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Effect of leukemia inhibitory factor on embryonic stem cell differentiation: implications for supporting neuronal differentiation[1]

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Key words

leukemia inhibitory factor; embryonic stem cells; neural fate; embryoid bodies

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Abstract

Aim: Leukemia inhibitory factor (LIF), a pleiotropic cytokine, has been used extensively in the maintenance of mouse embryonic stem cell pluripotency. In this current work, we examined the effect of the LIF signaling pathway in embryonic stem (ES) cell differentiation to a neural fate. **Methods:** In the presence of LIF (1000 U/mL), the production of neuronal cells derived from embryoid bodies (EB) was tested under various culture conditions. Inhibition of the LIF pathway was examined with specific inhibitors. The effects of cell apoptosis and proliferation on neural differentiation were examined. ES cell differentiation into three-germ layers was compared. **Results:** Under various culture conditions, neuronal differentiation was increased in the presence of LIF. Blocking the LIF-activated STAT3 signaling pathway with specific inhibitors abolished the neuronal differentiation of ES cells, whereas inhibition of the LIF-activated MEK signaling pathway impaired the differentiation of ES cells toward a glial fate. LIF suppressed cell apoptosis and promoted cell proliferation during ES cell differentiation. LIF inhibited the differentiation of ES cells to both mesoderm and extraembryonic endoderm fates, but enhanced the determination of neural progenitors. **Conclusion:** These results suggest that LIF plays a positive role during the differentiation of ES cells into neuronal cells.

Introduction

Leukemia inhibitory factor (LIF) is capable of maintaining embryonic stem (ES) cells in a pluripotent state through promoting self-renewal or suppressing stem cell differentiation[1–5]. It has become a standard protocol to use LIF to maintain murine ES cell pluripotency, whereas withdrawal of LIF allows ES cells to undergo cell differentiation[1–7]. Upon withdrawal of both LIF and feeder cells, ES cells are able to differentiate spontaneously into various cell types in three primitive layers[8–11].

As a pleiotropic factor, LIF has been proven to have a wide array of actions during the process of neural progenitor cell differentiation into neuronal and glial cells[12–19]. In the central nervous system, the LIF signaling pathway synergistically functions with other signaling pathways to inhibit the differentiation of neural stem cells along a glial lineage[12,13]. However, LIF downstream factor STAT3, in combination with other factors, selectively promotes fetal neural stem cell differentiation toward a glial fate[14–16]. During neural crest cell differentiation, LIF enhances the survival of sensory neurons and stimulates their formation from neural crest progenitor cells[17–19]. When ES cells are cultured in a chemically defined serum-free medium designed for neuronal cell culture, LIF alone is insufficient to sustain ES cell renewal[5]. Furthermore, when cultivated in low-density and serum-free conditions, ES cells with neural progenitor properties can be identified. It seems that LIF is critically required under these conditions for ES cells to undergo neuronal colony formation[20]. These observations form the

basis for the hypothesis that LIF might have a role in mediating the neural development of ES cells.

ES cell transition from pluripotency towards a specific cell type involves the actions of multiple cytokines. Removing LIF from culture medium seems to allow ES cells to differentiate into nearly all kinds of cell types, but it is unclear if LIF plays a role during ES cell differentiation towards a specific cell type[8–11,21]. In previous studies, either in retinoic acid (RA)-induced or non-induced ES cell differentiation, LIF was removed from culture medium. However, whether LIF plays a role in inhibiting neural differentiation during this process remains unknown[22–24,27,28].

To better understand whether LIF potentially exerts a role during the conversion of ES cells to a neuronal fate, embryoid bodies (EB) were obtained from ES cells and plated for further culture in a serum-containing medium either with (LIF+) or without LIF (LIF–), and then analyzed for cell differentiation.

Materials and methods

ES cell culture and embryoid body formation The mouse ES cell line D3 was obtained from the ATCC (American Type Culture Collection) organization, and was maintained in an undifferentiated state with feeder cells in the presence of LIF (ESGRO; Chemicon USA)[1–5]. The standard culture medium used in this work for maintaining ES cells contained the following components: high glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 15% fetal calf serum (Hyclone USA or Sijiqing China), 0.1 mmol/L β-mercaptoethanol (Gibco USA), 1 mmol/L sodium pyruvate (Gibco USA), 1% non-essential amino acids (Gibco, USA), 2 mmol/L glutamine (Gibco, USA), 0.1 mg/mL penicillin-streptomycin (Gibco, USA). LIF was added into the culture medium at a final concentration of 1000 U/mL before use. For cell differentiation induction, ES cells were dispersed into a single cell suspension with 0.25% trypsin (Gibco, USA). Hanging drops, each of which contained approximately 1000 cells in a 20 μL volume of culture medium, were maintained for 3 d on the lids of dishes filled with phosphate-buffered saline (PBS)[22–24]. EB that formed in the hanging drops were then plated and cultured on uncoated Petri dishes or cover-slips (Fisher) for 3 d. Prior to hanging drop culture, the expression of pluripotent marker genes Oct3/4, alkaline phosphatase (AP) and SSEA-1 was examined to ensure that the ES cells were indeed in an undifferentiated state (data not shown)[1–2]. The optimal procedure, shown in Figure 1A, includes suspension of ES cells for 3 d to form an EB (3 d EB), followed by plating EB cells for an additional 3 d (6 d EB). To assess the potential role of LIF during EB differentiation, the same concentration of LIF as that used in ES cell-maintaining medium was either included (LIF+, 1000 U/mL) or excluded (LIF–) in culture medium during the suspension and plating processes.

EB treatment For RA induction, 6 d EB were treated with 10^{-6}–10^{-5} mol/L all-trans RA (Sigma) for an additional 3 d and then transferred to fresh medium without LIF[22–24]. To block the LIF downstream signaling pathway, MEK1/2 specific inhibitor PD98059 (Calbiochem, USA) was dissolved into 100% EtOH and was added into culture medium with LIF at a final concentration of 2 μmol/L during either EB formation or EB plating; JAK/STAT3 specific inhibitor cucurbitacin I (Calbiochem, USA) was also dissolved into 100% EtOH and used to inhibit JAK/STAT3 function at a concentration of 300 nmol/L. The 6 d EB were fixed and analyzed with immunostaining.

Immunocytochemical assays SSEA-1 was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, USA). The monoclonal antibody Tuj1, a neuron-specific marker, was obtained from Sigma (St Louis, MO, USA), and the antibodies GFAP (Dako), a glial marker, and the antibodies against GATA-4, an extraembryonic endoderm marker, and brachyury, a mesoderm marker, were from Santa Cruz (Santa Cruz, CA, USA). For immunocytochemical assays, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 10 min, permeabilized with 0.1% Triton X-100/PBS for 1 min, washed with PBS 3 times, and blocked with 5% normal goat serum in PBS for 1.5 h. Samples were then incubated with primary antibodies in 10% NGS (normal goat serum)-PBS at 4°C for 12 h. After extensive washing with PBS, samples were incubated with secondary antibodies in 10% NGS-PBS for 1 h, washed again with PBS and then mounted in mowiol (Sigma USA). Specimens were analyzed under a confocal microscope (Zeiss L510 or Leica confocal microscope).

Cell proliferation and apoptosis assays Bromodeoxyuridine (BrdU; Sigma USA) was used to label proliferating cells in 5 d, 8 d (RA treated for 2 d) and 10 d (RA treated for 3 d and cultured for an additional 1 d) EB. BrdU was added into the culture medium at a concentration of 10 nmol/L for 12–16 h. Cells were washed with PBS for 10 min and fixed with 4% PFA for 15–20 min, then washed with PBS again for 15 min. Samples were then incubated in 2 N HCl for 5–0 min, followed by PBS washing for 15 min. EB were rinsed with $Na_2B_4O_7$ (0.1 mol/L) for 5–10 min to neutralize the HCl. Samples were then immunostained with BrdU antibodies and analyzed with a confocal microscope. TUNEL (Promega, USA) staining was performed according to the manufacturer's

instructions. Specimens were mounted by mowiol and analyzed with a confocal microscope.

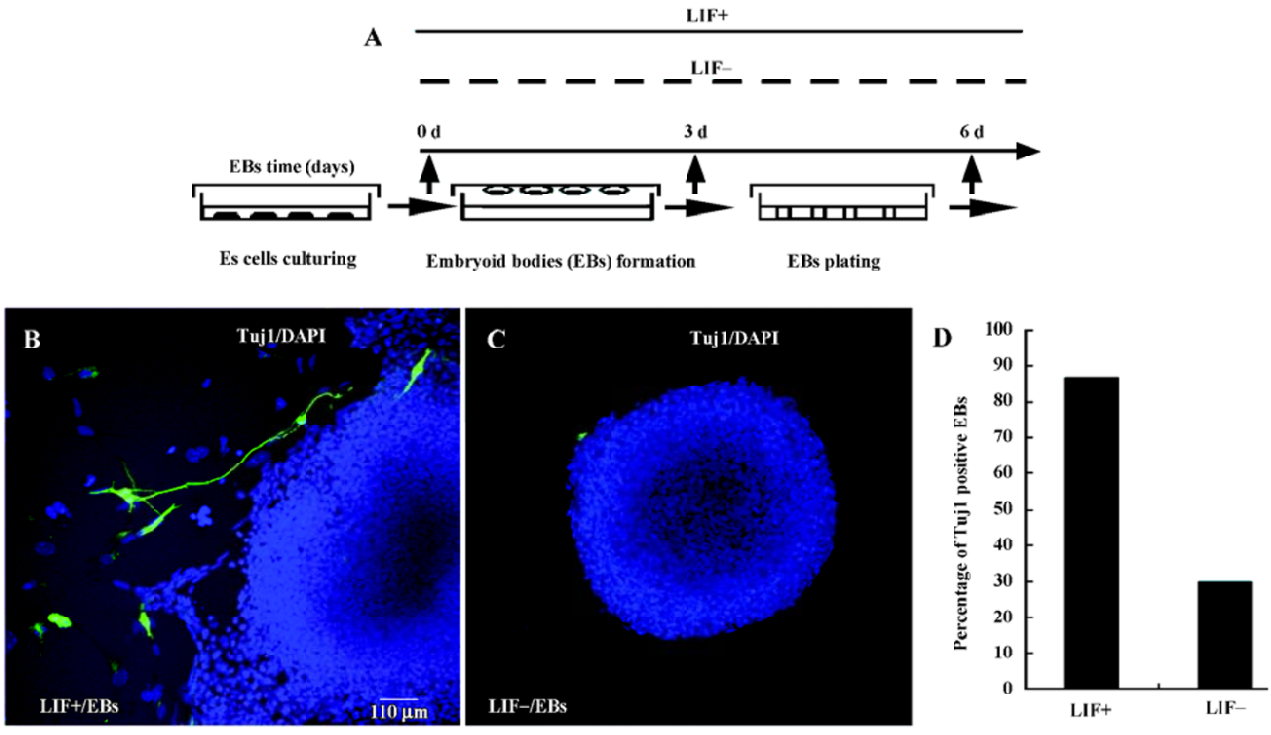
Statistics of apoptosis and proliferation EB were scanned into stacks of images (5 μm/images) with a confocal laser microscope. The middle image for each EB was used, and the number of TUNEL- or BrdU-labeled cells in the image was automatically counted by IPP (Image-Pro-Plus) software using the same parameters. The area of EB on each image was enclosed with the lasso or marquee tool of Photoshop software. We read out the pixel value of the enclosed area in a histogram. The number of BrdU- or TUNEL-labeled cells divided by the pixel value is the relative density of BrdU- or TUNEL-labeled cells per image, respectively. Thirty EB were counted in each case. Student's *t*-test was used on these data.

Results

Production of neuronal cells from ES cells was enhanced by LIF under various differentiation conditions ES cells in hanging culture formed spheroid aggregates, known as EB, that can differentiate into a large variety of cell types[8,9,21–29]. In previous reports, EB formation and differentiation occurred under LIF-free conditions[22–24]. However, studies regarding neural progenitor cell differentiation and ES cell neuronal fate commitment imply that LIF might play roles in ES cell neuronal transition. To assess this potential function we cultured LIF under various conditions and investigated neuronal differentiation thereafter.

The expression of neuronal specific marker Tuj1 was analyzed by immunostaining primary 6 d EB cultured in either LIF+ or LIF– medium. As shown in Figure 1, numerous Tuj1-positive cells were seen surrounding or within LIF+ 6 d EB (Figure 1B), whereas Tuj1-positive cells were rarely observed in LIF– 6 d EB (Figure 1C). The percentage of the primary EB that contained Tuj1-positive cells in LIF+ medium (27/31) was approximately two times greater than that for EB cultured in LIF– medium (12/40) (Figure 1D). Moreover, the average number of Tuj1-positive cells per EB was only 2 in LIF– medium (Figure 1E), whereas LIF+ EB had an average of 8.5 Tuj1-positive cells per EB, with up to 20 positive cells in some extreme cases (Figure 1E; these results were from 6 replicated experiments). To test whether different batches of sera, different numbers of cell passages, or different cell lines could potentially influence the result, we repeated the whole experiment with different batches of sera, with ES cells with various numbers of passages (8, 12, 15, 20 passages), and with the feeder-free E14.1 ES cell line, and obtained similar results (data not shown)[30,31]. LIF is thus strongly implicated in promoting the differentiation of ES cells to neuronal cells.

To verify whether LIF has dose-dependent or time-window effects on ES cell differentiation to neuronal cells, we cultured EB in media containing different concentrations



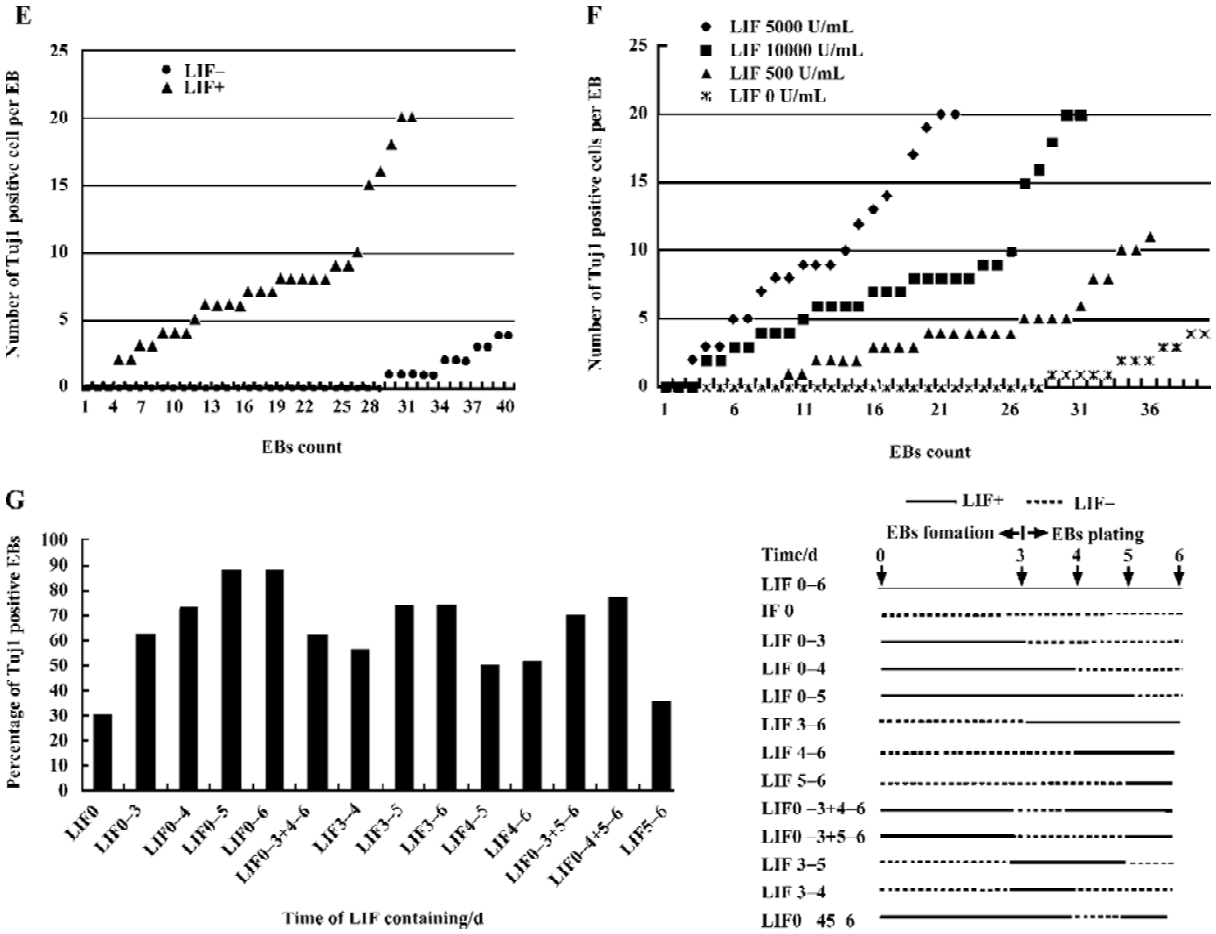


Figure 1. Neuronal differentiation is enhanced in EB cultured with LIF. (A) Schematic presentation of experimental protocol used for EB differentiation (0 d, 3 d and 6 d indicates the duration of EB culture). (B, C) Tuj1 expression in the primary 6 d EB was detected through indirect fluorescence immunostaining. Tuj1-positive cells were detected in the peripheral area of EB in the presence of LIF (B), but were rarely seen in the absence of LIF (C). (D) The percentage of EB containing Tuj1-positive cells increases under LIF+ conditions. EB were cultured with either LIF+ or LIF–conditions and the number of 6 d EB containing Tuj1-positive cells was counted. (E) The number of Tuj1-positive cells per EB is increased in the presence of LIF. Out of 31 primary 6 d EB counted in (E), 27 EB were positive for Tuj1 in the presence of LIF. The Tuj1-positive cells within each EB are represented by triangles. In the absence of LIF, out of 40 primary EB counted in (E) only 12 EB were Tuj1 positive, and Tuj1-positive cells in each EB are represented by stars. (F) LIF treatment produced a dose-dependent effect on neuronal differentiation. EB were cultured in a LIF concentration gradient of 5000 U/mL, 1000 U/mL, 500 U/mL and 0 U/mL, respectively. The Tuj1-positive cells per EB in cultures with higher concentrations of LIF outnumber those in cultures with lower concentrations (5000 U/mL>1000 U/mL>500 U/mL>0 U/mL). (G) LIF has a positive role in neuronal differentiation of ES cells. LIF was added to the culture medium at different time points as illustrated in the table G. In 6 d EB the number of Tuj1-positive cells per EB was counted. Compared with EB cultured in LIF+ medium, regardless of when LIF was added, the LIF– (LIF0) EB had the least capacity for neuronal conversion. EB cultured in LIF-containing medium for 5 d (LIF0–5) had the highest capacity for neuronal conversion, similar to the EB in LIF-containing medium for 6 d (LIF0–6).

of LIF (5000 U/mL, 1000 U/mL, 500 U/mL or 0 U/mL). The numbers of Tuj1-positive cells within each EB were counted (the experiment was repeated 3 times). The Tuj1-positive cells per EB when treated with a higher concentration of LIF outnumber those observed when treated with a lower concentra-tion, indicating that the enhancement of neuronal differentiation is indeed dose-dependent within the limited concentration threshold (Figure 1F). To investigate the step at which LIF exerts its action in the course of EB plating, LIF (1000 U/mL) was included in the culture medium at different time points. We found that neuronal differentiation was obviously enhanced by the addition of LIF between d 0 and d 5 of culture, especially between d 3 and d 4 (Figure 1G). Compared with EB cultured in LIF+ medium, irrespective of when LIF was added, the LIF– EB had the least neuronal conversion capacity (Figure 1G).

To investigate whether cell-cell interactions could result in the promotion of neuronal conversion in LIF+ medium, we cultured ES cells on cover slides at low density for 6 d to form a monolayer either with or without LIF. After 6 d in culture, some ES cells in LIF+ medium differentiated into Tuj1-positive cells, either in groups or individually (Figure 2A), although the ratio was very low. In contrast, there was no Tuj1-positive cell observed in the culture with LIF-free medium (Figure 2B). In classical RA-induced neuronal differentiation experiments, ES cells are cultured under LIF– conditions, although there is no evidence indicating that LIF inhibits the neuronal differentiation of ES cells[22–24]. To test the potential possibility that LIF also has a positive effect on the RA-induced neuronal differentiation of ES cells, we carried out RA induction assays. We found that LIF did enhance neuronal differentiation in the presence of RA, as evidenced by the fact that LIF+7 d EB (with RA induction for 1 d) generated more Tuj1-positive cells (Figure 2C) than did LIF–7 d EB (Figure 2D). Our results strongly imply that the LIF signaling pathway has a positive role during the neuronal differentiation of ES cells.

LIF signaling pathway is involved in the differentiation of ES cells to a neural fate LIF plays various roles in different cell types through gp130-mediated JAK/STAT3 and ERK/MEK signaling pathways[32–37]. If LIF does have a positive role in the transition of ES cells to a neuronal fate, this can be validated by inhibition of the LIF-activated signaling pathway. The MEK and STAT3 pathways can be inhibited by different specific chemical inhibitors[37,38]. When the JAK/STAT3 pathway was blocked by the specific inhibitor cucurbitacin I (300 nmol/L)[38] during EB formation or EB plating, neuronal differentiation was largely abolished (Figure 3B). Furthermore, when ES cells were treated with MEK1/2 specific inhibitor PD98059[37] (2 mmol/L) during either EB formation or EB plating, Tuj1-positive cells appeared within or surrounding EB, but these Tuj1-positive cells had a round shape without a long neurite (Figure 3A). Thus, blocking either LIF downstream signaling pathway interfered with LIF-enhanced ES cell neuronal differentiation. However, glial fibrillary acidic protein (GFAP)-positive glial cells are slightly reduced in the presence of LIF (Figure 3C,3D). Inhibition of the STAT3 pathway increased the production of GFAP-posi-

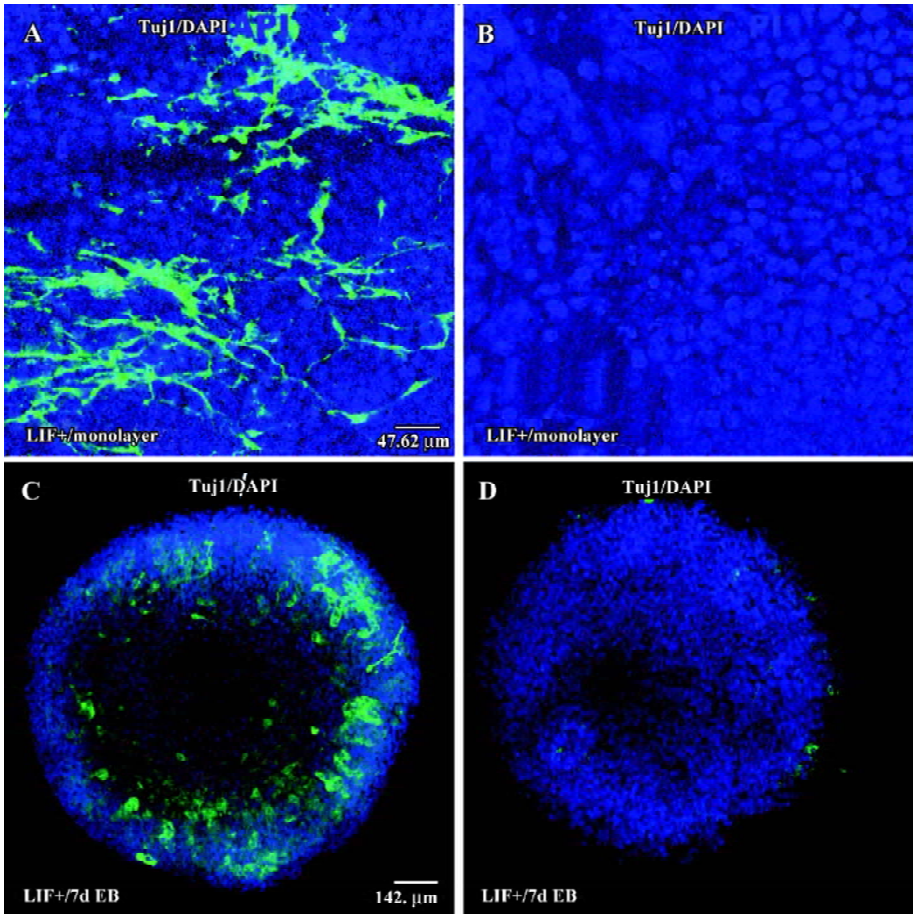


Figure 2. Neuronal conversion is enhanced in either ES cell monolayer culture or RA-induced neural differentiation of ES cells. ES cells were cultured for 6 d to form a monolayer in either LIF+ or LIF– medium. Cells were fixed with 4% PFA and subjected to Tuj1 immunostaining. (A,B) In the presence of LIF, Tuj1-positive cells, although in small amounts, were observed (A). In the absence of LIF, Tuj1-positive cells were seen only very rarely (B). (C, D) Six-day EB were treated with RA for 1 d under LIF+ (C) or LIF– (D) conditions; 7 d EB (RA induction for 1 day, C,D) were fixed and immunostained with anti-Tuj1 antibody. The production of Tuj1-positive cells in (C) was dramatically increased in comparison with that in (D).

tive cells (Figure 3B), whereas there was no GFAP-positive cell observed when the MEK pathway was inhibited (Figure 3A). This result indicates that both LIF-activated signaling pathways are involved in the regulation of glial differentiation from EB.

LIF inhibits cell apoptosis and enhances cell proliferation during EB differentiation To examine whether apoptosis and proliferation are also involved in the course of LIF-enhanced ES cell neuronal differentiation, TUNEL and BrdU incorporation assays[1,39] were performed in 5 d either in LIF+ (Figure4A,4D) or in LIF– medium (Figure4B,4E), respectively. In the presence of LIF, cell apoptosis was inhibited (Figure 4A–4C) and cell proliferation was enhanced (Figure 4D–4F) during both EB plating and RA treatment. However, LIF did not selectively suppress neuroectodermal precursor apoptosis and promote neuroectodermal precursor proliferation. In BrdU and TUNEL assays with nestin staining, we observed that nestin-positive cells exhibited signs of apoptosis and proliferation (data not shown).

LIF selectively enhances the commitment of neural progenitor from ES cells ES cells are capable of differentiating into various types of cells, whereas the differentiation of cardiomyocytes derived from EB can be hindered by LIF[29]. It remains unclear whether LIF selectively promotes the production of neuroectodermal precursor cells, or if LIF also has a role in mesoderm or extraembryonic endoderm cell lineage commitment. Under LIF+ conditions, a large number of nestin-positive cells appeared within or surrounding EB (Figure 5A), whereas EB cultured without LIF had a small number of nestin-positive cells (Figure 5B), indicating that LIF promotes the production of neural progenitors from ES cells. We also evaluated the expression levels of the mesoderm marker Bra (Brachyury) in 6 d EB cultured either with or without LIF[40]. The number of brachyury-positive cells in 6 d EB with LIF was significantly smaller than that for 6 d EB without LIF (Figure 5B,5C). In addition, it has been reported that ectopic GATA-4 expression is sufficient to induce ES cell differentiation into extraembryonic endoderm[41]. We found that in the absence of LIF the 6 d EB had a larger number of GATA-4-expressing cells surrounding the EB, whereas GATA-4 expression was dramatically reduced in 6 d EB with LIF (Figure 5D,5E). We therefore suggest that LIF

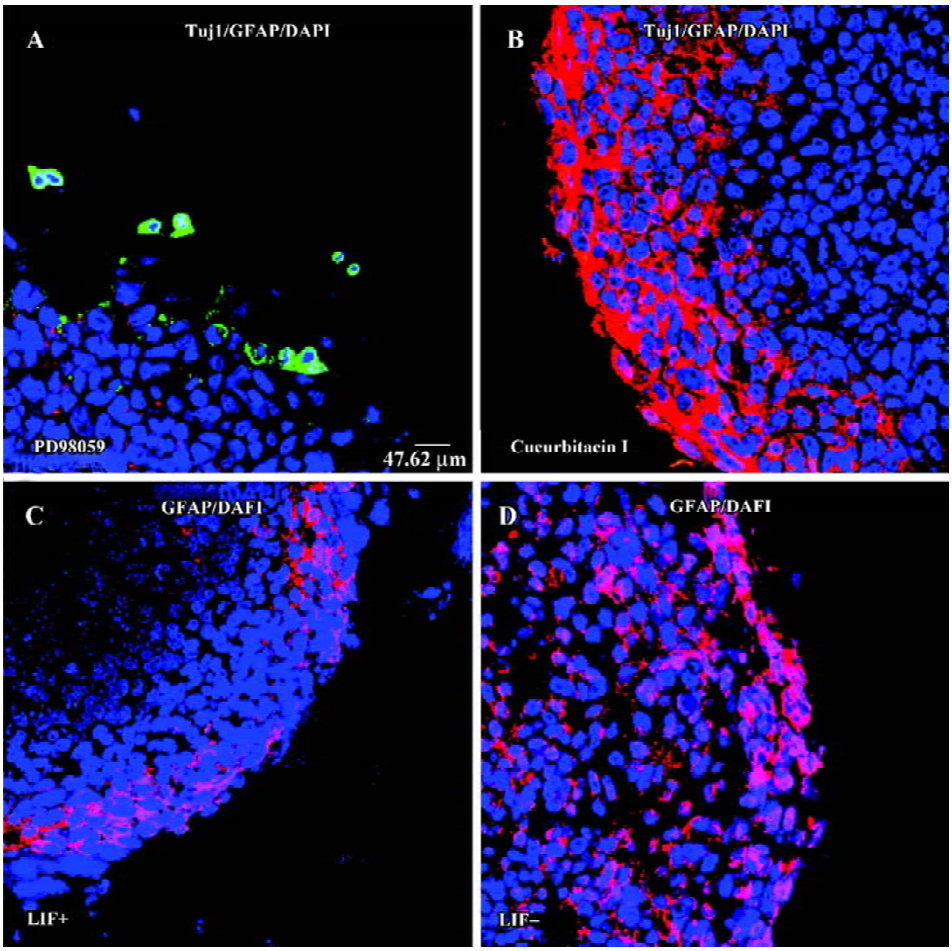


Figure 3. The LIF signaling pathway promotes conversion of ES cells to a neuronal fate. (A) Inhibition of LIF-activated MEK pathway with PD98059; Tuj1+ cells appeared within or surrounding EB, but there were no GFAP+ cells. (B) When the LIF-activated STAT3 pathway was blocked with cucurbitacin I, neuronal differentiation was largely abolished but the production of GFAP+ cells was not altered. (C) In LIF+ medium, EB produced a small number of GFAP-positive cells; (D) in LIF– medium, EB differentiated into a large number of GFAP cells.

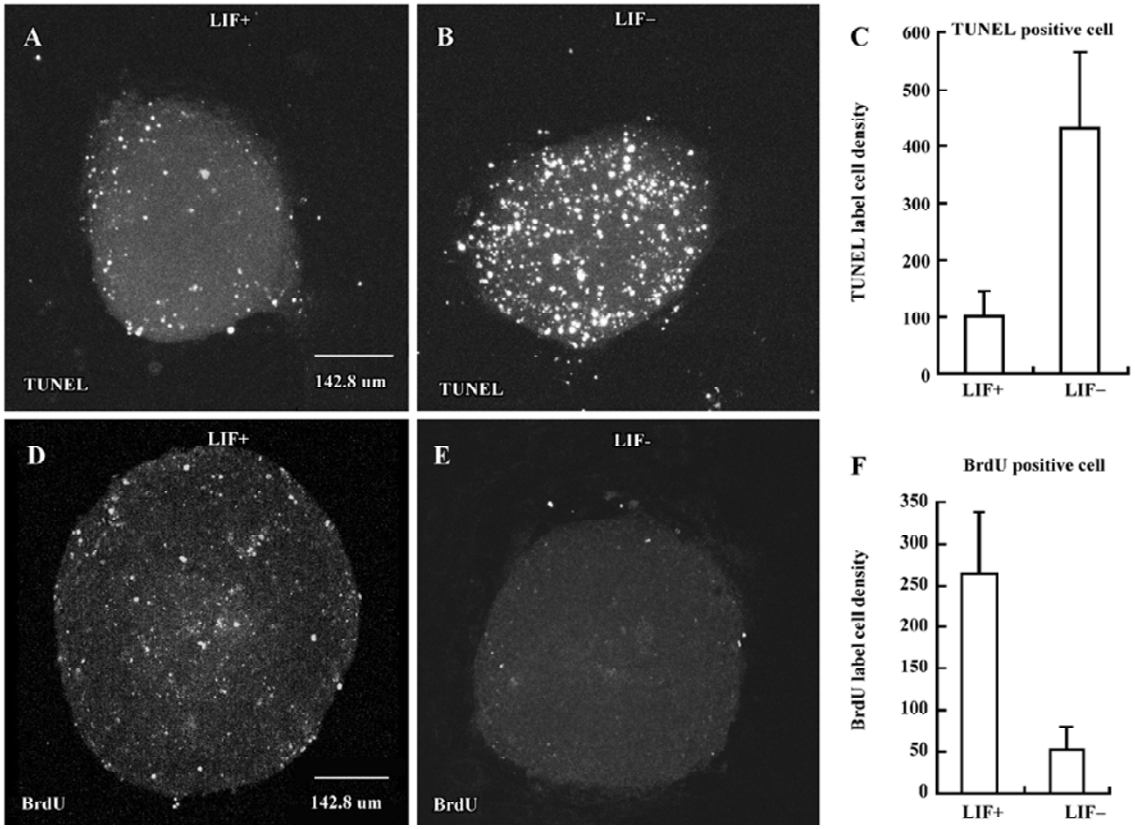


Figure 4. LIF inhibits cell apoptosis and promotes cell proliferation. The presence of LIF in culture medium inhibited cell apoptosis (A–C) and supported cell proliferation (D–F). (A–C) TUNEL assay demonstrated a suppression of apoptosis in the presence of LIF. (C) The density of TUNEL-positive cells in EB cultured without LIF was greater than that in EB with LIF ($P<0.05$). (D–F) A BrdU labeling assay indicated that the presence of LIF in culture medium supported cell proliferation. (F) The density of BrdU-positive cells in EB with LIF was greater than that for EB without LIF. The method used to count cell density is the same as used in the TUNEL assay.

potentiates the differentiation of ES cells to a neural fate, and might suppress both the mesoderm and extraembryonic endoderm fates.

Discussion

In this work, we observed that neuronal differentiation of ES cells was enhanced in the presence of LIF. This enhancement cauld be abolished when the JAK/STAT3 pathway was inhibited. Furthermore, inhibition of the MEK signaling pathway impaired the differentiation of ES cells toward a glial lineage. During this process, LIF inhibited cell apoptosis and promoted cell proliferation, and EB differentiation to form both mesodermal and extraembryonic endodermal cells ws inhibited, whereas the production of neural progenitor cells was increased. Thus our observations imply that the LIF signaling pathway is involved in ES cell differentiation into a neuronal cell fate, and provide cues for further investigations of this pathway.

During early vertebrate embryonic development, neural induction has been thought to be a default process. In the default model, the inhibition of the BMP (bone morphogenetic proteins) signaling pathway is necessary for neural cell fate determination during early embryonic development[42–44]. However, it remains unknown whether neural induction requires the participation of other signaling pathways other than simply BMP depletion. The clue that LIF might be involved in ES cell neural differentiation comes from the fact that in low-density and serum-free culture conditions, ES cells with neural progenitor properties were identified[20]. Under these culture conditions, LIF was required for ES cells to undergo neuronal colony formation. In the present report we provided evidence that LIF could enhance the production of neural progenitor cells from ES cells, and support the neuronal differentiation of EB in a dose-dependent manner (Figure 1). Furthermore, in the presence

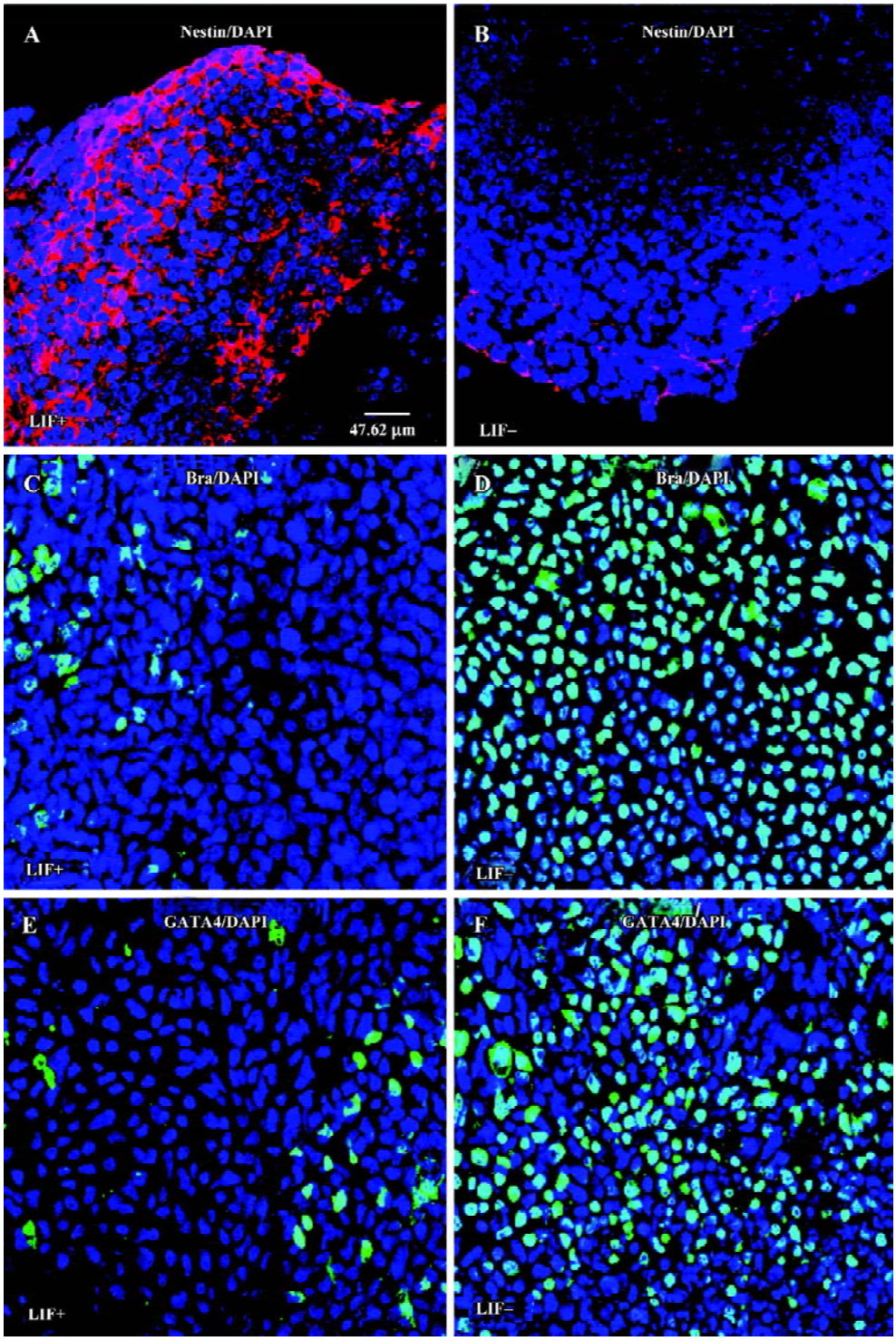


Figure 5. The expression of both mesodermal and extra-embryonic endodermal genes was suppressed and neural progenitors were enhanced in the presence of LIF. Six-day EB cultured either with or without LIF were fixed and immunostained with antibody against neural progenitor marker nestin (A,B), mesoderm marker brachyury (C,D), or extraembryonic endoderm marker GATA-4 (E,F). (A) In the presence of LIF, EB produced a large number of nestin-positive cells within EB; (B) a small number of nestin cells appeared within LIF– EB. Compared with EB cultured in the absence of LIF (D,F), EB cultured with LIF (C,E) had a reduced number of cells expressing both mesoderm and extraembryonic endoderm markers.

of LIF, the commitment of ES cells to both mesodermal and extraembryonic endodermal fates was suppressed (Figure 5). The LIF pathway also regulates neural differentiation into neuronal and glial fates. Our results not only indicate that LIF signaling is indeed involved in ES cell neural commitment, but also strongly suggest that the LIF pathway has multiple functions in different stages of ES cell differentiation.

A recent study has shown that LIF/Stat3 cooperated with BMP/Smad signaling to maintain ES cells in an undifferentiated state[5]. It seems that the balance between LIF/Stat3 and BMP/Smad signaling is critical for the choice between sustaining pluripotency or lineage commitment of ES cells. Thus the activation of the LIF signaling pathway reported here may account for promoting more ES cells to adapt to a neural cell fate, whereas LIF withdrawal leads to an elevated BMP signaling activity, which further stimulates ES cells to differentiate into mesodermal and endodermal cells. It would be of great interest to further investigate how BMP and LIF signaling pathways cross-talk during ES cell neural commitment.

Multiple lines of evidence indicate that LIF plays various roles in different types of neural stem cell differentiation[12–19]. In the central neuronal system, the LIF signaling pathway synergistically cooperates with other signaling pathways to inhibit neural stem cell differentiation to a glial fate[12,13]. However, LIF downstream factor STAT3, together with BMP downstream effector SMAD1, selectively promotes the glial differentiation of fetal neural stem cells[14–16]. Furthermore, during neural crest cell differentiation, LIF promotes sensory neuron differentiation and survival[17–19]. The LIF pathway requires cooperation with different factors to achieve its function as a pleiotropic cytokine. In our experiments, inhibition of the LIF-activated STAT3 pathway did not interfere with the production of GFAP+ cells, but abolished the differentiation of Tuj1+ cells from ES cells. This result is consistent with the finding that Ngn1 inhibits gliogenesis through inhibiting the activation of STAT transcription factors during neural differentiation[12]. Furthermore, blocking the LIF-activated MEK pathway abolished the glial differentiation of ES cells. Our results suggest that LIF signaling might interact with other pathways to exert various effects in the differentiation of different neural cell types. In addition, we found that LIF unselectively inhibited cell apoptosied and supports cell proliferation during ES cell neural differentiation. However, we consider that it would be worthwhile to further investigate the possibility that LIF affects the survival and proliferation of neuronal cells by using more specific cell markers.

LIF is not the only factor that has dual roles in ES cell culture. A recent report indicates that Oct3/4 also has dual roles in murine ES cell culture: sustaining ES cell self-renewal and taking part in ES cell fate determination. The precise level of Oct4 in ES cells controls the commitment of ES cells to undergo 3 different cell fates[1]. Ectopic expression or overexpression of Oct3/4 also appears to enhance neuronal differentiation in SDIA (stromal cell-derived inducing activity)-induced ES cell differentiation[45,46]. However, it is unclear whether LIF signaling interacts with the Oct3/4 signaling pathway in ES cell neuronal differentiation, but this certainly warrants further investigation in the future.

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