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# Involvement of Oct3/4 in the enhancement of neuronal differentiation of ES cells in neurogenesis-inducing cultures

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## SUMMARY

**Oct3/4 plays a critical role in maintaining embryonic stem cell pluripotency. Regulatable transgene-mediated sustained Oct3/4 expression in ES cells cultured in serum-free LIF-deficient medium caused accelerated differentiation to neuroectoderm-like cells that expressed Sox2, Otx1 and Emx2 and subsequently differentiated into neurons. Neurogenesis of ES cells is promoted by SDIA (stromal cell-derived inducing activity), which accumulates on the PA6 stromal cell surface. Oct3/4 expression in ES cells was maintained by SDIA whereas without it expression was promptly downregulated. Suppression of Oct3/4 abolished neuronal differentiation even after stimulation by SDIA. In contrast, sustained upregulated Oct3/4 expression enhanced SDIA-mediated neurogenesis of ES cells. Therefore, Oct3/4 appears to promote neuroectoderm formation and subsequent neuronal differentiation from ES cells.**

Supplemental data available online

Key words: Oct3/4, SDIA, ES cells, Neurogenesis, LIF, Mouse

## INTRODUCTION

During mammalian development, the initial step in the generation of the nervous system is the process of neural induction. However, little is known about the regulatory mechanisms governing mammalian neural induction. Mouse embryonic stem (ES) cells, which can contribute in vivo to the formation of all tissues, have provided a useful means to answer this question. It has recently been reported that neurogenesis of ES cells in vitro without the formation of embryoid bodies and retinoic acid treatment can be achieved by SDIA (stromal cell-derived inducing activity), which accumulates on the surface of the stromal cell line PA6 (Kawasaki et al., 2000). In character, ES cells resemble the inner cell mass (ICM) cells formed on embryonic day (E) 3.5, from which ES cells are derived (Bradley et al., 1984). The neural fate of the ICM cells in vivo is thought to be determined later in development on E6.5-E8 during gastrulation, when neural precursor cells start to be formed (Schoenwolf and Smith, 1990). The time course of neural marker induction in SDIA-stimulated ES cells in vitro was shown to correlate well with that seen in the embryo (Kawasaki et al., 2000). Thus, the neurogenesis-inducing system with SDIA is thought to mimic the neurogenesis that occurs in vivo, but its molecular basis remains unknown.

Addition of leukemia inhibitory factor (LIF) is sufficient to establish and maintain mouse ES cells without feeder cells in the presence of fetal calf serum (Nichols et al., 1990), and that activation of the transcription factor STAT3 in the signaling is sufficient to maintain the undifferentiated state of mouse ES cells (Matsuda et al., 1999; Niwa et al., 1998). Besides STAT3, another transcription factor, Oct3 (also known as Oct4; hereafter referred to as Oct3/4) is known to be important for the maintenance of ES cells. Oct3/4 is a POU-family transcription factor which has been confirmed to function specifically in pluripotent cell populations (Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1990b). Oct3/4 expression has been detected in the oocyte, blastocyst and embryonic ectoderm before gastrulation, is down-regulated in the neural tube from approximately E8.5, and thereafter becomes restricted to germ cells (Rosner et al., 1990; Scholer et al., 1990a). Targeted gene disruption has revealed the essential role of Oct3/4 in mouse development (Nichols et al., 1998). Oct3/4-deficient embryos fail to initiate fetal development by a loss of the establishment of a pluripotent population of cells in the preimplantation embryo. These cells give rise to tissue-specific stem cells in mammalian embryogenesis, but very few experimental studies have suggested possible mechanisms underlying early neural differentiation.

Oct3/4 has been suggested to be a candidate for the master regulator of initiation, maintenance and differentiation of pluripotent cells. A recent investigation using the conditional Oct3/4 repression or expression system in ES cells has revealed

that the precise level of this transcription factor is important for the maintenance of stem cell self-renewal (Niwa et al., 2000). It remains an interesting question whether up- or down-regulation of Oct3/4 affects neuroectoderm differentiation of ES cells.

In this report, we show that sustained upregulation of Oct3/4 in ES cells in serum-free LIF-deficient medium leads to efficient neuroectoderm formation and subsequent neuronal differentiation. We further show that ES cells cultured on a PA6 monolayer to induce neurogenesis continue to express Oct3/4. It is thus suggested that Oct3/4 promotes neuronal differentiation of ES cells under neurogenesis-inducing conditions.

## MATERIALS AND METHODS

### Cell culture

Undifferentiated ES cells (EB5, ZHTc6 and ZHBTc4) were maintained in an undifferentiated state on gelatin-coated dishes as described previously (Kawasaki et al., 2000; Niwa et al., 1998; Niwa et al., 2000). For differentiation, ES cells were cultured on a fixed monolayer of PA6 feeder cells or poly-L-ornithine and fibronectin (O/F)-coated dishes in G-MEM medium supplemented with 10% KSR (Gibco-BRL), 2 mM glutamine, 1 mM pyruvate, 0.1 mM nonessential amino acids, and 0.1 mM 2-ME. PA6 cells were grown to confluency, fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature, and rinsed with PBS several times before plating ES cells on them. In this SDIA-stimulation method, ES cells were plated on fixed PA6 cells at a density of $2\times10^4$ cells per 6 cm dish. In the ZHTc6 cell differentiation culture without PA6, the cells were seeded at a density of $1\times10^5$ cells per 6 cm O/F-coated dish in the ES-maintaining medium overnight, washed twice with PBS, and then cultured in N2-supplemented DMEM/F-12 medium in the presence or absence of 10% FBS with or without 1 μg/ml of tetracycline-HCl (Sigma) on O/F-coated dishes. In some cases 10 ng/ml of Bmp2 (Yamanouchi Pharmaceutical) or 150 ng/ml of BmpβR-Fc (R&D Systems) were added to the medium, and the Bmp2- or BmpβR-Fc-containing medium was exchanged for fresh medium every other day till the end of the culture.

### Immunocytochemistry

Immunofluorescent staining was performed with the following antibodies: an anti-MAP2 monoclonal antibody (Sigma) and an anti-nestin polyclonal antibody (provided by K. Yoshikawa, Osaka Univ.). The following secondary antibodies were used: an Alexa 488-conjugated goat anti-mouse IgG antibody (Molecular Probes), and a rhodamine-conjugated donkey anti-rabbit IgG antibody (Chemicon, Temecula, CA). The cells were counterstained with Hoechst 33258 to identify the nuclei. Images were obtained using fluorescence microscopy (AX70 microscope; Olympus, Tokyo).

### RNA analysis

For RT-PCR analysis, we performed oligo(dT)-primed reverse transcription on aliquots (5 μg) of total RNA and used 1/100 of the resultant single strand cDNA products for each PCR amplification. Primer sets, with which all cDNAs were amplified in a quantitable range, are listed in Table 1.

## RESULTS

### Upregulation of Oct3/4 expression in ES cells induces effective neurogenesis under serum-free LIF-deficient conditions

For the maintenance of ES cell self-renewal it has been demonstrated that Oct3/4 expression must remain within plus or minus 50% of the normal level, and that up- or down-regulation of Oct3/4 beyond this range triggers differentiation into primitive endoderm/mesoderm and trophectoderm, respectively (Niwa et al., 2000). Since neuroectoderm differentiation of ES cells under such conditions has not been examined, we first attempted to analyze neuronal marker expression in ZHTc6 ES cells with a regulatable Oct3/4 transgene, which had been established previously (Niwa et al., 2000). For this, ZHTc6 cells were cultured in either the presence or absence of Tc on poly-L-ornithine/fibronectin (O/F)-coated dishes in N2-supplemented serum-free DMEM/F12 without LIF. We applied this culture condition to ES cells because this is the one under which we had routinely induced neuronal differentiation from fetal mouse neuroepithelial cells in vitro (Takizawa et al., 2001). As shown in Fig. 1A, a large number of MAP2-positive neurons emerged in the culture without Tc, in which ZHTc6 cells are known to exhibit sustained expression of Oct3/4 from the transgene. In contrast, only a small number of MAP2-positive neurons were differentiated from ZHTc6 cells with no transgene expression, when Tc was present in the medium. Cells positive for other neuronal markers, βIII tubulin and neurofilament-M, also emerged efficiently by the Oct3/4 expression. Astrocytes and oligodendrocytes, however, did not appear within the 10-day culture, either with or without Tc (data not shown). The results suggest that sustained upregulation of Oct3/4 expression leads to efficient neuronal differentiation of ZHTc6 cells under the serum-free LIF-deficient culture condition. A time course study showed that continuous Oct3/4 upregulation in the culture without Tc led to earlier and more extensive differentiation of neurons (Fig. 1B). In this culture system, Oct3/4 did not induce any detectable apoptosis. These data imply that sustained upregulated expression of Oct3/4 accelerates the neuronal differentiation of ES cells under these culture conditions.

**Table 1. PCR primers used**

| Gene | Forward primer | Reverse primer | Size of product (bp) |
|---|---|---|---|
| Oct3/4 | atggctggacacctggcttc | ccaggttctcttgtctacctc | 1121 |
| Sox2 | acactgcccctgtcgcacatgtgagtcgacaa | ttgatatcatgtataacatgatggagacggagc | 976 |
| Otx1 | gcagcgacgggagcgcacca | tgctgctggcggcacttggc | 180 |
| Emx2 | ttcgaaccgccttctcgccg | tgagccttcttcctctag | 188 |
| Gata4 | ccgagcaggaatttgaagagg | gcctgtatgtaatgcctgcg | 469 |
| CK-17 | cctgctccagattgacaatg | cttgctgaagaaccagtcttc | 380 |
| Fgf5 | aaagtcaatggctcccacgaa | agaggctgtagaacatgatt | 464 |
| Gapdh | accacagtccatgccatcac | tccaccaccctgttgctgta | 452 |

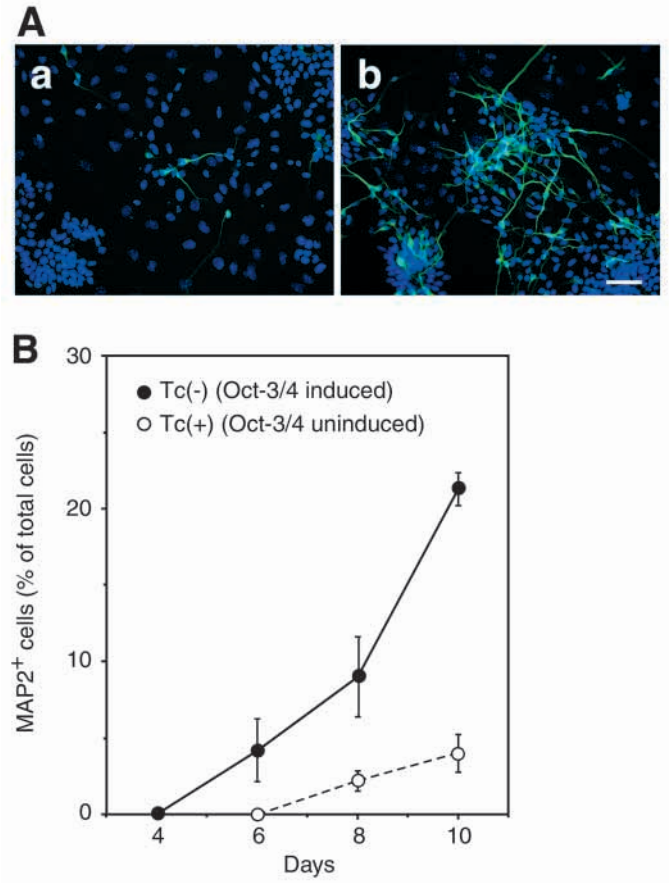


**Fig. 1.** Efficient neurogenesis of ES cells under serum-free LIF-deficient conditions. ZHTc6 ES cells were established (Niwa et al., 2000) in which expression constructs for a tetracycline (Tc)-regulated transactivator (tTA) transgene and a tTA-responsive Oct3/4 transgene were introduced. The ZHTc6 cells with no transgene expression in the presence of Tc showed induced Oct3/4 expression the level of which is comparable to that from the endogenous allele. Withdrawal of Tc triggers the transgene expression, which induces Oct3/4 expression at a level 50% above that of Oct3/4 expression in normal ES cells. (A) ZHTc6 cells were cultured in serum-free N2-supplemented DMEM/F-12 without LIF for 8 days on poly-L-ornithine/fibronectin (O/F)-coated dishes in the presence (a) or absence (b) of Tc. Cells were stained with an anti-MAP2 antibody (green) and Hoechst 33258 (blue). Upregulation of Oct3/4 induced by withdrawal of Tc promoted neurogenesis of ES cells. Scale bar, 50 μm. (B) ZHTc6 cells were cultured as in A with or without Tc for 10 days. The frequency of MAP2-positive cells with respect to the total number of cells was analyzed every 2 days. Vertical bars indicate the s.d.

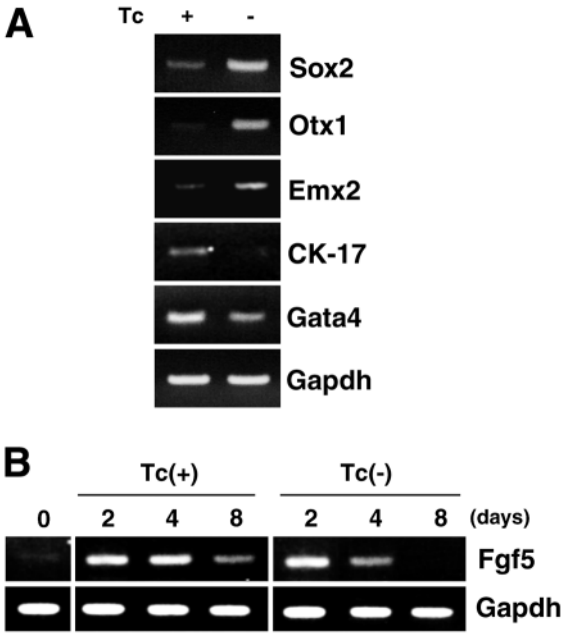


**Fig. 2.** Upregulation of Oct3/4 expression in ES cells induces neural marker gene expression under serum-free LIF-deficient conditions. (A) ZHTc6 cells were cultured for 8 days in the presence or absence of Tc. Total RNA was extracted from each culture and subjected to RT-PCR analysis for the genes indicated on the right. (B) RNA was extracted from each ZHTc6 cell culture on days 2, 4 and 8, and subjected to RT-PCR analysis for Fgf5 expression.

### Continuous upregulation of Oct3/4 expression induces neuroectoderm differentiation from ES cells under serum-free LIF-deficient conditions

We next examined the expression of neural and non-neural marker genes in ZHTc6 cells described above cultured with or without Tc. Sox2 is one of the earliest known transcription factors to be expressed in ICM and its expression continues throughout neural tube development (Wood and Episkopou, 1999). As shown in Fig. 2A, Sox2 mRNA was highly expressed in the differentiated ZHTc6 cells cultured without Tc to induce continuous upregulated expression of Oct3/4. The neural markers Otx1 (Acampora et al., 1998) and Emx2 (Simeone et al., 1992) were also obviously expressed in ZHTc6 cells cultured without Tc. To examine the non-neural cell fate of ZHTc6 cultured without Tc, we analyzed the expression of an epidermal marker, cytokeratin-17 (CK-17) (McGowan and Coulombe, 1998), and an early endodermal marker, Gata4 (Arceci et al., 1993). As shown in Fig. 2, CK-17 expression was not detectable in ZHTc6 cells cultured without Tc and Gata4 expression was lower than in ZHTc6 cells cultured with Tc. These results suggest that continuous expression of Oct3/4 may play a role in guiding the fate of ES cells towards neural differentiation under certain conditions where there are no neural inhibitors, such as those contained in serum, e.g. bone morphogenetic proteins (Bmps).

In mouse embryogenesis, the neuroectoderm is formed after the primitive ectoderm stage (Tam, 1989). The results described above suggest that sustained upregulation of Oct3/4 expression in ES cells might have accelerated the formation of primitive ectoderm and forced these cells to go beyond this transit stage towards further developmental stages. To evaluate whether sustained upregulation of Oct3/4 expression has any effect on the primitive ectoderm formation in our culture system, we examined the expression of Fgf5, a marker of primitive ectoderm (Hebert et al., 1991). In a time course study, the expression kinetics of Fgf5 in ZHTc6 cells cultured in the presence or absence of Tc was different (Fig. 2B). In the absence of Tc, the expression of Fgf5 was significant on day 2 but subsequently decreased and disappeared on day 8, whereas Fgf5 expression in the presence of Tc was significant from day 2 until day 4 and decreased but still remained

detectable on day 8. These results suggest that ES cells with sustained upregulated expression of Oct3/4 tend to go beyond the primitive ectoderm stage to the neuroectoderm stage more efficiently.

We next examined whether the Oct3/4-induced neuronal differentiation of ES cells is affected by anti-neurogenic factors. Neural inducers have been shown to act by antagonizing Bmps in the extracellular space (Hemmati-Brivanlou and Melton, 1997; Sasai and De Robertis, 1997). As shown in Fig. 3D, addition of 10 ng/ml Bmp2 resulted in complete suppression of the neuronal differentiation of ZHTc6 cells in serum-free LIF-deficient culture without Tc. Upregulated expression of Oct3/4 by omission of Tc does not therefore appear to sufficiently antagonize the Bmp signaling. Bmp antagonists such as chordin and noggin are known to be neural inducers (Hemmati-Brivanlou and Melton, 1997). We examined the effect of a BmpR-Fc fusion protein, which has the ability to block Bmp signaling, on the ZHTc6 cell culture. BmpR-Fc did not affect the neurogenesis from ZHTc6 cells either with or without Tc (Fig. 3E,F). Thus, the neurogenic effect of sustained upregulation of Oct3/4 does not appear to be simply due to the induction of Bmp-neutralizing factors. It should be noted that, in contrast to the serum-free conditions, no MAP2-positive cells were detected in the serum-containing culture regardless of the sustained upregulation of Oct3/4 expression (Fig. 3G,H). Addition of LIF also inhibited the Oct3/4-induced neuronal differentiation of ES cells even under serum-free conditions (K.S. and T.T., unpublished data), indicating that the withdrawal of both serum and LIF is necessary for Oct3/4-induced neurogenesis of ES cells.

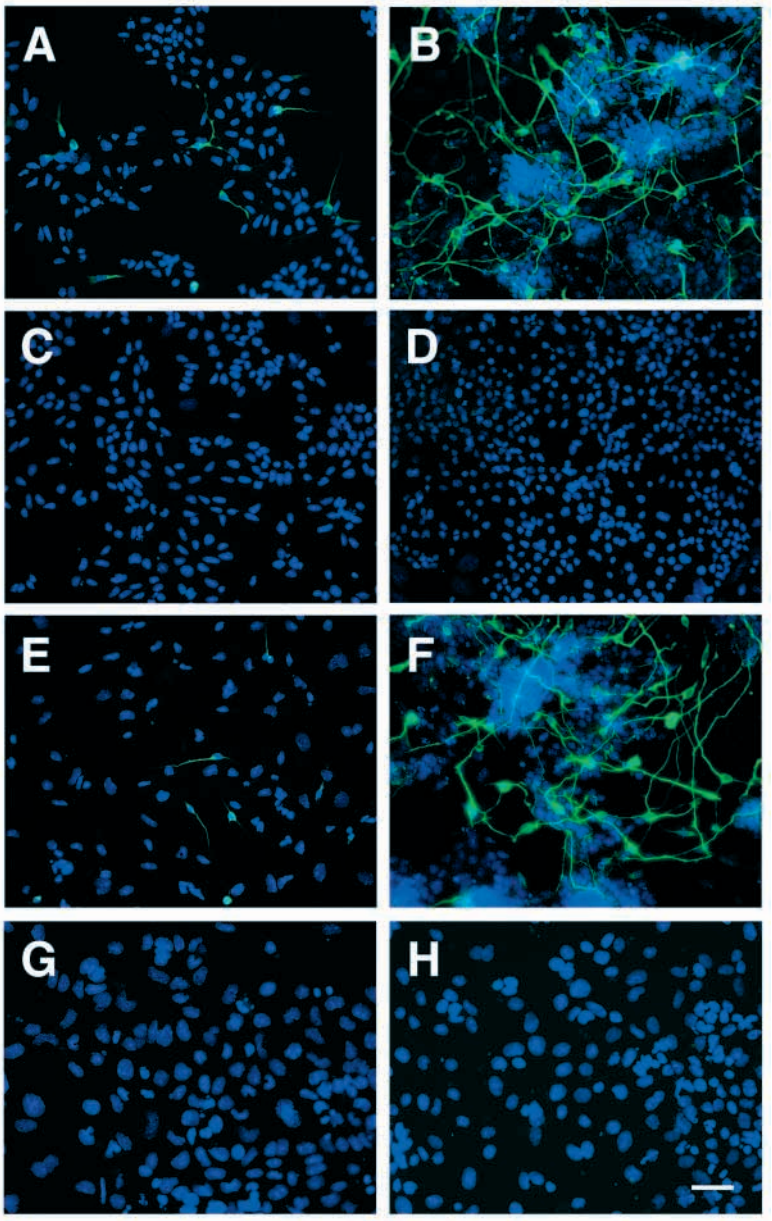


**Fig. 3.** Neurogenic function of Oct3/4 is not explained by the inhibition of cell autonomic Bmp signaling. MAP2 staining (green) of ZHTc6 cells cultured for 8 days with (A,C,E,G) or without (B,D,F,H) Tc. In some cultures, Bmp2 (10 ng/ml; C,D), BmpβR-Fc (150 ng/ml; E,F), or 10% serum (G,H) were added. Nuclei were stained with Hoechst 33258 (blue). Scale bar, 50 μm.

### Maintenance of Oct3/4 expression in mouse ES cells during SDIA-induced neurogenesis

The above results reminded us of the SDIA-induced neurogenesis of ES cells in which neurogenic differentiation is dramatically promoted by culture on PA6 stromal cells under serum-free LIF-deficient conditions. EB5 ES cells, which have normal Oct3/4 alleles rather than the Tc-regulated Oct3/4 transgene, were cultured in serum-free LIF-deficient medium on dishes precoated with either O/F or a monolayer of PA6 cells. As shown in Fig. 4A-F, when ES cells were cocultured on a PA6 monolayer, the cells expressed nestin, a marker of neural progenitors, very efficiently after 4 days. MAP2-positive neurons frequently emerged after 8 days, as has been previously reported (Kawasaki et al., 2000). When the cells were cultured on the O/F-coated dishes, only a few MAP2-positive cells were detected. When EB5 cells were cultured on a gelatin-coated dish (a negative control for the dish used for preparing the PA6 monolayer), the cells differentiated into neurons at a very low frequency, as in the case of the culture on the O/F-coated dishes (data not shown). Taken together with the observations of the ZHTc6 ES cell cultures, these results prompted us to examine the Oct3/4 expression during SDIA-induced neurogenesis. As shown in Fig. 4G, the Oct3/4 expression was rapidly downregulated in ES cells when the cells were cultured on O/F-coated dishes. In contrast, the expression of Oct3/4 was maintained when the cells were cultured on PA6 cells. Consistent with the previous report (Kawasaki et al., 2000), the expression of the mesencephalic dopaminergic neuron marker Nurr1 was effectively induced in ES cells cultured on PA6 cells (Fig. 4G).

### Maintenance of Oct3/4 expression is involved in SDIA-induced neurogenesis

Considering the above results, we wanted to know the consequences of suppression of Oct3/4 expression during SDIA-mediated neurogenesis using ZHBTc4 ES cells. This line of ZHBTc4 cells was established

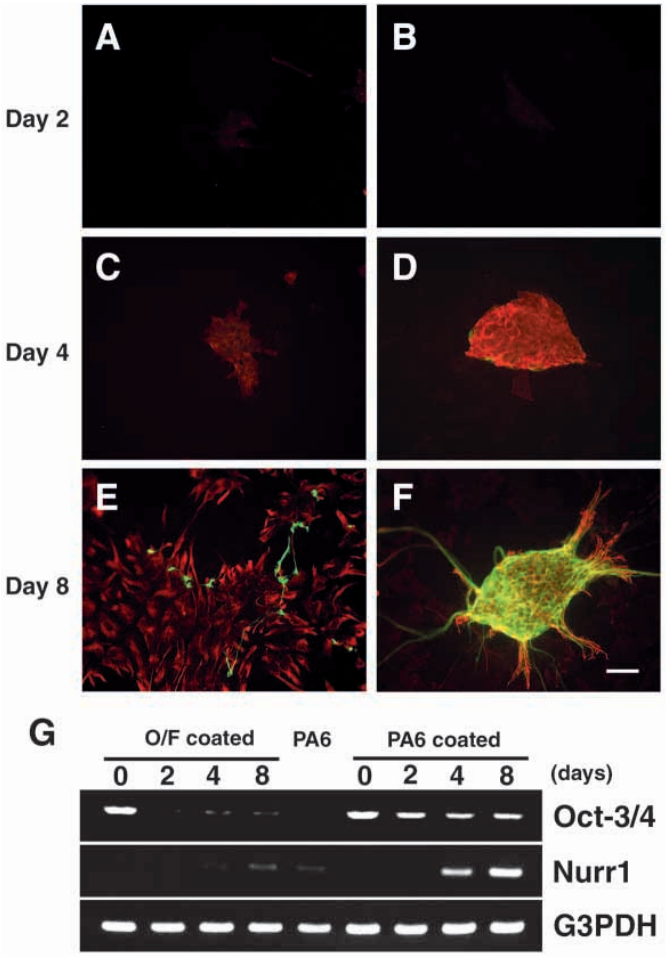


**Fig. 4.** Effective SDIA-induced neurogenesis of ES cells accompanied by maintenance of Oct3/4. Expression EB5 ES cells were cultured on O/F-coated dishes (A,C,E) or a monolayer of PA6 cells (B,D,F). Cells were stained with anti-nestin (red) and anti-MAP2 (green) antibodies on each day indicated in the figure. Scale bar, 50 μm. (G) RT-PCR analysis of the expression of Oct3/4 and a mesencephalic dopaminergic neuron marker, Nurr1, in EB5 ES cells cultured as in A-F.

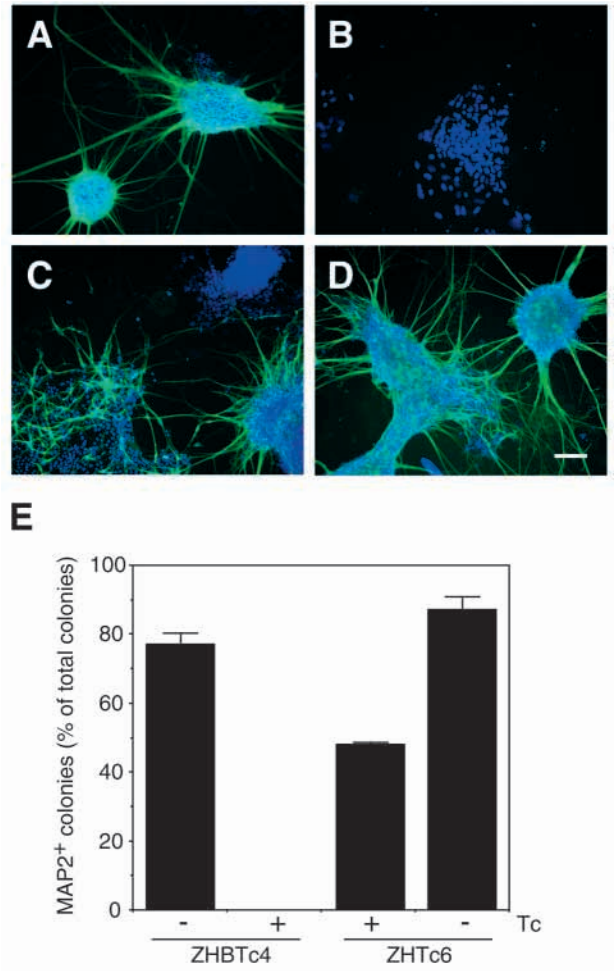


**Fig. 5.** Requirement of Oct3/4 for SDIA-induced neurogenesis. (A,B) ZHBTc4 and (C,D) ZHTc6 cells were cultured on a PA6 monolayer in serum-free LIF-deficient medium with (B,C) or without (A,D) Tc for 8 days. The cells were stained with an anti-MAP2 antibody (green) and Hoechst 33258 (blue). In ZHBTc4 Oct3/4 expression can be shut off by adding Tc. (E) MAP2-positive colonies were counted. Scale bar, 100 μm.

by disrupting the remaining endogenous *Oct3/4* allele in ZHTc6 cells using a targeting construct with a Tc-regulated transgene consisting of Oct3/4 driven by hCMV*-1 promoter. In ZHBTc4 cells, Oct3/4 expression is completely shut down by Tc (Niwa et al., 2000). The undifferentiated pluripotential state of ZHBTc4 cells can be maintained in serum-containing medium with LIF in the absence of Tc. When ZHBTc4 cells were stimulated with SDIA by culturing on a PA6 monolayer without Tc, the cells differentiated into neurons as effectively as ZHTc6 cells in the absence of Tc. Interestingly, there were no MAP2-positive cells when ZHBTc4 cells were cultured with Tc to shut off the Oct3/4 expression (Fig. 5B and the second column in 5E). These data indicated that Oct3/4 expression was essential for the generation of neurons by SDIA-mediated neurogenesis. The MAP2-negative cells were flat, which is reminiscent of trophectodermal cells, although we have not yet investigated these cells further. We also examined the effect of SDIA on the neurogenesis of ZHTc6 cells cultured without Tc to induce upregulated expression of Oct3/4 from the regulatable transgene. As shown in Fig. 5C, D and the right half of E, neurogenesis of ZHTc6 cells induced on a PA6 monolayer was promoted by the upregulation of Oct3/4 expression caused by the withdrawal of Tc. In order to show the time course of the appearance of nestin-positive cells and MAP2-positive cells from SDIA-stimulated ZHTc6 cells, the cells were stimulated by SDIA with or without Tc, and subjected to immunostaining every 2 days with anti-nestin and anti-MAP2 antibodies. As shown in Fig. 6, the sustained upregulation of Oct3/4 expression caused by Tc withdrawal further accelerated and enhanced the SDIA-induced differentiation of nestin-positive neural progenitors and MAP2-positive neurons.

We next attempted to determine the duration of the expression of Oct3/4 sufficient to mediate SDIA-induced neurogenesis. During the culture of ZHBTc4 cells on a PA6 monolayer, Tc was added to the medium on days 1, 2, 3, 4 and 5 until the end of the 8 day culture, to shut off the Oct3/4 expression on and after the respective time points (Fig. 7A,

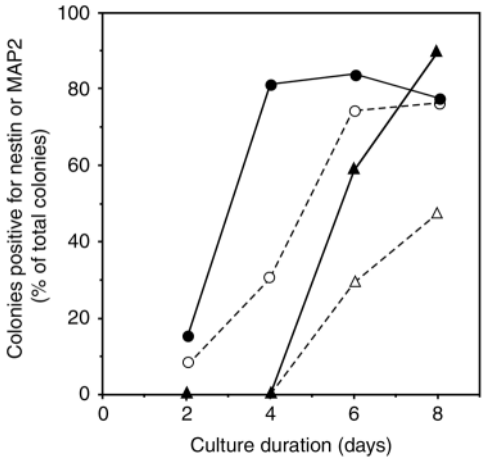


**Fig. 6.** Enhancement of SDIA-induced neurogenesis by upregulation of Oct3/4 expression. ZHTc6 cells were cultured with (open symbols) or without (closed symbols) Tc on a PA6 monolayer. The frequency of nestin-positive (circles) and MAP2-positive (triangles) colonies with respect to the total number of colonies on each day, as indicated, was calculated.

yellow bars). As shown in Fig. 7B, MAP2-positive colonies and nestin-positive colonies were consistently observed even when the Oct3/4 expression was shut off on and after day 5. No nestin- or MAP2-positive cells were detected in the culture in which Oct3/4 expression was shut off on and after day 1 (Fig. 7B). Although we could detect nestin- or MAP2-positive cells in the culture of ZHBTc4 cells in which the Oct3/4 expression was shut off on and after day 2 or day 3, the frequency of such colonies and their size were small (Fig. 7B,C). When the Oct3/4 expression was prolonged until day 4, the colony size as well as the number of cells positive for nestin or MAP2 was increased. Even under these culture conditions, however, the frequency of colonies containing nestin- or MAP2-positive cells with respect to the total number of colonies was approximately half of that in the culture without shutting off the Oct3/4 expression. Thus, Oct3/4 expression for 5 days from the beginning of the culture period appears to be necessary for SDIA to induce efficient neurogenesis in ES cells. To evaluate the susceptibility of ZHBTc4 cells to the anti-neurogenic cytokine Bmp2 under the SDIA-induced neurogenic culture conditions, the cells were cultured on a PA6 monolayer with Bmp2 added at different time points. As shown in Fig. 8, differentiation of nestin-positive cells and MAP2-positive cells was sensitive to Bmp2 when this cytokine was included in the culture on days 2, 3 or 4 until day 8. MAP2-positive colonies and nestin-positive colonies were observed when Bmp2 was added from day 5 on. It appears that a 5 day stimulation of ZHBTc4 cells by SDIA is sufficient for committing to neuronal lineages.

## DISCUSSION

Pluripotent ES cells can differentiate into a wide variety of cell types, and the differentiation of ES cells appears to be under the control of cell-intrinsic nuclear factors and cell-external cues. Regarding the cell-intrinsic factors, Oct3/4 has important roles in the maintenance of the pluripotentiality of ES cells (Nichols et al., 1998). When the Oct3/4 expression level goes beyond ±50% of the normal level, ES cells lose the pluripotency (Niwa et al., 2000). As for the cell-extrinsic cues, LIF is important for ES cell pluripotency, and under the LIF-deficient condition, PA6 cell-derived SDIA, for instance, has been shown to induce the neuronal differentiation of ES cells (Kawasaki et al., 2000). It is likely that PA6 cells provide ES cells with a cue to trigger neurogenic pathways but its molecular nature and intracellular signaling mechanisms are unknown. In the present study, we have shown that sustained

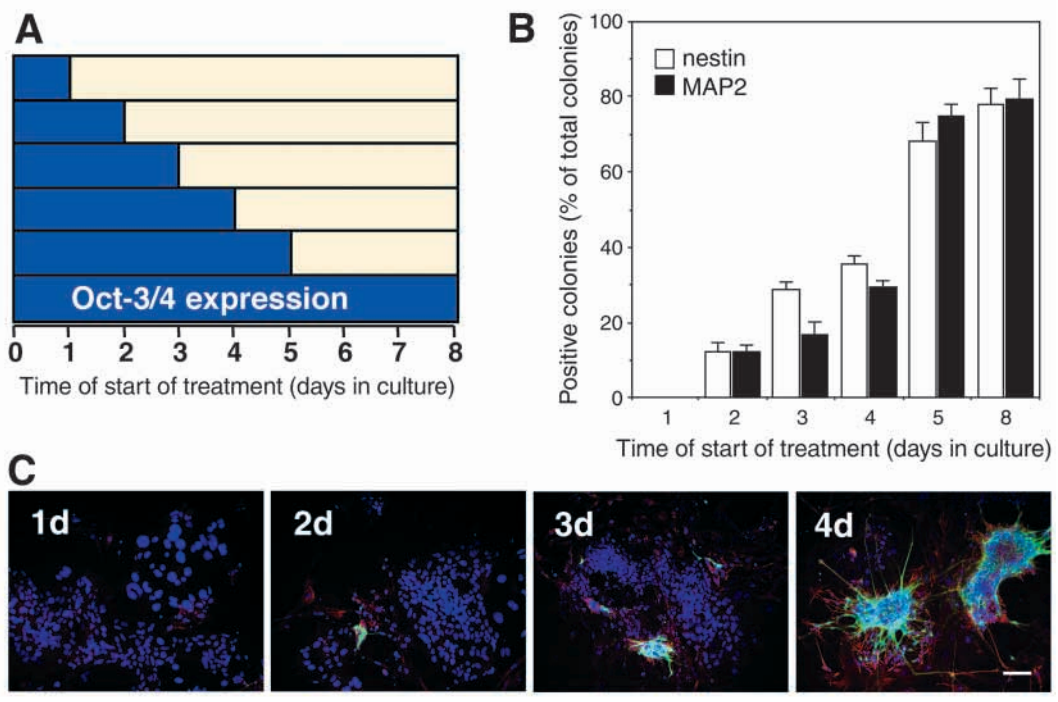


**Fig. 7.** Temporal requirement of Oct3/4 for SDIA-induced neurogenesis. ZHBTc4 cells were cultured on a PA6 monolayer for 8 days and stained with anti-nestin and anti-MAP2 antibodies.
(A) Tc was added to the medium on and after the indicated days to shut off the Oct3/4 expression until the end of culture.
(B) Frequencies of nestin- and MAP2-positive colonies with respect to the total number of colonies are indicated. Vertical bars indicate the s.d.
(C) Representative views of cultured ZHBTc4 cells immunostained on indicated days (anti-nestin, red; anti-MAP2, green). Scale bar, 100 μm.

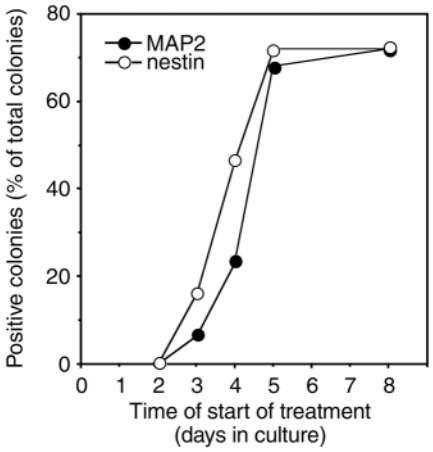


**Fig. 8.** Suppression of neural differentiation by Bmp2. ZHBTc4 cells were cultured on a PA6 monolayer for 8 days. Bmp2 (10 ng/ml) was added to the medium from the indicated days until the end of culture. On day 8, cells were stained for nestin and MAP2, and positive colonies were counted.

expression of Oct3/4 involves PA6-induced neurogenesis, and that forced upregulation of Oct3/4 in serum-free LIF-deficient medium efficiently induces neurogenesis of ES cells without feeder cells. This neurogenesis of ES cells was strongly suppressed by serum or Bmp2. However, addition of a Bmp-antagonist to the culture did not by itself enhance the neuronal differentiation. Thus, inhibition of Bmp signals is not the mechanism by which continuous Oct3/4 causes mouse ES cells to differentiate into neural lineages. It is suggested that Oct3/4 has an effect on ES cells to enhance their neuronal differentiation pathway under neurogenic culture conditions. It may be worth noting that the ZHBTc4 cells on O/F-coated dishes without SDIA, under serum-free LIF-deficient conditions showed efficient neurogenesis in the absence of Tc; conditions under which Oct3/4 is continuously expressed from the transgene (data not shown). When ZHBTc4 cells were stimulated by SDIA, the cells differentiated into neurons as effectively as ZHTc6 cells in the absence of Tc (Fig. 5), indicating that persistence, not up-regulation, of Oct3/4 induces neurogenesis. SDIA may contain a factor that cues ES cells into the neurogenic pathway as well as a signal that leads to sustained Oct3/4 expression. Although the signaling pathway that links SDIA and Oct3/4 remains to be elucidated, there are several orphan nuclear receptors that regulate the repression of Oct3/4 expression (Fuhrmann et al., 1999). In those genes, germ cell nuclear factor (GCNF) (Chen et al., 1994) contributes to the repression of Oct3/4 gene expression during early mouse embyogenesis (Fuhrmann et al., 2001). A possible mechanism whereby SDIA maintains Oct3/4 expression is that the signals from SDIA might control such negative regulators directly or indirectly. Although SDIA was reported to induce TH-positive dopaminergic neurons efficiently, we could not detect TH-positive neurons from ZHTc6 ES cells cultured on the O/F-coated dish without Tc. The level of Nurr1 expression in ZHTc6 cells that were forced to upregulate Oct3/4 expression was not as high as that observed in ES cells cultured on a PA6 monolayer (data not shown). Thus, it is suggested that SDIA promotes the differentiation of dopaminergic neurons via an unknown additional factor (or factors) besides the one that leads to the maintenance of Oct3/4 expression.

Recently, differentiation of ES cells into neuroectoderm-like cells was shown using HepG2 cell-conditioned medium (Rathjen et al., 2002). Maintenance of Oct3/4 expression was detected in ES cells during such neuroectoderm-like cell formation. Also, recent work on the zebrafish *spiel-ohne-grenzen* (*spg*) mutation, which causes abnomal hindbrain formation (Burgess et al., 2002; Reim and Brand, 2002), found that the *spg* mutation disrupts the *pou2* gene, which encodes a POU homeobox transcription factor. This work also showed by phylogenetic sequence analysis that pou2 is the zebrafish ortholog of mouse Oct3/4 and human POU5F1 and that mouse Oct3/4 can functionally replace zebrafish pou2 in a *spg* mutant rescue experiment. It is suggested that pou2 functions not only in an early developmental stage, like Oct3/4 in the inner cell mass of mammals, but also in a later stage when the brain primordium develops.

It was reported by Kawasaki et al. (Kawasaki et al., 2000) that the time course of neural marker induction by SDIA was, at least in part, reminiscent of that observed in the developing central nervous system (CNS). Others have also reported that neural stem cell formation from ES cells was preceded by a primitive neural stem cell stage (Tropepe et al., 2001). The term primitive neural stem cell has been used to describe a stem cell that retains a certain degree of pluripotency during the restricted early period of neural development when neuroectoderm is formed (Morrison et al., 1997). In our present study, sustained expression of Oct3/4 in ES cells promoted the formation of primitive ectoderm and subsequent neuroectoderm-like cells under serum-free conditions (Figs 1 and 2). In these processes, neural commitment seems to be determined during the first 5 days (Figs 7 and 8). The results of this study suggest that Oct3/4 is involved in the enhancement of neuroectoderm formation and subsequent neural differentiation.

It is known that the POU family of transcription factors can act as both transcriptional activators and repressors by cooperating with various co-factors. Several different co-factors for Oct3/4 have been reported. The Sry-related factor Sox2 was initially identified as a co-factor for Oct3/4 to activate the Fgf4 gene promoter (Yuan et al., 1995). Mouse Sox2 is expressed in the blastocyst ICM, embryonic ectoderm, and germ cells. After gastrulation, Sox2 is expressed in the neural tube from the earliest stage of its formation (Zappone et al., 2000). Sox2 is suggested to be essential for early neuroectoderm cells to consolidate their neural identity during the subsequent steps of neural differentiation. Oct3/4 and Sox2 are known to cooperate to activate the transcription of several genes, and it was recently reported that these two genes activate their own transcription (Tomioka et al., 2002). We have also found putative binding sites for Oct3/4 and Sox2 in both the promoter and enhancer regions of the *Sox2* gene, and observed the synergistic activation of the Sox2 promoter by Sox2 and Oct3/4 transcription factors in mouse E14.5 neuroepithelial cells (Supplemental data: http://dev.biologists.org/supplemental/). Sustained expression of Oct3/4 in ES cells under a LIF-deficient condition, as in our present study, may lead to the effective induction of Sox2 expression, which could be responsible for the effective induction of neuroectoderm formation. Thus, a possible role of sustained Oct3/4 expression in such a case could be to induce

Sox2 expression in cells that are on the way to neural differentiation under serum-free LIF-deficient condition where Smads and STAT3 signals are diminished. Although we have not yet clarified the relationship between Oct3/4 and other molecules concerned with neural differentiation, we speculate that Oct3/4 regulates the function of neural cell-inducing transcription factors in primitive neural development.

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