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Developmental stage-dependent self-regulation of embryonic cortical precursor cell survival and differentiation by leukemia inhibitory factor

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Abstract

Cortical precursor cells secrete soluble factors for their own survival and self-renewal. We show here that neural precursor cells isolated from embryonic rat cortices abundantly secrete leukemia inhibitory factor (LIF) and express its receptor components, gp130 and LIF receptor. LIF signaling is responsible for cortical precursor cell survival. As described previously, LIF caused astrocytic differentiation of cultured embryonic cortical precursor cells. LIF-mediated survival and astrocytic differentiation of cortical precursor cells were differentially regulated, depending on the developmental ages of embryos from which cortical precursors were isolated. LIF did not enhance the survival of cortical precursor cells isolated from later embryos (embryonic day 16, E16). Moreover, LIF-mediated astrocytic differentiation was not observed in early (E12) cortical precursors. Inhibition studies revealed that Janus-activated kinase/signal transducer and activator of transcription and phosphatidylinositol 3 kinase/Akt pathways participate in both the LIF-mediated effects. However, mitogen-activated protein kinase, another signal pathway activated by LIF, was specifically responsible for astrocytic differentiation. These findings collectively indicate that precursor cells self-regulate the sequential processes of brain development, such as early maintenance of the precursor cell population and later differentiation into astrocytes, via common LIF signaling.

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Abbreviations: LIF, leukemia inhibitory factor; LIFR, LIF receptor; E12–16, embryonic day 12–16; JAK/STAT, Janus-activated kinase/signal transducer and activator of transcription; PI3K/Akt, phosphatidylinositol 3 kinase/Akt; MAPK, mitogen-activated protein kinase; gp130, glycoprotein 130; CNS, central nervous system; CNTF, ciliary neurotrophic factor; SCM, stem cell-conditioned medium; bFGF, basic fibroblast growth factor; TuJ1, β-tubulin type III; GFAP, glial fibrillary acidic protein; DIV, day *in vitro*; EGF, epidermal growth factor; BrdU, bromodeoxyuridine; LDH, lactate dehydrogenase; N-CoR, nuclear receptor co-repressor; HBSS, Hank's balanced salt solution; EtBr, ethidium bromide.

Introduction

Neural precursor cells that arise from a single layer of neuroepithelium initially undergo cell division without commitment into their progenies,[1–3] followed by progressive differentiation into various types of neurons and glia in the late developmental stages. These sequential processes are precisely regulated in the developing brain.[4] A challenging issue in developmental neurobiology is the elucidation of the mechanisms underlying the regulation of timed sequential events. Environmental cues through diffusible signals[5–7] and cell–cell contacts[8,9] are active regulators of self-renewal and fate specification of neural precursor cells. Cytokines specific for certain developmental processes *in vitro* and *in vivo* mediate other or opposite phenomena, depending on intrinsic properties of cells progressively altered spatiotemporally in the developing brain.[10] Thus orchestration of the correct microenvironment with intrinsic properties of cells is critical for regulating a precise developmental schedule.

Leukemia inhibitory factor (LIF) is a member of the cytokine group that signals via binding to a heterodimeric complex of common glycoprotein 130 (gp130) and LIF receptor (LIFR) subunits.[11] A number of studies show that gp130/LIFR-mediated signaling has pleiotrophic action on different cell types in the nervous system. LIF acts as a survival factor for sensory and motor neurons in the mature nervous system (reviewed in Turnley and Bartlett[11]and Murphy *et al.*[12]). In the developing central nervous system (CNS), LIF[13,14] inhibits self-renewal of neural precursor cells and promotes differentiation into astrocytes, similar to other LIF/gp130-related cytokines such as ciliary neurotrophic factor (CNTF)[13–16] and oncostatin M.[17] However, recent data suggest an opposite effect of the signal in neural precursor cells, specifically the maintenance of cortical precursor cells[18,19] in the undifferentiated state by increasing their self-renewal and inhibiting progression to astrocytic lineage. The mechanism by which gp130/LIFR signal performs such opposite roles remains to be elucidated.

In this study, we initially show that undifferentiated proliferating precursor cells isolated from embryonic day 12 (E12)–E16 rat cortices abundantly release LIF and synthesize the receptor components, gp130 and LIFR,

and that expression of the receptors is positively regulated by the LIF ligand. We examine the putative roles of LIF-mediated signaling in survival, proliferation and differentiation of embryonic cortical precursor cells. Using culture conditions of clonal cell densities at which cell–cell interactions are minimized, we demonstrate that LIF is responsible for the survival of cortical precursor cells. Additionally, LIF treatment enhances the differentiation of cortical precursor cells into astrocytes. LIF-mediated survival and astrocytic differentiation are regulated differentially, depending on the developmental stage of cortical precursor cells. LIF does not induce astrocytic differentiation in cortical precursor cells isolated from early embryos (E12), whereas LIF-mediated survival is not observed in cortical precursor cells at the late developmental stage (E16). We show a distinct intracellular transduction pathway for LIF-mediated precursor cell survival and astrocytic differentiation, although common signal pathways for both LIF-mediated effects exist. These findings suggest that LIF, an autocrine/paracrine precursor cell factor, plays critical roles in regulating the timed developmental program in the embryonic brain.

Results

Embryonic cortical precursor cells release LIF and express its receptor subunits

We previously demonstrated that supplementation of conditioned medium prepared from confluent cultures for embryonic cortical precursor cells (stem cell conditioned medium: SCM) enhanced the survival and self-renewal of isolated single cells,[1] suggesting autocrine/paracrine action for the maintenance of embryonic neural precursor cell population. Based on data on the role of LIF in cell survival in the nervous system (reviewed in Murphy *et al*.[12]) and maintenance of neural precursors,[18,19] we evaluate the expression of cytokines and their receptors involved in LIFR/gp130 signaling to determine whether this pathway is responsible for the observed autocrine/paracrine survival and proliferation of cortical precursor cells. We initially performed semiquantitative and real-time RT–PCR analyses to determine the mRNA expression of LIF, CNTF and their receptor components in neural precursor cell-enriched cultures. Over 95% of cells were positive for nestin, an intermediate filament specific for undifferentiated neural precursor cells, after 4 days of *in vitro* expansion with mitogen basic fibroblast growth factor (bFGF) in passaged cultures for E14 cortical precursor cells (see Materials and Methods; left in Figure 1a). Differentiation was readily induced by withdrawing bFGF for 6 days. Consequently, 42 and 39% total cells were positive for β-tubulin type III (TuJ1; a neuronal marker) and glial fibrillary acidic protein (GFAP; an astrocytic marker), respectively ($n=12$; right in Figure 1a). Transcripts of cytokine LIF and its receptor components, gp130 and LIFR, were abundantly expressed in precursor cell-enriched cultures, and their expression was markedly decreased in differentiated cell cultures (Figure 1b). Real-time PCR analyses revealed that the mRNA levels of LIF, gp130 and LIFR in precursor cell-enriched cultures were 25.9 ± 5.1-, 16.5 ± 1.9- and 3.7 ± 2.2-fold greater than those in differentiated cultures, respectively ($n=5$, $P<0.001$; Figure 1b). On the other hand, CNTF mRNA was 41.4 ± 4.2-fold more abundant in differentiated cultures ($n=5$, $P<0.001$). Using Western blot analyses, we directly estimated the levels of LIF and CNTF in media conditioned in

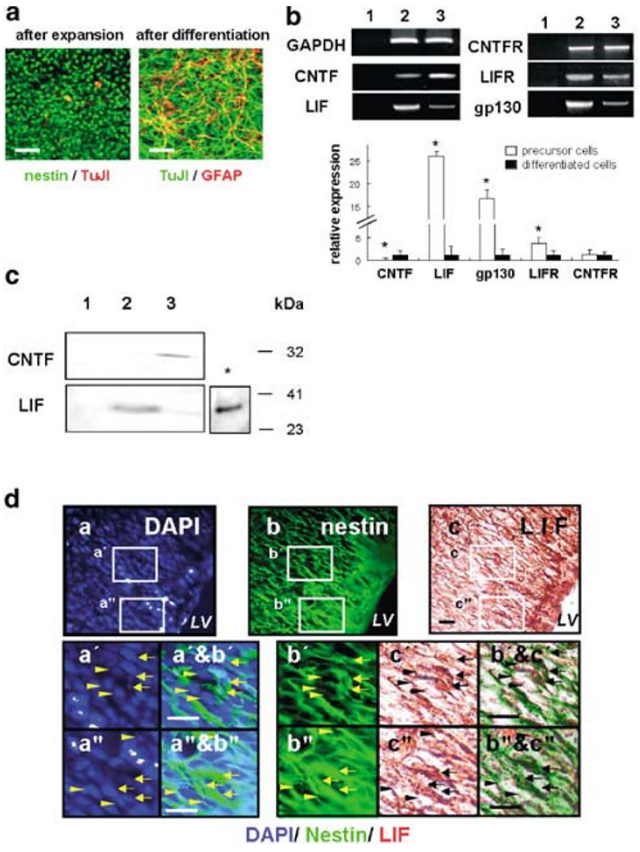

Figure 1 Expression of LIF and its ligands in undifferentiated cortical precursor cells. (**a**) Immunocytochemical properties of embryonic cortical precursor cell cultures after expansion (left) and differentiation (right). Most cells ($>95\%$) were immunoreactive to nestin (green, left), a marker protein specific for neural precursor cells, after 4 days of bFGF expansion in passaged cultures for E14 cortical precursor cells. Only 3% cells were positive for neuron-specific TuJ1 (red; left) and none were positive for GFAP, an astrocytic marker. On the other hand, approximately 80% cells acquired either a differentiated cell marker including 42% TuJ1-positive (green, right) and 39% GFAP-positive cells (red, right) after 6 days of differentiation, induced by bFGF withdrawal. Scale bar, 20 μm. (**b**) Semiquantitative and real-time PCR analyses for LIF, CNTF and their receptor subunits. The upper panels depict ethidium bromide (EtBr) staining of PCR products in cortical precursor cell cultures after expansion (lane 2) and differentiation (lane 3). Lane 1, negative controls from reactions without cDNA. Lower panel, real-time PCR analyses for identical genes. Each gene expression value was normalized to GAPDH. Boxes and bars represent mean and standard error values of five gene expression experiments on precursor cell-enriched cultures (left in (**a**)), relative to cultures enriched with neurons and astrocytes (right in (**a**)). *$P<0.001$. (**c**) Western blot analyses for LIF and CNTF in HBSS conditioned in precursor cell- (lane 2), differentiated cell-enriched cultures (lane 3) and negative control (unconditioned HBSS; lane 1). Proteins were extracted as described in Materials and methods. The equivalent of 2 ml HBSS media was loaded per well. *0.1 μg recombinant LIF control. (**d**) Immunohistochemistry for nestin (**b, b′, b″**; fluorescently stained) and LIF (**c, c′, c″**; stained with peroxidase reaction with AEC substrate) with DAPI cell nucleus counterstaining (**a, a′, a″**) in the same microscopic fields of the E14 rat basal cortical germinal zone. Lower panels, high-powered images in the white boxed areas. (**a′** & **b′** (**a″** & **b″**)) Merged image of nestin and DAPI staining. (**b′** & **c′** (**b″** & **c″**)) Nestin and LIF staining. LIF is colocalized in nestin+ cells (arrows). The arrowheads indicate cells negative for nestin and LIF. Secondary antibody alone was uniformly negative. *LV*, lateral ventricle. Scale bar, 20 μm

cultures for bFGF-proliferated precursor cells and differentiated cells (Figure 1c). Data obtained were consistent with those from RT–PCR analyses. Similar gene expression patterns for the ligands and receptor components of LIFR/gp130 signaling were also observed in precursor cell cultures isolated from E12 and E16 rat cortices (data not shown). Double labeling for nestin and LIF in E14 cortical tissue sections revealed that the two antigens were very closely localized (Figure 1d). These findings collectively suggest that LIF-mediated signaling is potentially activated in undifferentiated embryonic cortical precursor cells.

Ligand-induced upregulation of receptor expression has been demonstrated by a number of investigators.[20–22] Expression patterns of the receptor components, gp130 and LIFR, such as decreased levels in the differentiated condition, are similar to those of the LIF ligand (Figure 1b), suggesting that expression patterns of the LIF ligand and receptor components are linked. As expected, treatment with recombinant LIF elicited increased levels of gp130 and LIFR mRNAs in precursor cell cultures by 5.2 ± 0.56- and 2.7 ± 0.75-fold, respectively ($n = 5$, $P < 0.001$; Figure 2a), indicating ligand-mediated stimulation of receptor expression in LIFR/gp130 signaling. LIF treatment additionally induced an increase in the expression of gp130 proteins, as estimated by Western blotting (Figure 2b).

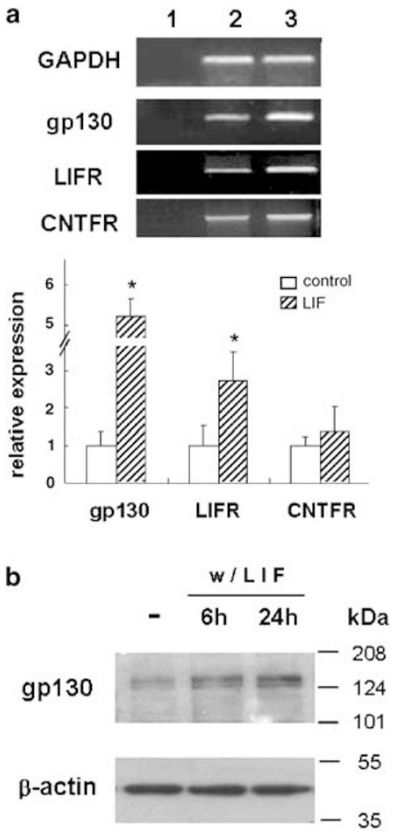

Figure 2 LIF ligand upregulates the expression of LIF receptor components. *In vitro*-expanded E14 cortical precursor cells were plated at a low cell density (8000 cells/cm^2) in N2 + bFGF, and further expanded for 3 days in the absence or presence of exogenous LIF. (**a**) The mRNA levels of the receptor components, gp130, LIFR and CNTFR, were analyzed after 3 days of bFGF proliferation by semiquantitative and real-time PCR. The upper panel represents EtBr staining of PCR products from samples without cDNA (lane 1), untreated control (lane 2) and LIF (20 ng/ml)-treated cultures (lane 3). The lower graph depicts mRNA expression of the receptor subunits in LIF-treated cells relative to those in untreated cells, analyzed by real-time PCR. *Significantly different from untreated control at $P < 0.001$ ($n = 5$ for all values). (**b**) Western blot analysis for gp130 in control and LIF-treated cultures. Cells were cultured for 3 days, similar to (**a**), except the duration of LIF treatment. Cells were treated with LIF for 6 h (lane 2) or 24 h (lane 3), prior to harvesting for protein preparation. The data represent immunoblots of six analyses from three independent experiments. The two distinct bands present in the gp130 immunoblot represent fully processed (upper) and precursor form (lower) of gp130 proteins[35]

LIF as a survival factor for neural precursor cells

To evaluate the role of LIF in the survival of cortical precursor cells, E14 rat cortices were dissociated into single cells and plated at the clonal densities 200–4000 cells/6 cm dish, either in the presence or absence of exogenous LIF (the concentration of exogenous LIF employed was 20 ng/ml in all experiments, unless otherwise specified) in bFGF-supplemented N2. As described previously,[1] cell survival was estimated by directly counting viable cells or fluorescent DAPI staining at 16–24 h after cell plating (day *in vitro* 1: DIV 1) and clone numbers at DIV 6. Cells underwent extensive death during the first day of culture and only 0–0.3% cells plated were viable at DIV 1. Over 98% of viable cells at DIV 1 were positive for nestin.[1] At all the cell densities tested, the numbers of viable nestin+ cells at DIV 1 were more than 7.5-fold greater in cultures treated with LIF, compared to untreated cultures ($n = 12$ experiments at each cell density, $P < 0.001$; Figure 3a). However, cell numbers in cultures treated with LIF at 2 h after plating were not significantly different from control cultures (997.2 ± 54.9 in LIF-treated *versus* 968.1 ± 41.9 in control cultures plated at 2000 cells/6 cm dish, $n = 6$, $P = 0.626$), suggesting that treatment with this factor does not affect plating efficiency. Since almost all cells at DIV 1 (except 2–5% cells in clusters) were single and isolated,[8] the viable cell number increase in LIF-treated cultures was not due to cell proliferation. Single isolated cells proliferated to form cell clusters (referred to as clones) as a result of the mitogenic action of bFGF. Consistent with survival at DIV 1, clone numbers of total cells plated (clone-forming units) at DIV 6 were at least eight-fold greater in cultures treated with LIF than those in untreated control cultures ($n = 12$, $P < 0.001$; Figure 3b). Supplementation of cultures plated at the clonal densities with SCM markedly enhanced the survival of E14 cortical precursor cells.[1] The SCM-mediated increase in survival at DIV 1 and clone-forming unit were blocked by anti-LIF blocking antibody (Figure 3a and b). These findings conclusively suggest an effect on the survival of nestin+ cortical precursor cells. Consistently, lower numbers of TUNEL+ cells at DIV 2, 4 and 6 were observed in LIF-treated cultures plated at 8000 cells/cm^2 (corresponding to 1.5×10^5 cells/6 cm dish), compared to untreated control cultures. The percentages of TUNEL+ cells were $48.3 \pm 4.7\%$ in LIF-treated *versus* $81.1 \pm 5.9\%$ in untreated control at DIV 2 ($n = 20$ for both values in four independent experiments, $P < 0.001$), $37.1 \pm 4.9\%$ in LIF-treated *versus* $68.1 \pm 5.2\%$ in control cultures at DIV 4 ($n = 20$, $P < 0.001$) and $25.2 \pm 3.9\%$ in LIF-treated *versus* $33.9 \pm 4.3\%$ in control cultures at DIV 6 ($n = 20$, $P < 0.05$; Figure 3c).

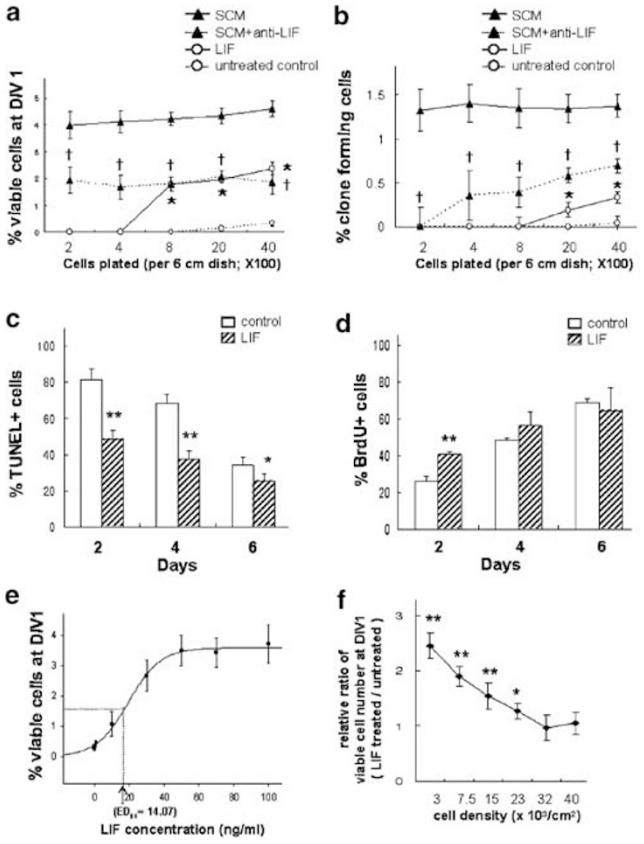

Figure 3 LIF as a survival factor for embryonic cortical precursor cells. Cells acutely dissociated from rat embryonic cortices were plated at various cell densities, and cultured in N2 with bFGF in the presence or absence of 20 ng/ml recombinant LIF. In some experiments, cultures were supplemented with SCM (see Materials and methods), and anti-LIF blocking antibody was added in the SCM-supplemented cultures to determine whether LIF is responsible for the SCM-mediated cell survival. (**a**) % viable cells at DIV 1. (**b**) Clone forming unit, % clone number of total cells plated after 6 days of bFGF expansion. Each value represents mean $\pm$S.E.M. of 12 independent experiments. In each experiment, two to four plates of control and LIF-treated cultures were counted. *Significantly different from untreated control at $P<0.001$; †significantly different from SCM-supplemented cultures at $P<0.001$. (**c**, **d**) TUNEL and BrdU incorporation assays. Cells were plated at 8000/cm^2 in N2 + bFGF and TUNEL and BrdU incorporation assays were performed at DIV 2, 4 and 6. *Significantly different from control values at $P<0.05$; **significantly different from control values at $P<0.001$. (**e**) Dose response of cell survival to LIF. Cells were plated at a density of 2000 cells/6 cm dish in the presence or absence of 0.1–100 ng/ml LIF, and viable cells were counted at DIV 1. The survival effect of LIF increased in proportion to exogenous LIF concentrations up to 100 ng/ml ($ED_{50}=14.07$ ng/ml). (**f**) Cell density effect on LIF-mediated survival. The data represent % survival at DIV 1 in LIF-treated cultures relative to those in untreated control cultures ($n=3$ for all values, *$P<0.05$ and **$P<0.001$)

Cell survival was proportional to exogenous LIF concentrations up to 100 ng/ml, and reached a plateau in cultures plated at 2000 cells/6 cm dish (ED_{50} value of LIF cell survival at DIV 1 was 14.07 ng/ml; Figure 3e). The survival effect of exogenous LIF was less significant in cultures plated at higher cell densities, and not significant at $>3.2\times10^4$ cells/cm^2, suggesting that cultures with high cell densities contain a sufficient quantity of endogenous survival factors, including LIF. Consequently, the effect of exogenous LIF was negligible (Figure 3f).

In contrast to bFGF and epidermal growth factor (EGF), LIF alone did not promote proliferation of E14 cortical precursor cells. In the absence of bFGF and EGF, average clone sizes (cell numbers assembled in a clone) at DIV 6 were 8.3 ± 0.9 cells in cultures treated with LIF *versus* 8.0 ± 1.2 cells in untreated control cultures ($n=24$, $P=0.793$), and 667.4 ± 28.7 cells in cultures treated with 15 ng/ml bFGF. LIF did not promote bFGF-induced proliferation of E14 cortical precursors, as estimated by clone size at 6 days of expansion. Average clone sizes were 645.7 ± 23.6 cells in LIF + bFGF treated and 706.5 ± 28.2 cells in cultures treated with bFGF alone ($n=42$ for each value, $P=0.184$). LIF also did not support precursor cell proliferation induced by EGF, another mitogen for neural precursor cells (data not shown). However, increased bromodeoxyuridine (BrdU) incorporation was observed at the early expansion period (DIV 2) in LIF-treated cultures (40.2 ± 2.2% of total cells were positive to BrdU in cultures treated with LIF + bFGF *versus* 26.0 ± 3.1% in cultures treated with bFGF alone, $n=12$, $P<0.001$). In contrast, BrdU incorporation in cells treated with LIF was not significantly different at DIV 4 (56.0 ± 6.7 *versus* 48.2 ± 0.9%, $n=12$, $P=0.052$), and even less than that in untreated cultures after 6 days of proliferation (64.5 ± 9.2 *versus* 69.1 ± 2.1%, $n=9$, $P=0.056$; Figure 3d).

Astrocytic differentiation of neural precursor cells by LIF

To clarify whether LIF induces or inhibits astrocytic cell fate determination and differentiation, precursor cells isolated from E14 cortices were cultured under various conditions in the presence or absence of LIF. Initially, cells dissociated from cortical tissues were plated at 2.0×10^4 cells/cm^2 and directly induced to be differentiated in N2 for 2 days. None of the cells were GFAP+, with the TuJ1+ cell population being 78.4 ± 6.5% in untreated control cultures, suggesting neurogenic potential of E14 cortical precursor cells. LIF treatment generated GFAP+ cells (1.8 ± 2.6% total cells) with a significant decrease in TuJ1+ cell population (72.2 ± 6.4%, $P<0.05$, $n=15$). Next, we evaluated LIF-mediated astrocytic differentiation using clonal analysis. Cells acutely dissociated from E14 cortices were clonally expanded with bFGF in the presence or absence of LIF for 3 days prior to differentiation by withdrawing bFGF. Consistent with results obtained from directly differentiated cultures, most clones (unpassaged (P0) clones) produced neurons only (neuron-only clone: 96.6 ± 0.7%) and none contained GFAP+ cells after 6 days of differentiation in the absence of LIF. In contrast, 6.0% of clones in the presence of LIF were positive for GFAP+ (neuron–astrocyte clone + astrocyte-only clone) as a result of the reduction in neuron-only clones (89.9 ± 1.0%, significantly different from the neuron-only clone in the untreated control at $P<0.001$). Based on the theory that the neurogenic potential of neural precursor cells is altered to multipotent and/or gliogenic following extensive cell division,[10] and therefore the effect of LIF on cell fate switch could be more clearly observed if clonal analysis is performed using cells that have undergone several cycles *in vitro*, cells isolated from E14 cortices were expanded to 70–80% confluency *in vitro* with bFGF, followed by clonal analysis (Figure 4a). As expected, 25% of the clones generated from cells expanded *in vitro* (passaged (P1) clones)

were positive for GFAP after 6 days of bFGF withdrawal. Exposure of neural precursor cells to LIF during the expansion period resulted in a significant increase in the astrocyte-only clone number at the expense of neuron-only clones. The percentage of astrocyte-only clones was 40.7±9.2% of the total clones in LIF-treated cultures *versus* 1.0±1.1% in untreated control cultures ($n=5$, $P<0.001$), while the neuron-only clone population was 19.1±1.0% in LIF-treated *versus* 53.0±1.0% in untreated cultures ($n=5$, $P<0.001$; Figure 4a). These results collectively suggest that LIF instructively induces embryonic cortical precursor cells to differentiate into astrocytes. The LIF-induced switch of the precursor cell fate into astrocytic lineage was further confirmed in cultures maintained with long-term cell expansion. As described above, embryonic cortical precursor cells themselves secrete LIF. Thus, exogenous LIF effects are not observed if cultures are confluent (Figure 3f). Cells dissociated from E14 cortices were expanded with bFGF in the presence or absence of LIF and passaged every 3 days to maintain less than 30% cell confluency. In parallel, passaged cells were expanded identically for 3 days, followed by differentiation in N2 for 6 days. As described above, the neurogenic potential of cortical precursor cells was normally switched to astrocytic potential over continued proliferation and passages in control cultures, estimated by immunocytochemical analyses for TuJ1 and GFAP in differentiated cultures (Figure 4b). Switching of the precursor cell fate to astrocytes occurred much earlier in cultures maintained with LIF. Specifically, in cultures passaged once (P1) and twice (P2), 56.6±8.7 and 69.2±4.5% LIF-treated cells were positive for GFAP upon differentiation, respectively, while only 0.8±1.3 and 3.2±2.6% cells were positive for GFAP in untreated cultures (Figure 4b).

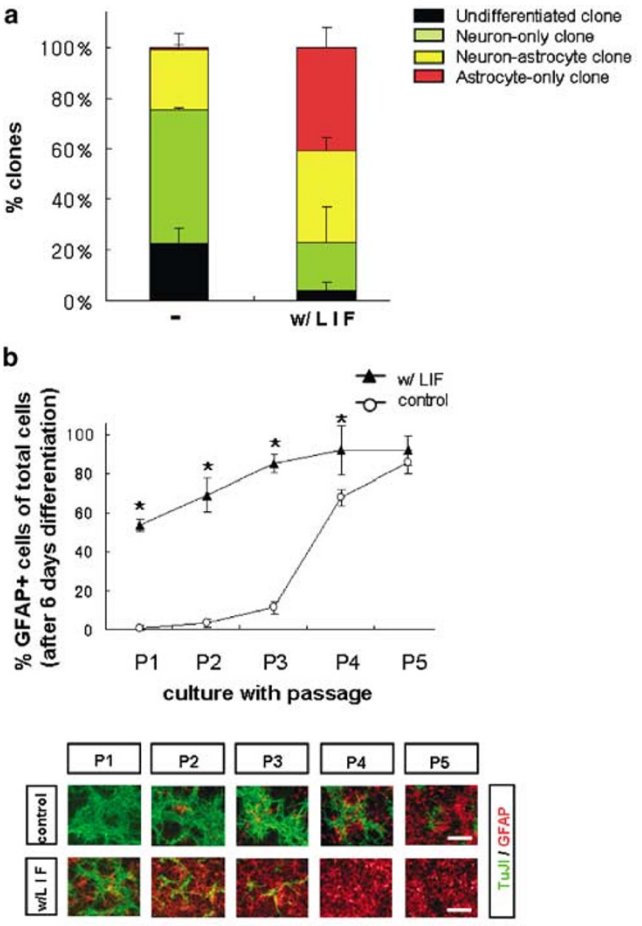

Figure 4 LIF-mediated astrocytic differentiation. (**a**) Clonal analysis of passaged (P1) culture. Cells from E14 cortices were expanded with bFGF. The *in vitro*-expanded cells were dissociated into single cells and re-plated at a density of 1000 cells/6 cm dish. Well-isolated single cells were marked and proliferated with bFGF to form clones for 3 days in the presence or absence of LIF. Phenotypes of clones were analyzed after 6 days of differentiation. (**b**) The switch from neurogenic to astrogenic potential of cortical precursor cells during long-term cell proliferation is facilitated by LIF. Proliferation of precursor cells from E14 cortices was maintained in the presence or absence of LIF for a long culture period with multiple cell passages. In parallel, each culture for passaged cells was differentiated for 6 days and the differentiation potentials of passaged cells were analyzed by immunostaining for GFAP and TuJ1. Passaged cultures are designated 'P' along with the passage number. For example, P1 represents culture after the first passage procedure. The lower panels display representative fluorescence images of Tuj1-positive (green) and GFAP-positive (red) cells from experiments described in (**b**). All data are taken from three independent experiments. Scale bar, 100 μm. *$P<0.001$ *versus* untreated control, two-way ANOVA

An increase in cell number in LIF-treated cultures compared to untreated cultures was observed for the first 6 days of bFGF expansion (before the second passage procedure, during P0–P1 cultures; Figure 5a). The number of TUNEL+ cells in LIF-treated cultures, compared to those in control cultures, was significantly less during early expansion (P0–P1; Figure 5b). However, the fold increase in cell number after the second passage (P2–P5 cultures) was significantly less in cultures maintained with LIF, compared to control cultures. Consistently, a smaller proportion of LIF-treated cells were positive for Ki67, an effective mitotic marker, in P2–P5 cultures (Figure 5c).

GFAP+ cells were not detected during early expansion (P0–P4), but only detected after the fifth passage (P5; Figure 5d). In contrast, early appearance and greater abundance of GFAP+ cells was observed in expanded cultures maintained with LIF (Figure 5d).

These findings collectively imply that LIF maintains self-renewal of embryonic cortical precursor cells at the early developmental stages, probably by enhanced cell survival, but decreases the expandability in late neural precursor cells that have undergone extensive division, probably by promoting astrocytic fate determination.

LIF-induced survival and astrocytic differentiation are differentially mediated depending on the developmental stage of cortical precursor cells

To determine whether LIF-induced precursor cell survival and astrocytic differentiation are dependent on the developmental stage, neural precursor cells were isolated from E12–E16 cortices and cultured in the presence or absence of LIF. LIF enhanced the survival of neural precursor cells isolated from E12 and E14 cortices significantly, as estimated by the viable cell number at DIV 1 (54.3±3.9% in LIF-treated *versus* 27.9±2.4% in control E12 cultures, 55.0±2.6 *versus* 38.3±4.1% in E14) ($n=15$ for each value, $P<0.001$;

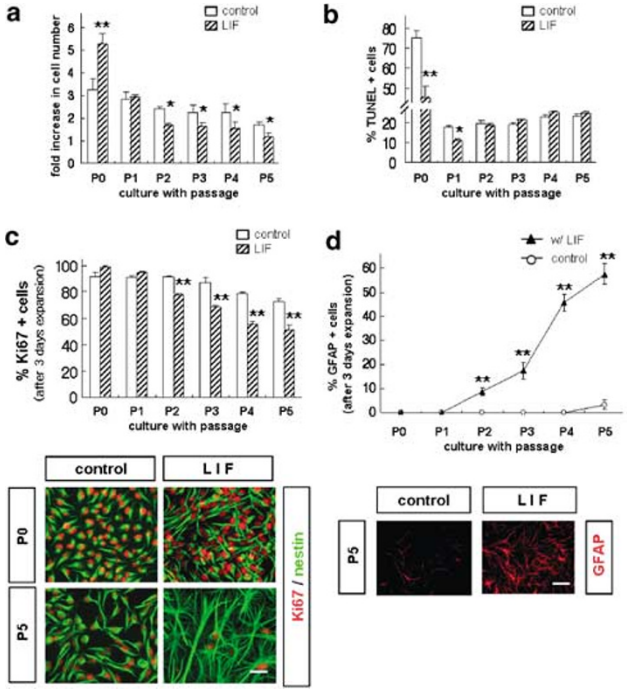

Figure 5 LIF effect on precursor cell maintenance. Cells from E14 cortices were cultured as described in Figure 4b. (**a**) Fold increase in cell numbers, as determined by the ratio of the cell number after 3 days of proliferation to the number of cells plated in each passaged culture. %TUNEL + apoptotic cells (**b**) and Ki67-expressing proliferating cells (**c**) were determined at every 3 days of expansion, right before each passage procedure, in cultures maintained with multiple passages. The lower panel in (**c**) shows representative images of Ki67 (red) and nestin (green) cells in control and LIF-treated cultures (P0 and P5). (**d**) Appearance of GFAP + cells during the *in vitro* expansion of cortical precursor cells. Immunocytochemistry was performed after 3 days of expansion in each passaged culture without subsequent differentiation steps. Scale bar, 20 μm. Significant differences from the respective control values were noted at $^{*}P<0.05$ and $^{**}P<0.001$

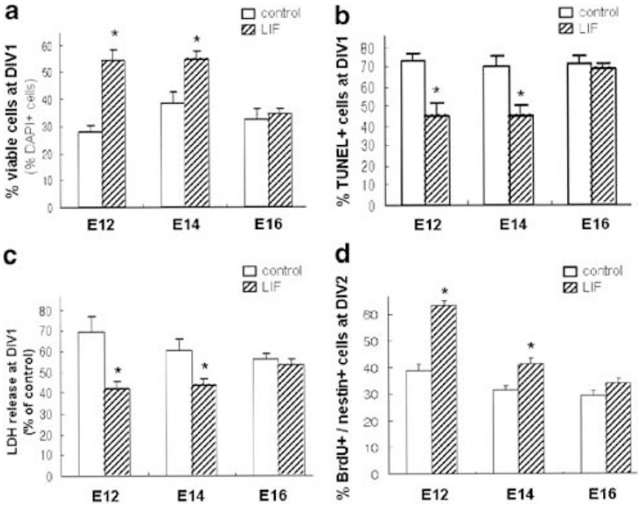

Figure 6 LIF-induced survival and proliferation depend on the developmental stage of cortical precursor cells. (**a**) Viable cell number, (**b**) %TUNEL + cell counting, (**c**) %LDH and (**d**) % BrdU + cells. Acutely dissociated E12, E14 and E16 cortical cells plated at 8000 cells/cm^2 were cultured in N2 + bFGF in the presence or absence of LIF. Survival indices (**a**–**c**) were estimated at DIV 1 and BrdU incorporation (**d**) at DIV 2. Results are presented as the mean ± S.E.M. percentage of DAPI +, TUNEL + and BrdU + cells in the total cell population, and percent LDH released relative to total LDH in culture ($n=15$ from three independent experiments, $^{*}P<0.001$)

Figure 6a). On the other hand, viable cell numbers were not significantly altered by LIF treatment in cultures for E16 cortical precursor cells (32.4 ± 4.2 *versus* 34.7 ± 1.9%, $n=15$, $P=0.347$). Consistently, no significant differences in the percentages of TUNEL + cells were observed in E16 cultures upon LIF treatment, while TUNEL + cells were significantly lower in LIF-treated E12 and E14 cultures, compared to untreated cultures (TUNEL + cell percentages in LIF-treated and untreated cultures were 45.6 ± 5.9 *versus* 73.2 ± 3.4% in E12, 44.9 ± 4.6 *versus* 69.5 ± 5.5% in E14, 68.7 ± 2.4 *versus* 70.8 ± 4.7% in E16 cultures; $n=4$, $P<0.001$ in cultures for E12 and E14 cells; $P=0.32$ for E16 cells; Figure 6b). The ineffectiveness of LIF on E16 precursor cell survival was further confirmed by lactate dehydrogenase (LDH) release. LDH release levels were 42.2 ± 3.4% in LIF-treated *versus* 69.6 ± 7.3% in untreated control E12 cultures ($n=4$, $P<0.001$), 44.0 ± 2.9 *versus* 60.3 ± 5.5% in E14 cultures ($n=4$, $P<0.001$) and 53.4 ± 3.0 *versus* 56.6 ± 2.2% in E16 cultures ($n=4$, $P=0.516$) (Figure 6c). Similarly, the numbers of proliferating precursors (% BrdU +/nestin + cells) at DIV2 were significantly greater in LIF-treated E12 and E14 cultures, but not E16 cultures (63.1 ± 1.7% in LIF-treated *versus* 38.7 ± 2.5% in untreated control E12 cultures, $n=12$, $P<0.001$; 41.1 ± 2.1 *versus* 31.3 ± 1.6% in E14 cultures, $n=12$, $P<0.001$; 33.8 ± 1.7 *versus* 29.4 ± 1.9% in E16 cultures, $n=12$, $P=0.16$; Figure 6d).

Next, we examined whether LIF-mediated astrocytic differentiation is additionally dependent on the developmental stage. Precursor cells from E12, E14 and E16 cortices were directly differentiated for 2 days in N2 in the presence or absence of LIF without the preceding bFGF expansion. Compared to the untreated control, GFAP + astrocytic cell numbers were significantly greater in LIF-treated cultures for E14 and E16 cortical cells (none contained GFAP + cells in the absence of LIF, while GFAP + cells were increased to 1.68 ± 1.62 and 31.8 ± 2.8% upon LIF treatment in the E14 and E16 cultures, respectively; Figure 7a). However, consistent with previous results,[23] E12 precursor cells did not undergo LIF-mediated increase in GFAP + cell number. A significantly greater number of cells in LIF-treated E14 and E16 cultures were positive for A2B5, a marker specific for glial progenitors[24] (Figure 7b), suggesting that LIF directs the fate of late neural precursors into astrocytic lineage, and does not merely facilitate GFAP expression in neural precursors or progenitors. On the other hand, no differences in A2B5 + cell numbers in the LIF-treated and control cultures were observed in E12 cultures. After 3 days of bFGF expansion, A2B5 cell populations were 54.1 ± 3.3% in LIF-treated *versus* 37.1 ± 2.6% in untreated control E16 cultures ($n=9$, $P<0.001$), 38.7 ± 1.5 *versus* 27.3 ± 1.6% in E14 cultures ($n=9$, $P<0.001$) and 3.2 ± 0.5 *versus* 3.1 ± 0.2% in E12 cultures ($n=9$, $P=0.633$).

These findings collectively suggest that LIF has two different actions on embryonic cortical precursor cells, depending on the developmental stage of the precursor cells, specifically, survival or maintenance at early brain development and astrocytic differentiation at late development.

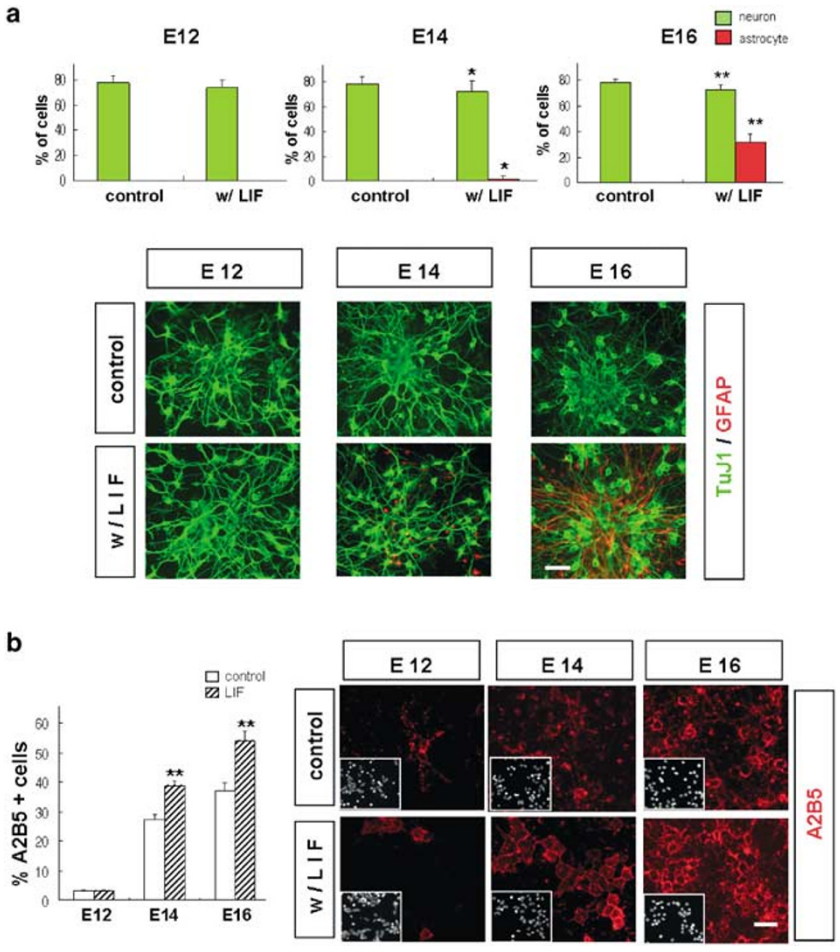

Figure 7 Developmental age-dependent astrocytic differentiation. (**a**) % GFAP + or TuJ1 + cells. Cells from different embryonic ages (E12, E14 and E16) were plated at 2×10^4 cells/cm^2 and were directly induced to differentiate for 2 days in N2 without the preceding bFGF expansion. The lower panel depicts the representative images of Tuj1 (green) and GFAP (red) immunostaining in control and LIF-treated cultures. (**b**) % A2B5 cells. Cells plated at 8000 cells/cm^2 were cultured in N2 + bFGF and immunostaining for A2B5, a surface molecule specific to glial progenitors, was performed after 3 days of expansion. Insets, DAPI nuclear staining of the same field. Results are presented as the mean ± S.E.M. of % GFAP +, TuJ1 + and A2B5 + cells in the total cell population ($n = 15$ from three independent experiments, $^*P < 0.05$ and $^{**}P < 0.001$). Scale bar, 20 μm

Intracellular signal pathways mediated by LIF-induced survival and astrocytic differentiation of neural precursor cells

As documented previously,[25–27] LIF induced the phosphorylation of STAT3, Akt and mitogen-activated protein kinase (MAPK) proteins in E14 cortical precursor cell cultures (Figure 8c), suggesting that Janus-activated kinase/signal transducer and activator of transcription (JAK/STAT), phosphatidylinositol 3 kinase/Akt (PI3K/Akt) and MEK pathways are the downstream transducers activated by LIF. To elucidate the intracellular pathways responsible for the LIF-mediated effects on survival and astrocytic differentiation of cortical precursor cells, each pathway was blocked with pharmacological inhibitors, such as the Jak family tyrosine kinase inhibitor AG 490,[28] the MEK inhibitor PD 98059[29] and the PI3K inhibitor LY 294002.[30] LY 294002 (10 μM) and AG 490 (15 μM) significantly blocked LIF-mediated increase in viable cell number at DIV 1 (2.35 ± 0.15% decreased to 0.82 ± 0.04 and 0.28 ± 0.07%, respectively, $n = 6$, $P < 0.001$, ANOVA with Tukey *post hoc* analysis; Figure 8a). However, PD 98059 (25 μM) did not have an effect on LIF-mediated cell survival (2.45 ± 0.1%, $n = 6$; ANOVA, $P = 0.442$), although the compound efficiently blocked LIF-induced phosphorylation of MAPK (Figure 8c). These inhibitors did not significantly affect cell survival in the absence of LIF at the concentrations tested (data not shown).

LY 294002 and AG 490 markedly blocked LIF-induced increase of astrocytic cells (the percentages of GFAP + astrocytic cells: 47.0 ± 4.3% in cultures treated with LIF alone *versus* 17.5 ± 1.6% with LIF + LY 294002, and 12.7 ± 1.9% with LIF + AG 490, $n = 5$, $P < 0.001$, ANOVA with Tukey *post hoc* analysis; Figure 8b). In contrast to ineffectiveness of the MEK inhibitor on the LIF-induced survival, the percentage of GFAP + astrocytic cells was significantly less in cultures treated with LIF + PD 98059 (30.9 ± 2.8%), compared to those treated with LIF alone. TuJ1 + neuron populations were significantly greater in the cultures treated with each blocker + LIF than with LIF alone (40.8 ± 1.4% with LIF alone *versus* 54.0 ± 1.2% with LIF + LY 294002, 51.9 ± 2.5% with LIF + AG 490 and 51.8 ± 2.2% with PD 98059, $n = 5$, $P < 0.05$; Figure 8b). These findings suggest that the MEK pathway is

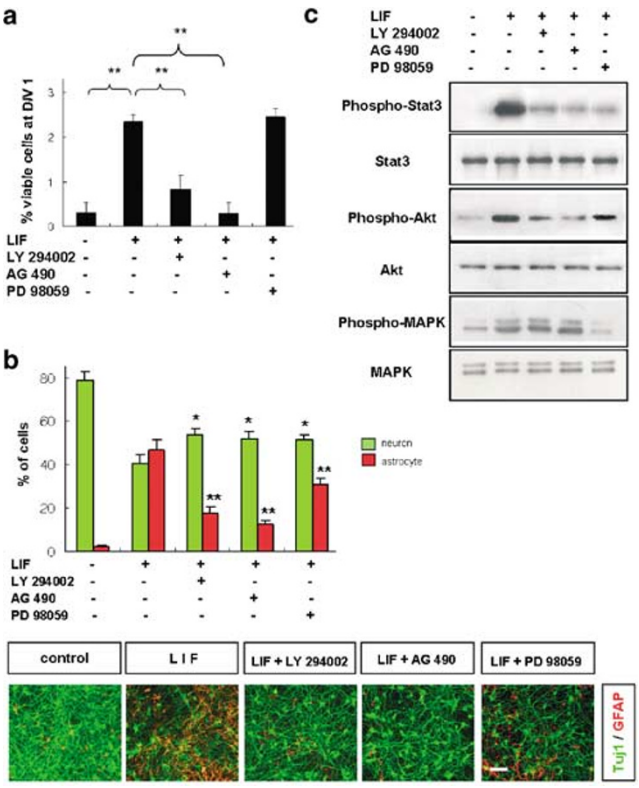

Figure 8 Intracellular pathways responsible for LIF-mediated precursor cell survival (**a**) and astrocytic differentiation (**b**). (**a**) Percentage of viable cell number at DIV 1. Cells isolated from E14 cortices were plated at a clonal density of 2000 cells/6 cm, cultured with LIF in the presence (+) or absence (−) of 25 μM PD 98059, 10 μM LY 294002 or 15 μM AG 490, and viable cells were counted at DIV 1. **Significantly different from LIF-treated cultures without the blocker at $P<0.001$, ANOVA with Tukey *post hoc* analysis, $n=6$. (**b**) Effect of Stat3, Akt or MAPK inhibition on LIF-mediated astrocytic differentiation. Acutely dissociated precursor cells were plated at 8000 cells/cm^2, and expanded for 3 days in the presence (+) or absence (−) of LIF during the last 24 h of expansion with (+) or without (−) the pharmacological inhibitors. After 6 days of differentiation, astrocytes and neurons were estimated by assessing the number of GFAP and Tuj1-immunoreactive cells. The lower panels depict fluorescence images of Tuj1-positive (green) or GFAP-positive (red) cells from the experiments described in (**b**). Scale bar, 20 μm. *$P<0.05$ and **$P<0.001$, compared to LIF-treated cultures, ANOVA with Tukey *post hoc* analysis, $n=5$ for all values. (**c**) LIF-induced activation of Stat3, Akt and MAPK. After 3 days of expansion, cultures were preincubated in the presence (+) or absence (−) of inhibitors for 5 min and either left untreated (−) or stimulated (+) with 20 ng/ml LIF for 30 min in the continuous presence of inhibitors. Cells were lysed and protein extracts were subjected to a Western blot analysis with anti-Stat3, anti-phospho-Stat3, anti-Akt, anti-phospho-Akt, anti-MAPK and anti-phospho-MAPK antibodies. Western blot results shown are typical examples from three independent experiments

specific for LIF-mediated astrocytic differentiation, whereas JAK/STAT and PI3K/Akt are common pathways for LIF-mediated survival and astrocytic differentiation of embryonic cortical precursor cells.

Discussion

Activated LIF-mediated signaling in embryonic neural precursor cells

In the present study, we demonstrate that LIF is an autocrine/paracrine factor for the survival and astrocytic differentiation of embryonic cortical precursor cells. Compared to neuron- and astrocyte-enriched cultures, LIF release (Figure 1c) and gp130 and LIFR expression (Figure 1b) were abundant in neural precursor cell-enriched cultures, suggesting that LIF-mediated signaling is active in embryonic neural precursor cells. These findings are consistent with previous studies that show mRNA expression of LIF and its receptor components in embryonic brain and cultures for neuroepithelium[31] and gp130 expression in the ventricular zone of E11–E15, which decreased in the ventricular zone but increased at later developmental stages in the subventricular zone in which the neural precursor cell pool resides.[19]

The regulation of receptor subunit expression by their respective ligands in gp130 signaling is a controversial issue. Following ligand binding, activated gp130 receptor-mediated signaling is negatively regulated by degradation or reduced cell-surface expression of the receptor subunits.[32,33] In contrast, several recently published studies have demonstrated enhanced synthesis of the gp130 receptor upon LIF treatment.[34,35] Consistently, treatment with exogenous LIF in this study led to enhanced expression of its receptor components in neural precursor cell-enriched cultures (Figure 2). The increase in LIF receptor expression in response to LIF is likely to be due to a direct stimulation of receptor expression in individual cells. However, it could also be a result of an increase in the proportion of undifferentiated precursors to differentiated cells by LIF action on precursor cell self-renewal, considering that LIF receptor mRNAs in undifferentiated precursors are more abundant than differentiated cultures (Figure 1b) and that the cultures do not represent complete pure populations of undifferentiated precursors.

LIF-mediated effects on cell survival and astrocytic differentiation of embryonic cortical precursor cells

In the present study, we show that the LIF-mediated signaling is responsible for the survival of embryonic neural precursor cells. However, earlier investigations[18,19] reported gp130-mediated proliferation of neural precursor cells instead of the survival effect of LIF. Cell survival and proliferation mutually affect each other, particularly if intimate cell–cell interactions are established, such as the *in vivo* state or *in vitro* culture at high cell densities. Thus a number of effects of growth factors, originally interpreted as stimulation of proliferation, may be the result of cell death inhibition. In the present situation, none of the parameters available to date (e.g., total cell number, BrdU+, Ki67+ cell count and TUNEL test) provided an absolute indicator for these activities. The best way to distinguish whether a cytokine has a survival or proliferation effect is to design experimental conditions in which cell–cell interactions are minimized. We evaluated LIF functions in embryonic neural precursor cells using *in vitro* culture with clonal cell densities at which interactions between cells through soluble signals and/or direct cell–cell contacts were minimized. LIF treatment significantly enhanced the survival of cortical precursor cells for 1 day in cultures at the clonal cell densities (Figure 3a), but the effect was minimized when the

number of cells was increased by seeding more cells, and no longer significant at $>3.2\times10^4$ cells/cm^2 (Figure 3f). Thus, previous studies demonstrating the ineffectiveness of gp130/LIFR-mediated signaling on precursor cell survival at the *in vivo* state or high-density culture system may be attributable to the low interest in the cell-density effect.

BrdU incorporation and cell expandability significantly increased in the early expansion period of E14 cortical precursor cultures (Figures 3d and 5a). The increase of these proliferation indices is likely to be attributable to the LIF-mediated survival on proliferating precursors, as described above. Previous studies report that gp130-mediated signals promote neural progenitor cell re-entry into the stem cell cycle without affecting the duration of the cycle.[19,36] Thus re-entering the cell cycle may be another possible mechanism for LIF-mediated increase in BrdU+ cells.

Another effect of LIF on astrocytic differentiation has been demonstrated under various conditions, such as cultures for embryonic cortical precursor cells with clonal cell densities, directly differentiated conditions and cultures with long-term cell expansion (Figures 4 and 5). We evaluated astrocytic differentiation by the acquisition of GFAP expression in the differentiated cultures. GFAP is generally a definite hall marker for the cells on astrocytic lineage. Given that GFAP+ cells in the subventricular zone of the adult brain are designated 'adult neural stem' or 'precursor' cells,[37] questions have been raised whether GFAP-expressing cells in primary cultures also have neural stem cell potentials. However, it has been demonstrated that embryonic neural stem cells do not express GFAP *in vivo* or *in vitro*, although the predominant neural stem cells from adult brain express GFAP.[38] Consistently, GFAP was not colocalized in nestin+ cells in the cultures for cortical precursors isolated from embryonic brain and GFAP expression was detected only after the induction of precursor cell differentiation (Figure 1a). We also addressed the detection of A2B5+ glial progenitor cells in embryonic cortical precursor cultures. A2B5 is a surface molecule specific to common glial progenitors for astrocytes and oligodendrocytes. However, oligodendrocytic differentiation is absent in embryonic brain and rare in primary cultures for embryonic neural precursor.[16] Furthermore, it has been demonstrated that fetal brain-derived A2B5+ cells give rise to mainly GFAP+ astrocytes.[39] These previous findings provide a rationale in employing A2B5 for evaluating the acquisition of astrocytic fate in embryonic neural precursor cultures. Astrocytic differentiation, estimated by GFAP+ cell number, was enhanced in the presence of LIF with a reduction in the number of differentiated TuJ1+ neurons produced (Figure 4). Short-term administration of LIF at early differentiation period (for 12 h at day 1 of differentiation) also effectively enhanced the astrocytic differentiation, whereas the exposure of the cells to LIF at day 6 of differentiation was not effective in increasing GFAP+ cell number (data not shown). These findings suggest that LIF does not merely induce GFAP expression but instructs neural precursor cells to progress to the astrocytic lineage. The LIF effect on astrocytic fate determination was supported by the finding that A2B5+ cell population was greater in LIF-treated E14 and E16 cortical precursor cultures (Figure 7b).

The LIF-mediated effect on cell survival was not observed in late (E16) cortical precursor cells, whereas the compound was ineffective in inducing the differentiation of early (E12) cortical precursor cells into astrocytes. This suggests that the LIF effect on the survival of embryonic neural precursor cells is restricted to the early developmental stage, while LIF has an effect on astrocytic differentiation only during late development. These differential actions of LIF depending on the developmental stage were also observed during the *in vitro* expansion of precursor cells isolated from E14 cortices. During the early period of *in vitro* cell expansion, LIF enhances cell viability. Accordingly, the fold increase in cell number was significantly greater in cultures treated with LIF, compared with control cultures (Figure 5a and b). However, if LIF is added during the late period of cell expansion, survival is not significantly altered. In contrast to the early period of expansion, the fold increase in LIF-treated cultures was smaller to that with control cultures during the late expansion period (Figure 5a). Due to the earlier appearance and greater number of GFAP+ cells during the expansion period in LIF-treated cultures, the decreased expandability is possibly attributable to the LIF effect on directing precursor cell commitment into astrocytic lineage.

Intracellular pathways of LIF-mediated neural precursor cell survival and astrocytic differentiation

Following ligand binding, the gp130-associated tyrosine kinase, JAK, phosphorylates STAT proteins (mainly STAT3) and the protein tyrosine phosphatase SHP-2.[40] Phosphorylated STAT3 monomers are dimerized and translocated to the nucleus where they bind specific DNA response elements in target gene promoters.[41] Phosphorylation of SHP-2 results in activation of the MEK/MAPK and PI3K/Akt signaling pathways.[26,27] All these pathways have been implicated in promoting cell survival to varying extents. Recent studies demonstrate that JAK/STAT and PI3K/Akt act as important intermediaries of cytokine-induced survival in motor neurons[42] and sensory neurons.[43] While MEK/MAPK signaling plays an important neuroprotective role following axotomy,[44] oxidative stress[45] and exposure to various toxic stimuli,[46,47] the pathway makes only a minimal contribution to neurotrophin-mediated neuronal survival.[48,49] Consistently, inhibition experiments performed in this study showed that LIF-induced neural precursor cell survival is mediated through activation of the JAK/STAT and PI3K/Akt, but not MEK/MAPK pathway (Figure 7a). Previous studies have shown that PI3K is the most relevant pathway mediating the survival-promoting effects of several trophic factors such as BDNF[50] and insulin.[51] Thus, JAK/STAT, an upstream activator of PI3K,[25,26] may be required for activating the PI3K pathway in LIF-mediated precursor cell survival. However, there is evidence for the induction of Bcl-X_L by a JAK-regulated survival pathway that is independent of activation of PI3K/Akt signaling.[52] Thus, a PI3K-independent role of JAK/STAT signaling in the survival of neural precursor cells cannot be excluded.

JAK/STAT is the major signal transducer mediating gp130/LIFR-mediated signal to astrocytic differentiation.[53] The MAPK pathway is required early in the astrocytic differentiation process and positively coupled with JAK/STAT for astrocytic differentiation *in vitro*.[54] In addition, the involvement of the PI3K/Akt pathway in CNTF-mediated astrocytic differentiation has been discussed. The nuclear receptor co-repressor (N-CoR), defined as a regulator of nuclear receptor-mediated repression, controls the differentiation of neural precursor cells into astrocytes.[55] CNTF activates PI3K/Akt-dependent phosphorylation of N-CoR and eventually causes astrocytic differentiation via redistribution of N-CoR to the cytoplasm. Consistent with these data, our inhibition studies show that all the JAK/STAT, MAPK and PI3K/Akt pathways are responsible for LIF-mediated astrocytic differentiation.

In conclusion, we reveal developmental stage-dependent roles of LIF in cultures for embryonic cortical precursor cells, which may partially explain how the embryonic brain regulates the timed developmental sequences, maintenance and differentiation of neural precursor cells.

Materials and Methods

Cell culture

Cells from rat embryonic cortices (gestation day 12, 14 or 16; day of conception = day 0) were mechanically dissociated in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS) and plated on fibronectin (1 μg/ml, Invitrogen, Carlsbad, CA, USA)-coated 6 cm culture dishes (featuring a 2 mm grid on the cell growth surface; Corning, NY, USA) or 12 mm glass coverslips (Bellco, Vineland, NJ, USA). Cell proliferation was induced with bFGF (15 ng/ml; R&D systems, Minneapolis, MN, USA) in serum-free defined medium (N2).[16] The mitogen was withdrawn in the medium to promote differentiation. In some experiments, bFGF-expanded cells, without direct induction of differentiation, were passaged by trypsinization and re-plated onto freshly coated dishes or coverslips for subsequent culture. For clonal analysis, dissociated cells were plated at 1000 cells/6 cm dish. After 6 h of settling, well-isolated cells were marked with a 3 mm circle marker (Nikon, Tokyo, Japan) on the bottom of the plate. Only cells growing within the marked circles were analyzed as clones. SCM was prepared as described previously[7] and in some experiments added to the medium at a concentration of 50% (v/v). The following reagents were added to cultures at the given concentrations: LIF (20 ng/ml, or for dose response, 0.1–100 ng/ml; ESGRO™; Chemicon, Temecula, CA, USA), anti-LIF blocking antibody (diluted to 1 : 1000; R&D systems), EGF (15 ng/ml; R&D systems), PD 98059 (25 μM), AG 490 (15 μM) or LY 294002 (10 μM; Calbiochem, San Diego, CA, USA).

Immunostaining

Cultured cells or cryosectioned cortical slices were immunostained after fixation with 4% paraformaldehyde/0.15% picric acid in PBS, except in the case of A2B5 immunostaining. Cortical sections from the E14 rat brain were prepared as described previously.[56] Primary antibodies and their dilutions are as follows: nestin anti-rabbit, 1 : 50 (Martha Marvin and Ron McKay, NIH, Bethesda, MD, USA), TuJ1 anti-rabbit, 1 : 2000 (Babco, Richmond, CA, USA) and anti-mouse, 1 : 500 (Babco), GFAP anti-mouse, 1 : 100 (ICN Biochemicals, Costa Mesa, CA, USA), BrdU anti-rat, 1 : 100 (Accurate Chemical, Westbury, NY, USA), Ki67 anti-mouse, 1 : 200 (Novocastra Laboratories Ltd., Newcastle, UK) and anti-LIF anti-goat, 1 : 1000 (R&D systems). Except LIF immunostaining, immunoreactive cells were visualized using fluorescent-labeled secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Peroxidase staining was performed using a biotinylated secondary antibody and Vectastain ABC peroxidase and AEC substrate kits for the detection of LIF antibody (Vector Laboratories, Burlingame, CA, USA). VECTASHIELD^R with DAPI mounting medium was used for nuclear counterstaining (Vector Laboratories). For A2B5 immunostaining, live cells were incubated with an A2B5 antibody (1 : 200, anti-mouse, Chemicon), and visualized with a Cy3-conjugated secondary antibody. Coverslips were fixed and mounted for microscopic examination.

TUNEL, LDH and BrdU assay

The TUNEL assay was performed using an *in situ* cell death detection POD kit (Roche Ltd., Basel, Switzerland), following the manufacturer's protocol. Viability was additionally measured with the LDH assay. LDH activity was measured with a Cytotox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Results were expressed as a percentage of maximum LDH release obtained on complete cell lysis following 1.0% Triton X-100 treatment. Fresh N2 medium was taken as the negative control (0%). Cells were pulsed with 10 μM BrdU (Roche) for 1.5 h before fixation. Incorporation of BrdU was detected using anti-BrdU antibody (Accurate Chemical), as described above.

Semiquantitative and real-time RT–PCR analysis

Total RNA was prepared with the Tri- reagent (MRC Inc., Cincinnati, OH, USA) followed by cDNA synthesis using the Superscript II kit (Invitrogen), based on the manufacturer's instructions. PCR was performed with *Taq* polymerase using standard protocols. Primer sequences, annealing temperature and $MgCl_2$ concentrations are as follows: CNTF receptor, tatgcctgtttccaccgtgac, attcgagagctccacatgct, 56°C, 1.5 mM; LIF receptor, aggacgtcaattcaacagtcg, tttcttgccaccacactgatg, 56°C, 1.5 mM; and gp130, tgcctcaacttggattcaggt, tcacagtgccatcttcttgct, 56°C, 1.5 mM. Primer sequences and PCR conditions for glyceraldehyde phosphate dehydrogenase (GAPDH), CNTF and LIF are published in an earlier report.[7] Real-time PCR was performed on the iCycler iQ™ (Bio-Rad, Hercules, CA, USA) using SYBR Green (Molecular Probe, Inc., Eugene, OR, USA), according to the manufacturer's instructions. For the quantification of relative gene expression, intercalated SYBR fluorescence was measured in real time during the extension step. All gene expression values were normalized to GAPDH.

Western blot analysis

Proteins secreted from cultured cells were collected in 4 ml HBSS for 12 h.[7] Proteins were denatured by boiling in 2 $\times$ Laemmli buffer containing 10% β-mercaptoethanol, and electrophoresed on 10% SDS–polyacrylamide gels. The equivalent of 2 ml HBSS media was loaded per well. In the cases of total cell lysates, 10 μg protein per each sample was electrophoresed and transferred to a nitrocellulose membrane, which was incubated in 5% bovine serum albumin to block nonspecific binding. The blot was probed with an anti-LIF goat antibody (diluted to 1 : 2000; R&D systems), anti-CNTF mouse antibody (1 : 300; Chemicon), anti-gp130 rabbit antibody (1 : 200; SantaCruz Biotechnology, Inc., SantaCruz, CA, USA), anti-STAT3 rabbit antibody (1 : 1000), anti-phospho-STAT3 (Tyr705) rabbit antibody (1 : 1000), anti-Akt rabbit antibody (1 : 1000), anti-phospho-Akt (Ser473) rabbit antibody (1 : 1000), anti-p44/42 MAPK

mouse antibody (1 : 1000) or anti-phospho-p44/42 MAPK (Thr202/Tyr204) mouse antibody (1 : 1000; all from Cell signaling Technology, Inc., Beverly, MA, USA) followed by peroxidase-conjugated anti-goat IgG (SantaCruz), anti-rabbit IgG (New England Biolab. Inc., Beverly, MA, USA) or anti-mouse IgG (New England Biolab.) with all for 1 : 2000 dilution. Bands were visualized by enhanced chemiluminescence (ECL detection kit, Amersham Pharmacia Co., Buckinghamshire, UK).

Statistical analysis

Statistical comparisons were performed using SPSS software (version 11.0; SPSS Inc., Chicago, IL, USA). One- or two-way ANOVA, followed by Tukey *post hoc* comparison was applied where appropriate. All the results are presented as mean $\pm$ S.E.M. and the null hypothesis was rejected on the basis of $P < 0.05$.

Acknowledgements

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