Neurobiol. 22, 469–481, Review.
[2] Connor, F., Wright, E., Denny, P., Koopman, P. and Ashworth, A. (1995) Nucleic Acids Res. 23, 3365–3372.
[3] Takamatsu, N., Kanda, H., Tsuchiya, I., Yamada, S., Ito, M., Kabeno, S., Shiba, T. and Yamashita, S. (1995) Mol. Cell. Biol. 15, 3759–3766.
[4] Narahara, M., Yamada, A., Hamada-Kanazawa, M., Kawai, Y. and Miyake, M. (2002) Biol. Pharm. Bull. 25, 705–709.
[5] Hagiwara, N., Klewer, S.E., Samson, R.A., Erickson, D.T., Lyon, M.F. and Brilliant, M.H. (2000) Proc. Natl. Acad. Sci. USA 97, 4180–4185.

[6] Jones-Villeneuve, E.M.V., McBurney, M.W., Rogers, K.A. and Kalnins, V.I. (1982) J. Cell Biol. 94, 253–262.
[7] Nakakura, E.K., Watkins, D.N., Sriuranpong, V., Borges, M.W., Nelkin, B.D. and Ball, D.W. (2001) Brain Res. Mol. Brain Res. 95, 162–166.
[8] Farah, M.H., Olson, J.M., Sucic, H.B., Hume, R.I., Tapscott, S.J. and Turner, D.L. (2000) Development 127, 693–702.
[9] Hamada-Kanazawa, M., Ishikawa, K., Nomoto, K., Uozumi, T., Kawai, Y., Narahara, M. and Miyake, M. (2004) FEBS Lett. 560, 192–198.
[10] Gao, X., Bian, W., Yang, J., Tang, K., Kitani, H., Atsumi, T. and Jing, N. (2001) Biochem. Biophys. Res. Commun. 28410, 98–103.
[11] Itoh, F., Nakane, T. and Chiba, S. (1997) Tohoku J. Exp. Med. 1823, 27–36.
[12] Smolich, B.D. and Papkoff, J. (1994) Dev. Biol. 166, 300–310.
[13] Tang, K., Yang, J., Gao, X., Wang, C., Liu, L., Kitani, H., Atsumi, T. and Jing, N. (2002) Biochem. Biophys. Res. Commun. 2931, 67–73.
[14] Sporn, M.B. and Roberts, A.B. (1991) Mol. Endocr., 53–57.
[15] Glozak, M.A. and Rogers, M.B. (2001) Exp. Cell Res. 268, 128–138.
[16] Fujita, E., Soyama, A., Kawabata, M. and Momoi, T. (1999) Cell Death Differ. 6, 1109–1116.
[17] Glozak, M.A. and Rogers, M.B. (1998) Exp Cell Res. 2421, 65–73.