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# Induction of Midbrain Dopaminergic Neurons from ES Cells by Stromal Cell–Derived Inducing Activity

Neurotechnique

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## Summary

**We have identified a stromal cell–derived inducing activity (SDIA) that promotes neural differentiation of mouse ES cells. SDIA accumulates on the surface of PA6 stromal cells and induces efficient neuronal differentiation of cocultured ES cells in serum-free conditions without use of either retinoic acid or embryoid bodies. BMP4, which acts as an antineuralizing morphogen in *Xenopus*, suppresses SDIA-induced neuralization and promotes epidermal differentiation. A high proportion of tyrosine hydroxylase-positive neurons producing dopamine are obtained from SDIA-treated ES cells. When transplanted, SDIA-induced dopaminergic neurons integrate into the mouse striatum and remain positive for tyrosine hydroxylase expression. Neural induction by SDIA provides a new powerful tool for both basic neuroscience research and therapeutic applications.**

## Introduction

During the last decade, much progress has been made in the molecular understanding of early neural differentiation in *Xenopus*. Neural inducer molecules, such as Chordin, Noggin, and Follistatin, were identified, and several intracellular mediators of neural differentiation have been characterized (for review, see Hemmati-Brivanlou and Melton, 1997; Sasai and De Robertis, 1997; Sasai, 1998). By contrast, relatively little is known about regulatory factors in mammalian neural induction. One main reason for this is that good experimental systems for in vitro neural differentiation are still lacking in mice (i.e., something comparable to the animal cap assay commonly used in *Xenopus* studies).

Mammalian ES cells can differentiate into all embryonic cell types when injected into blastocyst-stage embryos (Bradley et al., 1984). This pluripotency of ES cells can be partially recapitulated in vitro by floating culture of ES cell aggregates, or embryoid bodies (EBs). After a few weeks of culture without LIF, EBs frequently contain ectodermal, mesodermal, and endodermal derivatives (for review, see Keller, 1995). Recent studies have shown that a superphysiological dose (up to 1 μM) of retinoic acid (RA) promotes neural differentiation in EBs (Bain et al., 1995; Li et al., 1998). Although this method can produce a good proportion of neural cells, it has two apparent problems. First, it is difficult to analyze and control each regulatory step of differentiation in this method because EBs contain many different kinds of cells, including mesodermal and endodermal cells. Second, RA, a strong teratogen, is supposed to perturb neural patterning and neuronal identities in EBs as it does in vivo (Soprano and Soprano, 1995; Sucov and Evans, 1995). For instance, RA treatment of early embryos causes suppression of forebrain development. Precise specification of a particular neuronal characteristic, such as neurotransmitter choice, is crucial when induced neurons are to be used for therapeutic applications or basic neuroscience research. It is therefore preferable to avoid RA treatment unless RA induces the particular type of neurons of one's interest.

In this report, we introduce an efficient system for in vitro neural differentiation of mouse ES cells in a serum-free condition that requires neither EBs nor RA treatment. We also discuss the possibilities for therapeutic application of this method, by which dopaminergic neurons are efficiently produced.

## Results

### Stromal Cells Produce Factors Supporting Neural Differentiation of Mouse ES Cells

By using a coculture system, we screened various primary culture cells and cell lines for activities promoting neural differentiation of ES cells under serum-free conditions (see the Experimental Procedures). Most of the cell types screened, including mouse embryonic fibroblasts (MEF), MDCK, and COS cells, did not significantly induce neural markers such as NCAM (pan-neural) in the overlying ES cells (Figure 1A; arrows, negative colonies; inset, phase contrast image of colonies). However, some stromal (or mesenchymal) cells promoted neural differentiation when used as feeders. A low but significant number of NCAM-positive colonies were observed in the presence of OP9 (stromal line derived from mouse calvaria) and NIH3T3 (an embryonic fibroblast line) cells (data not shown). PA6 cells (stromal cells derived from skull bone marrow; Kodama et al., 1986) induced remarkably efficient neural differentiation when cocultured with ES cells (Figure 1B), resulting in 92% of colonies ($n = 200$) becoming NCAM-positive by day 12.

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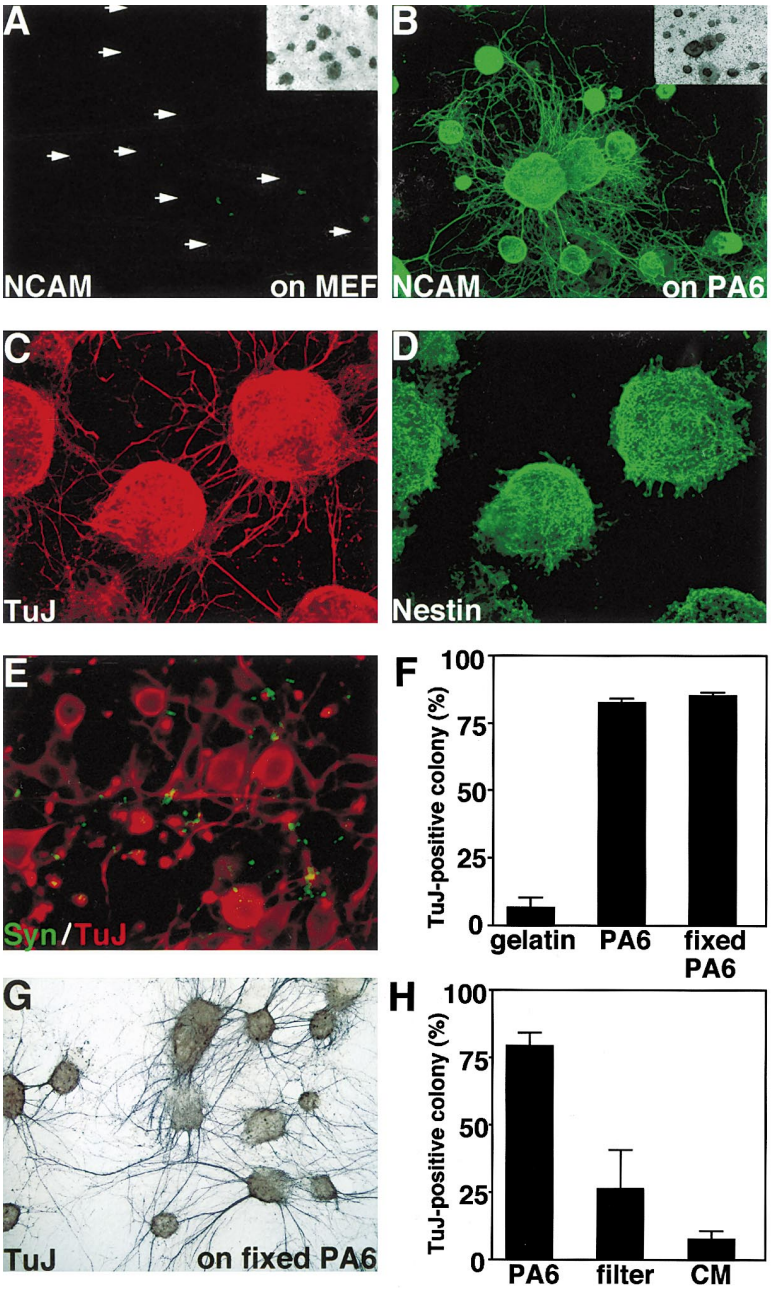


Figure 1. Neural-Inducing Factors Produced by PA6 Stromal Cells

(A and B) NCAM staining of ES cell colonies grown from a single cell for 8 days on MEF and PA6 feeder cells, respectively. Inset, phase contrast view.

(C and D) PA6-induced ES colonies double-stained with TuJ and nestin antibodies, respectively.

(E) The presynapse-specific marker synaptophysin (Syn, green) was detected on the induced neurons (TuJ, red).

(F) Percentages of TuJ-positive ES cell colonies on gelatin, PA6, and PFA-fixed PA6 after 8 days of induction.

(G) TuJ staining of ES cells cultured on PFA-fixed PA6 for 8 days.

(H) PA6 cells induce the neuronal marker TuJ in cocultured ES cells even when separated by a filter membrane (filter). The medium conditioned with PA6 for 3 days (CM) did not induce significant neural differentiation in ES cells cultured on gelatin. Arrows, negative colonies in immunostaining.

In these colonies, the majority of cells were stained with either the neuronal marker TuJ (β-tubulin type III; Figure 1C) or the neural precursor marker nestin (Figure 1D). Very few colonies contained GFAP-positive cells (2%, n = 200). The TuJ-positive neurons also expressed other neuronal markers such as MAP2 and neurofilament (data not shown), and the presynaptic marker synaptophysin (green; Figure 1E) was detected on the induced neurons (red, TuJ). To confirm that nestin-positive cells were neural precursors, cells were double-stained with nestin and RC2, a marker for neuroepithelium and radial glia (Misson et al., 1988). All of the nestin-positive cells were costained with RC2 antibody (n = 200; data not shown). We next tested whether neural differentiation induced by PA6 was accompanied by mesodermal induction. In contrast to the high NCAM-positive percentage, very few colonies expressed mesodermal markers such as PDGFR α, Flk1, and MF20 (all < 2% colonies, n = 100; data not shown) (Bader et al., 1982; Nishikawa et al., 1998). This is consistent with a previous report showing that PDGFR α and Flk-1 are induced in ES cells cocultured with OP9 cells but not in those cocultured with PA6 cells (Kataoka et al., 1997). Thus, PA6 can promote neural differentiation of cocultured ES cells without inducing mesodermal markers.

We next studied the role of PA6 in this induction. When cultured on a gelatin-coated dish in the same medium but without PA6, ES cells differentiated into neurons at a low frequency (Figure 1F, lane 1) compared with the efficient rate obtained with ES cells cultured

on PA6 (lane 2). This suggests that PA6 has an active role in the promotion of neural differentiation of ES cells. Interestingly, PA6 cells retained the neural-inducing activity even after being fixed with paraformaldehyde (PFA) (Figure 1F, lane 3, and Figure 1G). Furthermore, a similar activity became evident on other PFA-fixed stromal cells, including MEF, OP9, and NIH3T3 cells (data not shown). These cells without fixation, as mentioned above, showed weak neural-inducing activity if any at all. It is unlikely that the appearance of neuralizing activity is simply due to artifacts resulting from PFA fixation as this property was not observed in COS, MDCK, and 3Y1 cells regardless of fixation status (data not shown). This suggests that PA6-like inducing activity is present in a large variety of stromal cells. We speculate that stromal cells other than PA6 simultaneously produce inducing factors and inhibitory factors, the latter being inactivated upon PFA fixation and missing in PA6. We named this neural-inducing activity SDIA (stromal cell–derived inducing activity).

As fixed cells can no longer secrete factors, the neural-inducing activity of PA6, at least in part, should be mediated through something on the surface of stroma cells (i.e., membrane-anchored molecule[s] or surface matrix component[s]). We then tested whether direct physical contact between ES cells and PA6 cells was essential for the induction. ES cell colonies were cultured on gelatin-coated dishes and separated from cocultured PA6 cells by a 0.4 $\mu$m filter membrane. In the absence of physical contact, PA6 cells were still able to induce significant neural differentiation of ES cells cultured on gelatin (Figure 1H, lane 2), indicating that PA6 cells produce soluble inducing factor(s). However, PA6-conditioned medium could not elicit significant induction (Figure 1H, lane 3).

Together with the PFA fixation data, these results suggest two possibilities as to the molecular nature of SDIA. One is that SDIA consists of two different neural-inducing factors, a cell surface-anchored factor and a labile soluble factor. Another scenario might be that SDIA is mediated by secreted factors, which are secondarily tethered to the cell surface, as exemplified by Wnts and FGFs (Iozzo, 1998; Bernfield et al., 1999). At present, we cannot exclude either possibility, but the latter appears to be supported by our preliminary data that preincubation of PA6 with heparin before fixation removes the inducing activity from the cell surface (see the Experimental Procedures), as reported for Wnts (Bradley and Brown, 1990).

### BMP4 Suppresses Neural Differentiation and Promotes Epidermogenesis of SDIA-Treated ES Cells

In *Xenopus*, BMP4 inhibits neural differentiation of animal cap ectoderm and promotes epidermogenesis (Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995). Neural inducers have been shown to act by antagonizing BMP in the extracellular space (Hemmati-Brivanlou and Melton, 1997; Sasai and De Robertis, 1997). We therefore tested whether BMP4 has a similar effect on the neural differentiation of mouse ES cells induced by SDIA. Addition of 0.5 nM BMP4 resulted in clear suppression of NCAM and nestin induction ($<$4%, n $=$ 100; Figures 2A–2D). Since it was previously shown that BMP4, under different conditions, could induce mesodermal differentiation (Johansson and Wiles, 1995; Kanatsu and Nishikawa, 1996), we examined whether the suppression of neural fate by BMP4 was accompanied by induction of nonneural ectoderm or mesoderm. BMP4 treatment of ES cells on PA6 significantly increased the number of colonies positive for the nonneural ectoderm marker E-cadherin (Takeichi, 1988) (16% without BMP4, 75% with BMP4, n $=$ 100; Figures 2E and 2F) without inducing mesodermal markers such as PDGFR $\alpha$, FLK1, or MF20 significantly (all $<$ 5%; data not shown). Furthermore, after 11 days of culture, BMP4 induced Keratin 14 (early epidermal marker; Fuchs and Green, 1980) –positive colonies (0% without BMP4, 34% with BMP4, n $=$ 100; Figures 2G and 2H). However, as compared to efficient E-cadherin induction in BMP4/SDIA-treated ES cells, Keratin 14 induction was less dramatic (compare Figures 2F and 2H). We reasoned that BMP4/SDIA-treated ES cells became nonneural ectoderm (or early epidermal precursor) cells that were not mature enough to express Keratin 14 efficiently. Therefore, we attempted to promote maturation by adding serum, which is a mixture of various growth factors. Addition of serum during the last 3 days of induction promoted further epidermogenesis and generated large colonies with strong Keratin 14 signals (47%, n $=$ 100; Figures 2I and 2J). These results suggest that BMP4, in concert with SDIA, regulates an ectodermal binary decision for either epidermal or neural fate in mouse ES cells, as it does in *Xenopus*. A time course study (Figure 3A) showed that neural differentiation in ES cells was most sensitive to BMP4 during days 3–5 of the induction period. This correlates well with the observation that mouse ES cells resemble the inner cell mass (E4) in character (Bradley et al., 1984) and that neural fate is thought to be determined during gastrulation (E6.5$\sim$E8) (Kaufman and Bard, 1999), when the BMP-neural inducer antagonism is believed to play a role.

One important question that arises is whether PA6-derived factors act by antagonizing BMP4 in a similar manner as Chordin and Noggin (Hemmati-Brivanlou and Melton, 1997). Northern blot and RT-PCR analyses showed that PA6 cells do not express significant levels of mouse *Chordin*, *Noggin*, or *Cerberus* (data not shown). In our preliminary studies, neither transfection of pCMV-Chordin plasmid into ES cells nor addition of Follistatin protein (up to 5000 ng/ml) to culture medium induced significant neural differentiation of ES cells. To obtain more direct evidence, we actively blocked BMP signaling by administering neutralizing BMPR-Fc receptobody, which mimics the anti-BMP activities of neural inducers (Figure 3B). BMPR-Fc (150 ng/ml) efficiently reversed the inhibitory effect of BMP4 (0.5 nM) on SDIA-induced neural differentiation (lanes 3 and 4). However, the presence of BMPR-Fc did not affect the extent of neural differentiation in the ES cells cultured on PA6, fixed PA6, or gelatin (lanes 1, 2, and 5–8). BMPR-Fc did not affect neural differentiation of ES cells on live MEF (lanes 9 and 10). (This may suggest that lack of inducing activity in MEF cannot be explained by production of BMP-like activity.) These results demonstrate that inhibition of BMP signaling is not sufficient to mimic SDIA.

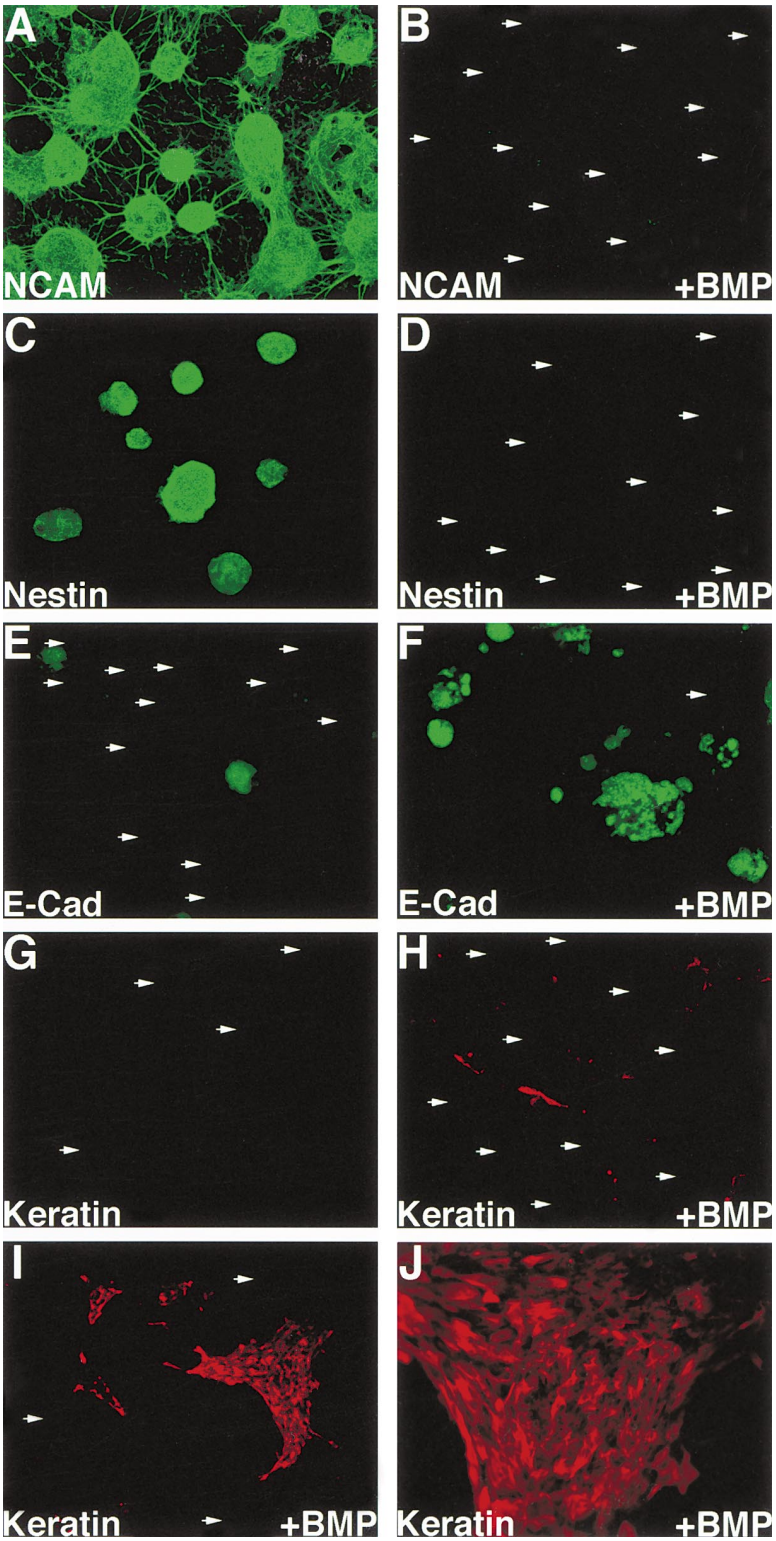


Figure 2. BMP4 Negatively Regulates Neural Induction by SDIA and Promotes Epidermogenesis

ES cells cultured on PA6 cells for 8 days (A–F) or 11 days (G–J) were stained with NCAM (A and B), Nestin (C and D), E-cadherin (E and F), or Keratin 14 (G–J) antibodies. In (B), (D), (F), and (H)–(J), 0.5 nM BMP4 was added to the medium. In (A)–(H), cells were cultured in serum-free differentiation medium with or without BMP4. In (I) and (J), 10% FCS was added during the last 3 days of the 11 day induction. (J) is an enlarged view of a colony in (I). Arrows, negative colonies in immunostaining.

Taken together, BMP antagonism is unlikely to explain the neuralizing activity of SDIA.

The molecular nature of SDIA remains to be identified. Our preliminary study has shown that none of the following treatments promoted neural differentiation of ES cells; bFGF, FGF8, Shh, a combination of FGF8 and Shh, HGF, EGF, PDGF, LIF, or LiCl (mimicking Wnt signaling). As SDIA is not detected in the conditioned medium (Figure 1H), it may be necessary to identify the SDIA-producing gene by functional expression screening.

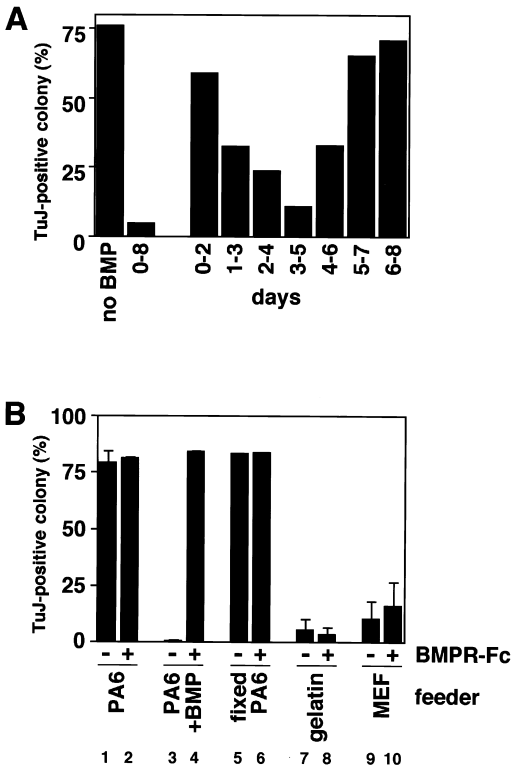


Figure 3. Suppression of Neural Differentiation by BMP4: Temporal Requirement and Blocking Study

(A) Time window of susceptiveness to antineuralizing activity of BMP treatment. ES cells were cultured on PA6 for 8 days, and TuJ-positive colonies were counted. BMP4 (0.5 nM) was added to the medium during the days indicated below the bar.

(B) BMPR-Fc (150 ng/ml) was added to the medium (+) or not (−). Although BMPR-Fc sufficiently antagonized antineuralizing activity of 0.5 nM BMP4 (PA6 + BMP), BMPR-Fc did not cause significant change in neuronal differentiation of ES cells cultured on PA6, fixed PA6, MEF, or gelatin.

### A High Proportion of Dopaminergic Neurons in SDIA-Induced Neurons from ES Cells

Immunohistochemical analyses of the characteristics of SDIA-induced neurons revealed that 92% of colonies contained tyrosine hydroxylase (TH)–positive neurons ($n = 200$; Figure 4A). This value was much higher than those for GABAergic, cholinergic, and serotonergic neuron markers (43%, 28%, and 7%, respectively). To estimate the efficacy of dopaminergic neuron induction at the cell level, the numbers of TuJ-, nestin-, and TH-positive cells were counted in randomly selected ES cell colonies on induction day 12. TuJ-positive neurons and nestin-postive cells represented 52% ± 9% and 47% ± 10% of total cells, respectively. TH neurons occupied 30% ± 4% of TuJ-positive neurons ($n = 5050$; i.e., 16% of total cells). This value is again significantly higher than percentages of GABAergic, cholinergic, and serotonergic neurons in TuJ-positive neurons (18% ± 8%, 9% ± 5%, and 2% ± 1%, respectively) at the cell level.

A time course study showed that TH-positive neurons appeared between days 6–8 of the induction period, following the appearance of nestin and tubulin markers (Figure 4B). These TH-positive neurons proved to be dopaminergic as they were negative for DBH (dopamine-β-hydroxylase; marker for norepinephrine and epinephrine neurons) even after 13 days of induction (Figure 4B). Consistent with the TH staining, the mesencephalic dopaminergic neuron markers *Nurr1* (Zetterstrom et al., 1997) and *Ptx3* (Smidt et al., 1997) were induced in SDIA-treated ES cells (Figure 4C).

To further confirm the generation of dopaminergic neurons, we examined dopamine production in the induced neurons by using reverse phase HPLC. In response to a depolarizing stimulus (56 mM $K^+$), ES cell–derived neurons released a significant amount of dopamine into the medium (7.7 pmol/$10^6$ cells; the dopamine peak is shown as DA in Figure 4D). Dopamine derivatives, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were also detected (2.5 pmol and 4.0 pmol/$10^6$ cells, respectively). These data show that functional neurons producing dopamine were generated with this method.

Although RA treatment of EBs is a commonly used method for inducing neural differentiation (Bain et al., 1995), such efficiency in the induction of dopaminergic neurons has not been previously reported. We treated EBs with 1 μM RA as described previously (4–4+ method) (Bain et al., 1995) and examined the dopaminergic marker TH. Less than 1% of EBs contained cells positive for TH. Interestingly, RA treatment (0.1–1 μM; days 4–8) suppressed SDIA-mediated induction of TH-positive neurons without reducing the number of TuJ-positive neurons (data not shown). These results indicate that the absence of RA from the culture medium is required for successful induction of dopaminergic neurons.

### Implantation of SDIA-Induced Neurons into the Mouse Brain

The data above indicate that the in vitro neural differentiation system mediated by SDIA may prove a good source of mesencephalic-type dopaminergic neurons, which are necessary for cell transplantation therapy of Parkinsonism. Therefore, we next tested whether SDIA-treated ES cells could be integrated into the mouse striatum after implantation. ES cell colonies were cultured on PA6 cells for 12 days, and detached en bloc from the feeders by a mild protease treatment without EDTA. In order to enrich for postmitotic neurons by eliminating mitotic cells, the SDIA-treated ES cells were treated with mitomycin C (MMC) before grafting.

The isolated ES cell colonies were then implanted into the mouse striatum, which had been treated with 6-hydroxydopamine (6-OHDA). 6-OHDA largely depleted dopaminergic neuron projections in the nigro-striatal system on the injected side (Figures 5A and 5B). Ipsilateral implantation of SDIA-induced neurons significantly restored TH-positive areas in and around the DiI-positive graft (Figures 5C and 5D). Control medium injection did not cause any changes in TH-positive regions (data not shown). Confocal microscopy images of the graft are shown in Figures 5E and 5F. After implantation of $4 \times 10^5$ SDIA-treated ES cells, $3.9 \pm 0.6 \times 10^4$ grafted cells (DiI; Figure 5E) were found in the brain on day 15, and 74% of them were TuJ-positive neurons (data not shown). Among them, $1.3 \pm 0.1 \times 10^4$ TH-positive neu-

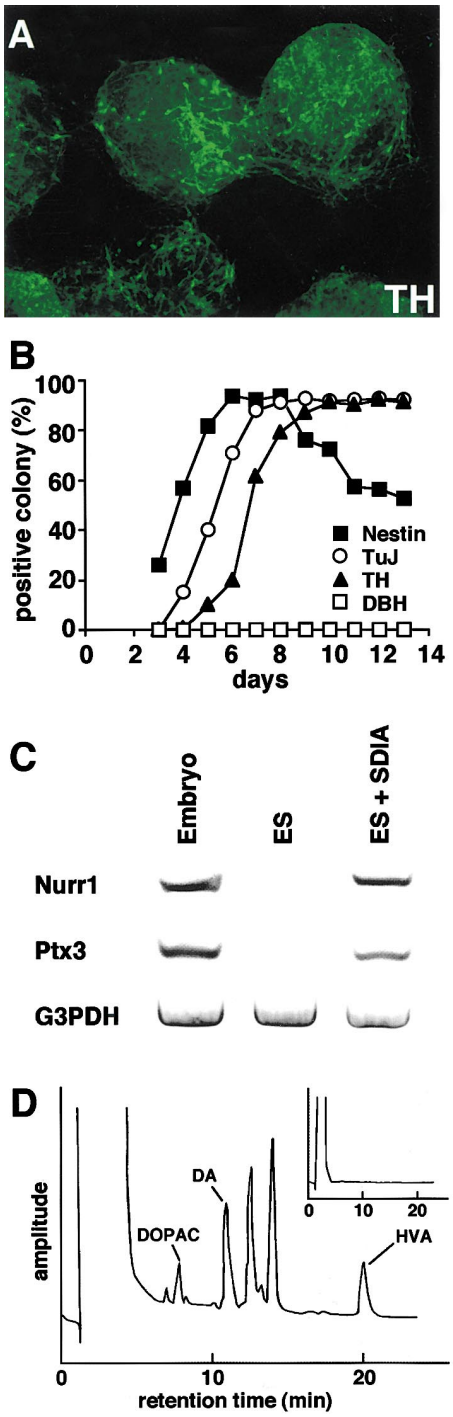


Figure 4. SDIA-Induced Neurons Contain a High Proportion of Dopaminergic Neurons

(A) Immunostaining of SDIA-induced neurons (day 10) with anti-TH antibody.

(B) Time course study of appearance of ES cell colonies with TH, DBH, TuJ, and Nestin-like immunoreactivities. Nestin appeared on day 3, TuJ on day 5, and TH between day 6–8 (the first induction day defined as day 0). DBH-positive neurons were not observed.

(C) RT-PCR analysis with markers (*Nurr1* and *Ptx3*) of mesencephalic dopaminergic neurons (day 12).

(D) Detection of dopamine secreted by SDIA-induced neurons. Reverse phase HPLC pattern with electrochemical detection is shown. DA, DOPAC, and HVA indicate peaks of dopamine, 3,4-dihydroxyphenylacetic acid and homovanillic acid, respectively. The inset shows chromatography of control medium (conditioned with feeder cells only).

rons (green in Figure 5F) were present. As the injected ES cell colonies initially contained $6 \times 10^4$ TH-positive cells, the survival rate of TH-positive neurons was ~22% after these procedures. No teratoma formation was observed in the grafted tissue by histological analyses ($n = 5$; data not shown).

## Discussion

### Neuronal Differentiation Induced by SDIA

In this study, we have introduced a new method for generating neurons from ES cells by using PA6-derived SDIA. This method is technically simple, and the induction is efficient and speedy. The SDIA method does not involve EB formation or RA treatment, and each differentiating colony grows from a single ES cell in two dimensions under serum-free conditions. Because of these features, the SDIA method has advantages over the EB/RA methods when used for detailed analyses of differentiation, as exemplified by the role of BMP4 in this study (Figures 2 and 3).

We found that mesencephalic dopaminergic neurons can be efficiently produced by our method. Recently, it was reported that dopaminergic neurons could be generated from neural precursor cells amplified from EBs (Lee et al., 2000). In contrast to our single-step method, they used the following four steps for the production of dopaminergic neurons: (1) generation of EBs without RA treatment in a serum-containing medium; (2) selection of nestin-positive cells; (3) expansion of nestin-positive cells with bFGF; and (4) induction of neuronal differentiation in serum-free medium. Since only small numbers of neural precursors are present in RA-untreated EBs, one needs to select and amplify nestin-positive cells for a long time (14 days) in this method before inducing differentiation (>24 days in total) (Lee et al., 2000). The avoidance of RA treatment concurs with our finding that RA is a negative factor for dopaminergic neuron generation. This four-step method produced TH-positive neurons at an efficiency of ~7% of TuJ-positive neurons in conventional medium. When Shh, FGF8, and ascorbate were added, the production increased to ~30%, which is comparable to the efficiency of our method.

The molecular basis of dopaminergic neuron induction in the SDIA method remains to be understood. We have tested those factors that have been implicated in the regulation of dopaminergic differentiation (Hynes and Rosenthal, 1999), such as FGF8, Shh, IL1, IL11, LIF, GDNF, or neutralizing antibodies of FGF8 and Shh. So far, we have not observed any marked effects on the induction of dopaminergic neurons. Interestingly, as discussed above, the efficiency of dopaminergic neuron induction in the SDIA method is as high as the maximum efficiency obtained in the four-step method with Shh, FGF8, and ascorbate treatment. One possible explanation for this is that the differentiation of dopaminergic

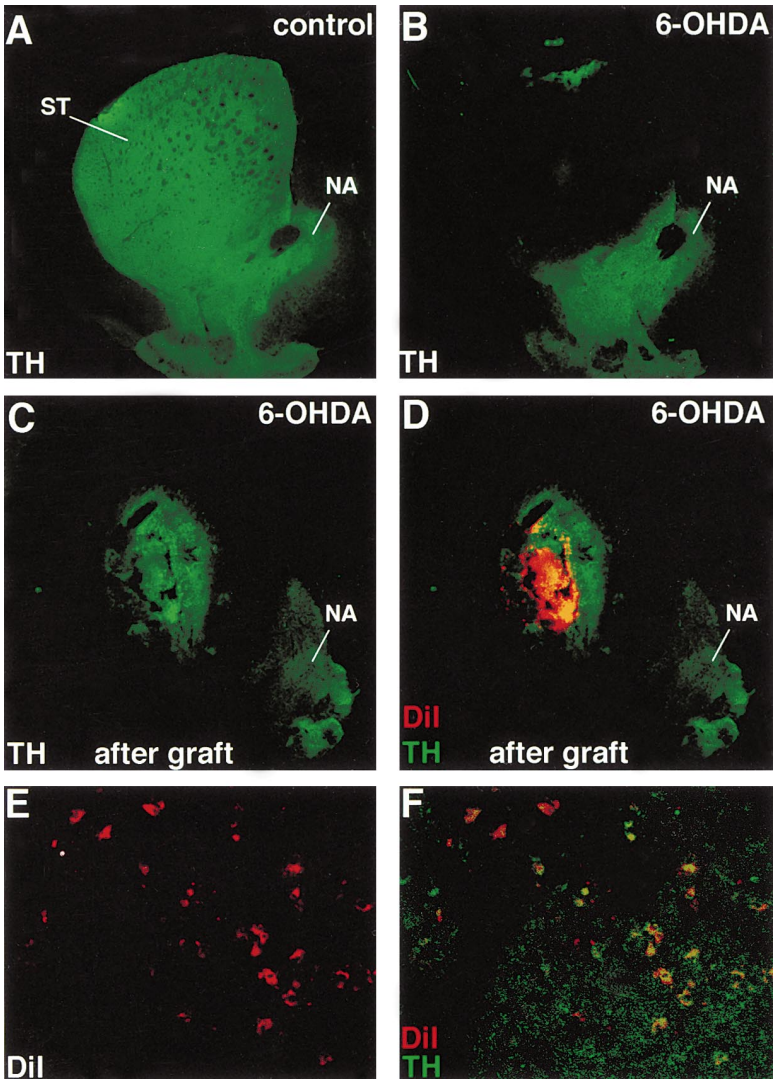


Figure 5. Integration of SDIA-Induced Dopaminergic Neurons in the Mouse Striatum

(A) TH staining of normal mouse striatum.

(B) Injection of 6-OHDA depleted TH-like immunoreactivity from the striatum (1 week after drug treatment).

(C and D) SDIA-induced ES cells were implanted into 6-OHDA-lesioned striatum 3 days after drug treatment. TH-positive region (green) recovered in and around the implanted cells (red, [D]) in the striatum 1 week after grafting. In our experiment, DiI labeled efficiently the cell bodies but not the axons. As a result, TH signals in the neurites of the grafted neurons expanded beyond the DiI positive area.

(E and F) Confocal microscopy images of the graft after 2 weeks. TH-positive neurons (green) in the striatum were derived from grafted ES cells (red, DiI). In (F), in addition to cell bodies, TH signals were also found in numerous neurites (green speckles) generated in the graft.

ST, striatum; NA, nucleus accumbens.

neurons in the SDIA system may be regulated somehow in an intrinsic manner (e.g., the default status), although further study is needed to address this question.

The SDIA-mediated in vitro differentiation method should provide a convenient experimental system for many aspects of neuroscience research, such as neural development, cell biology, neuropharmacology, and electrophysiology.

### Possible Roles of SDIA-like Activity in Early Neural Development

The time course of neural marker induction by SDIA (Figure 4B) is reminiscent of that observed in the developing central nervous system (CNS), at least in part. For instance, in this method, TH-positive neurons appear on the induction days 6–8, while TH expression is first detected in the fetal mouse midbrain on E11.5 (Foster et al., 1988). Given that ES cells behave like the inner cell mass (E4), the period required for TH induction in vitro correlates well to that seen in the embryo.

One important question is whether an SDIA-like activity functions in vivo during early neurogenesis. In vertebrates, the organizer is thought to play an important role in the induction and patterning of the CNS (Sasai and De Robertis, 1997). In mice, the primitive node is shown to be the organizer, and heterotopic graft of the node induces a secondary axis, including ectopic formation of neural tissues (Beddington, 1994). Interestingly, the CNS tissues can form in the mouse embryo even in the absence of the organizer. Most CNS structures form in the *HNF3*$\beta^{-/-}$ mouse, which lacks the node (Klingensmith et al., 1999). Furthermore, *noggin*/*chordin*-compound null mutant mice show severe defects in the forebrain, but not in the CNS tissues posterior to the midbrain (Bachiller et al., 2000). These results suggest that some factors other than the classical organizer-derived neural inducers (i.e., BMP antagonists) play an important role in the induction of mammalian neural tissues.

In our experiments, SDIA-induced neural differentiation of ES cells was strongly suppressed by a low dose of BMP4 (Figure 2). However, addition of a BMP4 blocking agent alone did not sufficiently induce neural differentiation (Figure 3B), although low levels of BMP2 and BMP4 are present in ES cells (Johansson and Wiles, 1995;

Winnier et al., 1995). Thus, inhibition of BMP signals is essential but not sufficient for mouse ES cells to differentiate into neural cells. One possible explanation is that some additional factors such as SDIA are required for neural differentiation before mouse cells make the neural/epidermal binary decision in a BMP-dependent manner. Consistent with this idea, the SDIA-treated cells acquire their highest sensitivity to BMP4 subsequent to the onset of nestin expression (day 3; Figures 3A and 4B). This indicates that SDIA has already exerted some effects (nestin induction) before the cells react to BMP signals.

An interesting observation regarding SDIA and BMP4 is that SDIA seems to suppress the mesodermalizing effects of BMP4 on ES cells. BMP4 promotes mesodermal differentiation of mouse ES cells and epiblasts cultured in serum-free media (Johansson and Wiles, 1995; Kanatsu and Nishikawa, 1996), as it does in the animal cap assay (Dale et al., 1992). However, when BMP4 is combined with SDIA, epidermogenesis, rather than mesodermal differentiation, is promoted (Figure 2). One explanation for this is that SDIA acts by stabilizing the ectodermal fate and protecting from mesodermalizing influences. If so, the following scenario might be applicable to the mechanism of neuralization occurring in SDIA-treated ES cells. First, ES cells cultured on PA6 move in an ectodermal direction under the influence of SDIA. SDIA-treated ES cells then adopt a default neural status unless they receive a sufficient level of BMP4 signals. In accordance with this model, explants of pre-streak epiblast (E 6.0) differentiate efficiently into neural cells on PA6 unless exogenous BMP4 is added to the culture media (H. K., K. M., and Y. S., unpublished data). However, as the molecular nature of SDIA remains to be elucidated, we must await further study to judge this proposition and to understand the relevant roles of SDIA in the embryo.

### Application to Cell Replacement Therapy

As this method produces a high yield of mesencephalic dopaminergic neurons, SDIA-induced neurons may provide a noninvasive alternative to embryonal brain tissues and neural stem cells for neuronal replacement therapy of Parkisonism (Lindvall, 1999; Gage, 2000). Long-term survival of SDIA-induced neurons and its functional consequences such as motor recovery are currently being investigated in parkinsonian mice. One advantage of using ES cells over neural stem cells is that genetic manipulations such as modifying histocompatibility are theoretically feasible by homologous recombination.

Before SDIA-treated ES cells can be applied to cell replacement therapy of Parkinsonism, important technical advances must be made at least in the following steps. First, we must test whether human ES cells differentiate into dopaminergic neurons in a manner similar to mouse ES cells. We infer that the same principles should be applicable to human cells, as the early development phase relevant to ES cell differentiation exhibits minimal differences across mammalian species in general. However, minor modifications may be necessary as some properties of human ES cells differ in culture conditions from mouse ones, such as LIF-independent growth (Thomson et al., 1998; Reubinoff et al., 2000). Second, we should develop an even more efficient way to enrich for dopaminergic neurons from the total population of differentiated ES cells. Sorting by flow cytometry or separating with magnetic beads should be feasible once appropriate surface antigens for early dopaminergic neurons become available. Third, we need to test the safety of ES cell therapy in long-term implantation studies. It may be beneficial to select only postmitotic neurons by eliminating dividing cells with MMC (and/or Ara C) before grafting to prevent tumor formation, as we did in this study.

To explore further possibilities for therapeutic application and basic neuroscience research, it will be worthwhile to study how to regulate the differentiation of SDIA-treated ES cells into many specific types of the CNS neurons.

### Experimental Procedures

#### Induction of Neural Differentiation of ES Cells

Undifferentiated ES cells (CCE or EB5) were maintained on gelatin-coated dishes in G-MEM supplemented with 1% FCS (JRH), 10% KSR (GIBCO-BRL), 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM pyruvate, 0.1 mM 2-mercaptoethanol (2-ME), and 2000 U/ml LIF (GIBCO-BRL). EB5 cells (a kind gift of Dr Hitoshi Niwa, Osaka University) carry the blasticidin S-resistant selection marker gene driven by the Oct3/4 promoter (active under the undifferentiated status) and were maintained in medium containing 20 μg/ml blasticidin S to eliminate differentiated cells. EB5 is a subline derived from E14tg2a ES cells (Hooper et al., 1987) and was generated by targeted integration of Oct-3/4-IRES-BSD-pA vector (Niwa et al., 2000) into the *Oct-3/4* allele.

For differentiation, ES cells were cultured to form colonies from a single cell on various feeder cells in G-MEM medium supplemented with 10% KSR, 2 mM glutamine, 1 mM pyruvate, 0.1 mM nonessential amino acids, and 0.1 mM 2-ME ("differentiation medium"). It was essential to remove serum from the media since the addition of FCS strongly inhibited neural differentiation. LIF also suppressed neural differentiation. ES cell colonies were grown at a density of $1 \times 10^3$ colonies per 3 cm dish. Medium change was performed on day 4 and every other day following that. EB5 and CCE behaved similarly in this study, and the data shown in this report were from experiments with EB5 cells. Reverse phase–HPLC analysis of dopamine was performed by using an electrochemical detector (the Monoamine Analysis System, Eicom Corp., Kyoto, Japan.) ES cells were cultured on PA6 for 8 days in differentiation medium and for an additional 6 days in G-MEM supplemented with N2, 100 μM tetrahydrobiopterin and 200 μM ascorbate, 2 mM glutamine, 1 mM pyruvate, 0.1 mM nonessential amino acids, and 0.1 mM 2-ME. After rinsing twice with HBSS, cells were incubated with HBSS containing 56 mM $K^+$ for 15 min. The medium was then collected, stabilized with 0.4 M perchloric acid and 5 mM EDTA, and kept at −80°C until being used for analysis.

#### Immunostaining and RNA Analysis

Immunohistochemistry was performed with the following antibodies; anti-NCAM, anti-TH, anti-GAD (GABAergic marker), and anti-VAChT (cholinergic marker) (Chemicon), TuJ (Babco), anti-nestin (Pharmingen), anti-GFAP and anti-synaptophysin (Sigma), RC2 and MF20 (Developmental Studies Hybridoma Bank), anti-PDGFR alpha and anti-Flk1 (Nishikawa et al., 1998), anti-E-cadherin (Takara), anti-Keratin 14 (Biomedia), anti-serotonin (Dia Sorin), and anti-DBH (PROTOS Biotech). Immunoreactivity of each antibody was confirmed with appropriate positive control tissues under the same conditions. RT-PCR was performed as described previously (Sasai et al., 1995), except for addition of 5% DMSO in the case of *Ptx3* cDNA detection to counter the high GC content. The primers used are as follows; *Nurr1*, TGAAGAGAGCGGACAAGGAGATC and TCTGGAGTTAAGAAATCGGAGCTG; *Ptx3*, AGGACGGCTCTCTGAA GAA and TTGACCGAGTTGAAGGCGAA; *G3PDH*, GACCACAGTCC ATGCCATCACT and TCCACCACCCTGTTGCTGTAG.

### PFA Treatment and Transfilter Assay of SDIA

PA6 cells were grown to confluency, fixed with 4% PFA for 15 min at room temperature, and rinsed with PBS several times. ES cells were seeded on the fixed feeder cells in a similar manner to live feeder cells. For pretreatment with heparin, PA6 cells were cultured for 2 days in the presence of 200 ng/ml heparin, rinsed with PBS, and then fixed with PFA.

For the filter assay, ES cells were seeded on gelatin-coated 6-well plates and PA6 cells were cultured on a filter membrane of cell culture inserts (Falcon).

### BMP Treatment

ES cells were cultured on PA6 cells in differentiation medium, and 0.5 nM BMP4 was added to the differentiation medium during the indicated days. BMP4 was freshly added at each medium change. For the epidermal differentiation assay, ES cells were cultured on PA6 for 8 days in differentiation medium and then for 3 days in the same medium or G-MEM supplemented with 10% FCS, 2 mM glutamine, 1 mM pyruvate, 0.1 mM nonessential amino acids, and 0.1 mM 2-ME. Keratin 14 was used as an epidermal marker, since it is the earliest epidermis-specific bona-fide marker while Keratin 8/18 expression starts earlier but is not limited to epidermis. BMP4 and BMPR-Fc were purchased from R&D Systems.

### Implantation Experiment into Mouse Striatum

For 6-OHDA treatment (Kaneko et al., 2000), 8- to 9-week-old 129/Sv mice were anesthetized with pentobarbital and fixed on a stereotactic device (Narishige). 6-OHDA was dissolved in PBS (8 $\mu$g/$\mu$l), and 0.5 $\mu$l was injected unilaterally with a glass needle at three sites into the striatum. All injection coordinates were as according to the atlas of Franklin and Paxinos (1997) using the bregma as a reference and were as follows: (A + 0.5, L + 2.0, V + 3.0) (A + 1.2, L + 2.0, V + 3.0) (A + 0.9, L + 1.4, V + 3.0). TH signals had largely disappeared from the injected striatum 3 days after 6-OHDA treatment, when differentiated ES cells were implanted into the ipsilateral striatum. Animal experiments were performed in accordance with institutional guidelines.

ES cells were cultured on PA6 cells for 8 days in differentiation medium and for an additional 4 days in G-MEM supplemented with N2 (GIBCO-BRL), 1 mM pyruvate, 2 mM glutamine, 0.1 mM nonessential amino acids, and 0.1 mM 2-ME. The change of culture medium during the last 4 days of culture increased the number of TH-positive neurons by ~30%. At the end of culture in differentiation conditions, the cells were incubated with MMC for 2 hr to eliminate mitotic cells, but not postmitotic neurons. ES colonies were detached en bloc from feeder cells by treatment with papain for 5 min at room temperature (Papain Dissociation Kit, Worthington). Clumps of differentiated ES cells were then labeled with 5 $\mu$g/ml CM-DiI in PBS + 4 mg/ml glucose for 20 min at room temperature. ES cell clumps were rinsed twice with G-MEM + N2, and then suspended in G-MEM + N2 at $4 \times 10^5$ cell equivalents/$\mu$l. By using a blunt-ended 26G Hamilton syringe, 1 $\mu$l of ES cell suspension was slowly injected into the striatum (A + 0.9, L + 2.0, V + 3.0) over a 3 min period. In controls, 1 $\mu$l of suspension medium was injected. In our experiment, the cell lineage tracer CM-DiI stained the cell bodies of the grafted neurons strongly but the neurites only weakly. In contrast, TH marker is stronger in the axons.

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