Non-cell-autonomous action of STAT3 in maintenance of neural precursor cells in the mouse neocortex

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The transcription factor STAT3 promotes astrocytic differentiation of neural precursor cells (NPCs) during postnatal development of the mouse neocortex, but little has been known of the possible role of STAT3 in the embryonic neocortex. We now show that STAT3 is expressed in NPCs of the mouse embryonic neocortex and that the JAK-STAT3 signaling pathway plays an essential role in the maintenance of NPCs by fibroblast growth factor 2. Conditional deletion of the STAT3 gene in NPCs reduced their capacity to form neurospheres in vitro, as well as promoted neuronal differentiation both in vitro and in vivo. Furthermore, STAT3 was found to maintain NPCs in the undifferentiated state in a non-cell-autonomous manner. STAT3-dependent expression of the Notch ligand Delta-like1 (DLL1) appears to account for the non-cell-autonomous effect of STAT3 on NPC maintenance, as knockdown of DLL1 by RNA interference or inhibition of Notch activation with a γ-secretase inhibitor abrogated the enhancement of neurosphere formation by STAT3. Our results reveal a previously unrecognized mechanism of interaction between the JAK-STAT3 and DLL1-Notch signaling pathways, as well as a pivotal role for this interaction in maintenance of NPCs during early neocortical development.

KEY WORDS: STAT3, Neural precursor cell, Neocortex, FGF2, Notch, DLL, Non-cell-autonomous

INTRODUCTION

The three major cell types of the mammalian central nervous system – neurons, astrocytes and oligodendrocytes – are derived from common neural precursor cells (NPCs) that reside in the ventricular zone (VZ) of the developing neuroepithelium (Anderson, 2001; Davis and Temple. 1994; Gage, 2000; McKay, 1997). In the early stages of brain development, NPCs primarily increase in number. They proliferate and generate neurons and glial cells in a sequential manner during mid-to-late gestation and postnatal development, respectively (McConnell, 1995; Qian et al., 2000). The final number of neurons in each region of the brain is determined by various factors, including the number of times that NPC's divide in the undifferentiated state before undergoing the neuronal differentiation that ultimately leads to cell cycle arrest. The maintenance of NPCs in the undifferentiated state is thus strictly regulated during brain development, although the underlying mechanisms are not fully understood. Such regulation is also an important determinant of the pool size of NPCs in the adult brain.

Maintenance of NPCs is supported by two types of extracellular cues: Notch ligands and secreted growth factors. Notch is a transmembrane receptor that is activated on cell-cell contact by its ligands delta-like and jagged expressed in neighboring cells (Artavanis-Tsakonas et al., 1999; Lai, 2004; Yoon and Gaiano, 2005). After such ligand binding, the intracellular domain of Notch is released as a result of cleavage by the presenilin (PS)-containing γ-secretase complex and translocates to the nucleus, where it activates the transcription factor RBP-J. Activation of Notch signaling results in the expression of target genes such as those for HES1 and HES5 (Artavanis-Tsakonas et al., 1999; Honjo, 1996; Kageyama and Nakanishi, 1997; Selkoe and Kopan, 2003; Yoon and Gaiano, 2005), both of which inhibit the activity and expression of proneural basic helix-loop-helix (bHLH) proteins. Misexpression of activated NOTCH1 or of HES1 or HES5 in the developing mouse forebrain inhibits neuronal differentiation and results in expansion of the NPC pool (Gaiano et al., 2000; Ohtsuka et al., 2001), whereas mice lacking PS1, PS2, NOTCH1, RBP-J, or both HES1 and HES5 manifest premature neurogenesis accompanied by a loss of NPCs (Cau et al., 2000; de la Pompa et al., 1997; Donoviel et al., 1999; Handler et al., 2000; Lutolf et al., 2002; Ohtsuka et al., 1999).

The abundance of NPCs can be estimated in vitro by culture of neural cells in suspension at a low density and determination of the efficiency of formation of ‘neurospheres’ that contain cells capable both of generating new neurosphere colonies on dissociation and of differentiating into neurons and glia (Reynolds and Weiss, 1996; Tropepe et al., 1999). Inactivation of the Notch signaling pathway by treatment with a γ-secretase inhibitor or by disruption of NOTCH1, PS1, RBP-J, or both HES1 and HES5 genes impaired the ability of mouse neuroepithelial cells (or embryonic stem cell-derived neural cells) to form passagable neurospheres (Chojnacki et al., 2003; Hitoshi et al., 2002; Ohtsuka et al., 2001), further supporting the notion that the Notch pathway is essential for NPC maintenance.

Various secreted growth factors are also thought to contribute to the maintenance of NPCs. The activity of fibroblast growth factor 2 (FGF2) in this regard has been widely taken advantage of to isolate and expand NPCs in a variety of in-vitro systems (Gritti et al., 1999; Kilpatrick and Bartlett, 1993; Mayer-Proschel et al., 1997; Reynolds et al., 1992; Reynolds and Weiss, 1992; Reynolds and Weiss, 1996; Tropepe et al., 1999; Vescovi et al., 1993). FGF2-responsive NPCs first appear in the mouse neocortex as early as embryonic day (E) 8.5, and an additional population of epidermal growth factor (EGF)-responsive NPCs arises from these FGF2-responsive cells between E11 and 13 (Burrows et al., 1997; Mayer-Proschel et al., 1997;
Tropepe et al., 1999). Mice lacking FGF2 exhibit a reduced density and number of neurons in the neocortex (Dono et al., 1998; Ortega et al., 1998; Raballo et al., 2000), whereas microinjection of FGF2 into the cerebral ventricles at early stages of mouse embryogenesis increased the number of rounds of division of NPCs, resulting in an increased number of neurons (Vaccarino et al., 1999). FGF2 signaling thus appears to contribute to NPC maintenance during embryogenesis, although the mechanism of this effect remains largely unknown.

We have now found that STAT3 (signal transducer and activator of transcription 3) is responsible for the maintenance of mouse embryonic neocortical NPCs by FGF2. STAT3 is a transcription factor that is activated as a result of phosphorylation on Tyr705 by tyrosine kinases such as Janus kinase (JAK). Such phosphorylation induces dimerization of STAT3 and its translocation to the nucleus, where it modulates transcription through interaction with specific DNA elements (Levy and Darnell, 2002). STAT3 promotes astrocytic differentiation in the postnatal mouse neocortex (Bonnin et al., 1997; Rajan and McKay, 1998), but its roles in the early embryonic neocortex have been unclear. We found that STAT3 is expressed specifically in NPCs at early stages of neocortical development, and that deletion of the STAT3 gene resulted in premature neuronal differentiation of neighboring cells. We also show that Delta-like1 (DLL1) is an essential component of the STAT3-mediated signaling pathway responsible for NPC maintenance, demonstrating a nexus between the Delta-Notch and JAK-STAT3 pathways that regulates the fate of NPCs.

MATERIALS AND METHODS
Expression constructs and reagents
The plasmids pMX-IRE5-EGFP and pMX-SV40-puro were kindly provided by Dr T. Kitamura. STAT3-C and Cre recombinase constructs were kindly provided by Drs J. F. Bromberg and S. Kato, respectively. The plasmids pMX-GFP and pMX-CΔ8 were constructed by inserting the coding sequences for enhanced green fluorescent protein (EGFP) or for the extracellular and transmembrane domains of human CD8 into pMX-SV40-puro, replacing the puromycin resistance gene. The coding sequence for STAT3-C was inserted into pMX-IRE5-EGFP to create pMX-STAT3-C-GFP or pMX-CΔ8 to create pMX-STAT3-CΔ8. The coding sequence for Cre recombinase was inserted into pMX-GFP or pMX-CΔ8 to create pMX-Cre-GFP and pMX-Cre-CΔ8.

All pharmacological signaling pathway inhibitors were obtained from Calbiochem (AG490, LY294002, U0126, L685,458).

Primary culture
Neuroepithelial cells were isolated as described previously (Hirabayashi et al., 2004). The cells were cultured in medium comprising 1:1 (v/v) mixture of Dulbecco’s modified Eagle’s medium and F12 medium (Gibco) supplemented with B27 (Invitrogen) with or without FGF2 (20 ng/ml, R&D) or EGF (20 ng/ml, Upstate Biotechnology).

Neuroepithelial cell culture
The cells were transferred to noncoated 100-mm dishes for 90 minutes to remove non-neural cells, and the remaining floating cells were plated on poly-D-lysine-coated dishes to yield a neuroepithelial cell culture, which was maintained in the presence of FGF2 (20 ng/ml). Infection of these cells with a retrovirus encoding GFP (pMX-GFP) and subsequent immunofluorescence analysis revealed that >90% of the nonfibroblastic GFP-positive cells expressed nestin (fibroblast-like cells were excluded from the analysis on the basis of their distinct morphology).

NPC culture
To obtain an NPC-enriched population, we plated the dissociated neuroepithelium directly on noncoated 100-mm dishes in culture medium containing FGF2 (20 ng/ml) and EGF (20 ng/ml) and cultured the cells for 3 days. The resulting neurospheres were then dissociated and plated on poly-D-lysine-coated dishes in culture medium containing FGF2 (20 ng/ml) to yield an NPC culture.

Immunoblot analysis
Immunoblot analysis was performed as described (Kamakura et al., 2004). Primary antibodies included rabbit polyclonal antibodies to STAT3 (1:1000 dilution, Upstate Biotechnology) or to the Tyr705-phosphorylated form of STAT3 (1:200, Cell Signaling), with mouse monoclonal antibodies to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000, Chemicon), or with goat polyclonal antibodies to DLL1 (1:100, C-20, Santa Cruz Biotechnology).

Neurosphere assay
Completely dissociated primary neuroepithelial cells were suspended in culture medium containing FGF2 (20 ng/ml) at a density of 5000 cells/ml, and 200 μl of the cell suspension were transferred to each well of a 96-well plate. The number of primary neurospheres was counted after culture for 7 days. About 5% of neuroepithelial cells prepared from E12.5 mouse neocortex generated neurospheres in the presence of FGF2, whereas no neurospheres formed in the absence of FGF2. About 90% of the primary neurospheres formed in the presence of FGF2 were multipotent, in that they were able to differentiate into both neurons and glia when plated on poly-D-lysine-coated dishes in the absence of FGF2 (data not shown).

For assay of the formation of secondary neurospheres, primary neurospheres formed at a cell density of 2.5 × 10^5 cells/ml in 6-well plates and culture medium containing FGF2 (20 ng/ml) were collected and dissociated. Portions (200 μl) of cell suspension (5000 cells/ml) were then transferred to each well of a 96-well plate in culture medium containing FGF2 (20 ng/ml) and EGF (20 ng/ml). The number of secondary neurospheres was counted after culture for 7 days.

Immunocytochemistry
Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and incubated with primary antibodies for 2 hours. Primary antibodies included rabbit anti-STAT3 (1:500 dilution, Medical and Biological Laboratories), mouse anti-STAT3 (1:500, Chemicon), mouse anti-CΔ8 (1:200, PharMingen), mouse anti-nestin (1:200, Becton Dickinson), mouse anti-βIII-tubulin (TuJ1) (1:500, Babco), rabbit anti-GFAP (1:2000, DAKO), and mouse anti-BrDU (1:50, BD). Immune complexes were detected with Alexa Fluor 488- or Alexa Fluor 594-conjugated goat secondary antibodies (Molecular Probes).

Immunohistochemistry
Immunohistochemistry was performed as described (Hirabayashi et al., 2004). For staining with anti-DLL1, antigen retrieval was accomplished by autoclave treatment of sections for 15 minutes at 105°C in 0.01 mol/l sodium citrate buffer (pH 6.0) and subsequent washing with Tris-buffered saline containing 0.05% Tween 20 (TBS-T). Primary antibodies included rabbit anti-STAT3 (1:100 dilution, Sigma), rabbit anti-STAT3 (1:500), mouse anti-STAT3 (1:1000), mouse anti-nestin (1:200), mouse anti-βIII-tubulin (1:1000), rabbit anti-SOX2 (1:500, Chemicon), mouse anti-MAP2 (1:500), HM-2, SIGMA), rabbit anti-Musashi (1:500, Chemicon), and rabbit anti-DLL1 (1:100, H-265, Santa Cruz Biotechnology). The sections were washed in TBS-T and then incubated for 1 hour at room temperature with donkey secondary antibodies conjugated with Alexa Fluor 488- or Alexa Fluor 594-conjugated goat secondary antibodies (Molecular Probes), or with biotin-conjugated secondary antibodies before exposure to reagents first of an ABC kit (Vector Laboratories) and then of a TSA kit (Molecular Probes).

In situ hybridization
In-situ hybridization on frozen sections was performed basically as described previously (Nomura and Osumi, 2004). For staining with anti-DLL1, antigen retrieval was accomplished by autoclave treatment of sections for 15 minutes at 105°C in 0.01 mol/l sodium citrate buffer (pH 6.0) and subsequent washing with Tris-buffered saline containing 0.05% Tween 20 (TBS-T). Primary antibodies included rabbit anti-STAT3 (1:100 dilution, Sigma), rabbit anti-STAT3 (1:500), mouse anti-STAT3 (1:1000), mouse anti-nestin (1:200), mouse anti-βIII-tubulin (1:1000), rabbit anti-SOX2 (1:500, Chemicon), mouse anti-MAP2 (1:500), HM-2, SIGMA), rabbit anti-Musashi (1:500, Chemicon), and rabbit anti-DLL1 (1:100, H-265, Santa Cruz Biotechnology). The sections were washed in TBS-T and then incubated for 1 hour at room temperature with donkey secondary antibodies conjugated with Alexa Fluor 488- or Alexa Fluor 594-conjugated goat secondary antibodies (Molecular Probes), or with biotin-conjugated secondary antibodies before exposure to reagents first of an ABC kit (Vector Laboratories) and then of a TSA kit (Molecular Probes).

In utero electroporation
Introduction of plasmid DNA into neuroepithelial cells of mouse embryos in utero was performed as described (Tabata and Nakajima, 2001). Plasmid DNA (pMX-GFP or pMX-Cre-GFP, at 1 ng/ml) was injected into the lateral...
ventricle of each littermate at E14.5. Two days after electroporation, the embryos were harvested and the brains were examined immunohistochemically.

**Chromatin immunoprecipitation (ChIP)**
ChIP assay was performed as described previously (Hirabayashi et al., 2004). Mouse anti-STAT3 (C-20) or control immunoglobulin G (Santa Cruz Biotechnology) was used. The sense and antisense primers for amplification of fragments of the DLL1 locus were 5′-TGGTAGGTGGAACCC-3′ and 5′-AGGCCAAAAGGTAACTATACATC-3′ (nucleotides –6777 to –6638, relative to the transcription start site), 5′-TGGTAGGTGGAACCC-3′ and 5′-CTGGACCTGCCTGCTC-3′ (nucleotides –2819 to –2639), 5′-CTGGACCTGCCTGCTC-3′ and 5′-ATGGACCTGCCTGCTC-3′ (nucleotides –2370 to –2211), 5′-ATGGACCTGCCTGCTC-3′ and 5′-TTCACCTGGTCGTCGTC-3′ (nucleotides –1486 to –1323), 5′-ACTCTCCAGGGCTCAATC-3′ and 5′-CTGTCAGCCTCCCTTCTC-3′ (nucleotides –1118 to –937), 5′-AGCGTGACCTGGGCCTGGGG-3′ and 5′-CGCTCATTACATCTGC-3′ (nucleotides –760 to –644), and 5′-GGTGCAGCAGCTGGTGATGCG-3′ and 5′-GGCGACTGACCTGGTGATGCG-3′ (nucleotides +4572 to +697), respectively.

**RT-PCR**
Total RNA was isolated from infected NPC cultures with the use of the TRIzol reagent (Invitrogen) and was subjected to RT with an oligo(dT)12.18 primer (Invitrogen) and ReverTra Ace reverse transcriptase (Toyobo). The abundance of target mRNAs was normalized relative to that of GAPDH mRNA. The sense and antisense primers, respectively, were as follows: GAPDH, 5′-TGGGTGAT-3′ and 5′-AGTACCCAGCCTGCCTA-3′; NOTCH1, 5′-TCAGAGGCCACATAGCTTC-3′ and 5′-AAGTTGTCATGGATGACCTT-3′; DLL1, 5′-TCAGAGGCCACATAGCTTC-3′ and 5′-TTAATGCGCTTCTGGAATGTG-3′; DLL1, 5′-TGGTAGGTGGAACCC-3′ and 5′-GGCGACTGACCTGGTGATGCG-3′; and GFAP, 5′-TCAGAGGCCACATAGCTTC-3′ and 5′-TCGACCTGATGGTTGATGCG-3′.

**Statistical analysis**
Quantitative data are presented as means ± s.e.m. from representative experiments that were repeated a total of more than three times with similar results. Values were compared with the unpaired Student’s t test. A P-value of <0.05 was considered statistically significant.

**RESULTS**

**JAK2 activity is required for NPC maintenance**
To identify signaling molecules that mediate NPC maintenance by FGF2, we performed an in-vitro colony-formation assay, known as a neurosphere assay, that has been widely applied to monitor the abundance of NPCs (Campos et al., 2004; Hitoshi et al., 2002; Molofsky et al., 2003; Weiss et al., 1996). In this assay, neuroepithelial cells are dissociated and cultured in suspension at a low density (<15,000 cells/ml) so that most of the resulting cell aggregates (neurospheres) are derived from single cells (Hulspas et al., 1997). We first examined whether signaling pathways mediated by the mitogen-activated protein kinase ERK and its upstream regulator MEK, by phosphatidylinositol 3-kinase (PI3K), or by JAK and STAT contribute to the FGF2-dependent formation of neurospheres with the use of pharmacological inhibitors. Neuroepithelial cells prepared from E12.5 mice were dissociated and cultured with FGF2 in the absence or presence of either the MEK inhibitor U0126 (10 μM), the PI3K inhibitor LY294002 (20 μM), or the JAK2 inhibitor AG490 (10 μM). Treatment of neuroepithelial cells with these concentrations of U0126, LY294002 or AG490 reduced the level of phosphorylation of ERK, Akt and STAT3, respectively, observed in the presence of FGF2 (data not shown), confirming that these reagents effectively blocked the corresponding signaling pathways. Culture of the cells for 7 days with AG490 resulted in marked inhibition of neurosphere formation, whereas LY294002 exhibited a smaller (but significant) inhibitory
effect and U0126 had no effect (Fig. 1A). These results suggest that JAK2 and PI3K contribute to FGF2-mediated formation of neurospheres.

To examine whether JAK2 and PI3K are involved in NPC maintenance, we next exposed primary neurospheres generated by incubation of neuroepithelial cells in the presence of FGF2 to the inhibitors during day 3 of the culture. The neurospheres were then dissociated and replated in the presence of FGF2 and EGF to allow the formation of secondary neurospheres in the absence of inhibitors (Fig. 1B). In this assay, >80% of the resulting secondary neurospheres generated neurons, astrocytes and oligodendrocytes when cultured under differentiation-promoting conditions (data not shown), indicating that most of the neurosphere-forming cells were multipotent NPCs. Exposure of the primary neurospheres to AG490, or to U0126 and LY294002 together, or to LY294002 alone resulted in a significant decrease in the number of secondary neurospheres (Fig. 1B; see Fig. S1A in the supplementary material). The proliferation rate of overall neuroepithelial cells was slightly inhibited by AG490, whereas the survival rate was not much affected (see Fig. S1C,D in the supplementary material). However, AG490 increased the percentage of neuron-only clones in a clonal assay (see Fig. S1B in the supplementary material). These results together suggest that the activity of JAK2 is required for FGF2-dependent NPC maintenance (which includes promotion of survival/proliferation and inhibition of differentiation of NPCs).

**STAT3 is required for NPC maintenance**

JAK2 activates members of the STAT family of transcription factors (Levy and Darnell, 2002). Among the members of this family, STAT3 is abundant and expressed in the brain (De-Fraja et al., 1998; Yan et al., 2004). We found that the expression of STAT3 in the forebrain of E13.5 and 16.5 mouse embryos overlapped in part with that of the NPC marker nestin (Fig. 2A-F,H,J). Nestin was exclusively expressed in cells in the VZ and in radial fibers, whereas STAT3 was expressed in these nestin-positive cells as well as in cells in the subventricular zone (SVZ) and the apical side of the intermediate zone (IMZ) (Fig. 2K-R). In-situ hybridization analysis revealed a distribution of STAT3 mRNA similar to that of the encoded protein (Fig. 2S). By contrast, the expression of STAT3 was not detected in cells positive for the neuronal marker TuJ1 (βIII-tubulin) (Fig. 2G,I), suggesting that STAT3 is abundant in NPCs and downregulated after neuronal differentiation.

Removal of FGF2 from the culture medium reduced the level of phosphorylation of STAT3 on Tyr705 in ‘NPC cultures’ (monolayer cultures obtained by plating primary neurospheres generated from the embryonic mouse brain. Brain sections from embryos at E13.5 (A-C,G,H-K,M,Q) or E16.5 (D-F,I,J,N-P,R) were immunostained with anti-STAT3 together with anti-nestin or anti-βIII-tubulin (TuJ1), as indicated. STAT3 was expressed in nestin-positive cells (A-F,H,J), but not in cells positive for the neuronal marker TuJ1 (G,I). Nestin was expressed in nestin-positive cells (A-F,H,J), but not in cells positive for the neuronal marker TuJ1 (G,I). K and N show the phase-contrast micrographs of the sections. STAT3 was expressed in cells in the VZ and apical side of the IMZ at E13.5 (K-M,Q) and in the VZ, SVZ and the apical side of the IMZ at E16.5 (N-P,R). Q and R show magnification of cells expressing STAT3 (arrow) in the VZ and IMZ in L and in the VZ, SVZ and IMZ in O, respectively. In-situ hybridization analysis of the E13.5 mouse brain revealed that STAT3 mRNA was present in the neuroepithelium of the telencephalic plate (S). Dorsal side is up in A-I,S and left in K-R. Scale bars: 400 μm in A,S; 40 μm in G,K,N,Q,R.
Fig. 3. Requirement of STAT3 for maintenance of FGF2-sensitive NPCs. (A) NPCs cultures prepared from E12.5 mouse neocortex were incubated in the absence of FGF2 for the indicated times, after which cell lysates were subjected to immunoblot analysis with antibodies to STAT3, to the Tyr705-phosphorylated form of STAT3 (Anti-phospho STAT3) and to GAPDH (loading control). Reduction of STAT3 phosphorylation by FGF2 deprivation suggests FGF2-dependent phosphorylation of STAT3, although it may also reflect the reduction of sphere-forming NPCs by FGF2 deprivation. (B) Neuroepithelial cells prepared from the neocortex of E12.5 Stat3flox/flox mice were infected with a retrovirus encoding Cre recombinase (Cre) or with a control retrovirus (Control). After culture in the presence of FGF2 (20 ng/ml) for the indicated times, the cells were lysed and subjected to immunoblot analysis. (C) Neuroepithelial cells from Stat3flox/flox mice were infected and then assayed for the formation of primary neurospheres in the presence of FGF2 (20 ng/ml). *P<0.01. (D) Neuroepithelial cells prepared from the neocortex of E12.5 wild-type mice were infected with retroviruses encoding both STAT3-C and GFP (STAT3-C) or GFP alone (Control). They were subsequently assayed for the formation of secondary neurospheres. *P<0.01.

(E-G,M) Neuroepithelial cells prepared from E12.5 wild-type mouse neocortex were infected and plated in the presence of FGF2 (20 ng/ml) for 2 days (E,F) or in the absence of FGF2 for 3 (G) or 4 (M) days. Then the cells were stained with Hoechst to identify apoptotic cells by counting cells exhibiting condensed and fragmented nuclei (F), or stained with anti-GFAP (G) or anti-βIII-tubulin (TuJ1) antibody together with anti-GFP (M), or incorporated BrdU for 2 hours and stained with anti-BrdU and anti-GFP (E). *P<0.02. (H-L) Neuroepithelial cells prepared from E12.5 Stat3flox/flox mouse neocortex were infected and plated in the presence of FGF2 (20 ng/ml) for 2 days (H,I) or a lower concentration of FGF2 (2 ng/ml) for 3 (J) or 4 (K,L) days. Then the cells were analyzed as in E-G,M. Scale bar: 80 μm (K). *P<0.01.
suggesting that STAT3 phosphorylation is, directly or indirectly, dependent on FGF2 in this culture. We then examined whether STAT3 is required for the FGF2-dependent maintenance of NPCs by performing conditional deletion of the STAT3 gene. ‘Neuroepithelial cell cultures’ (see Materials and methods) were prepared from the neocortex of E12.5 STAT3^floxed/floxed^ mice (Takeda et al., 1998), in which both Stat3 alleles are flanked by LoxP sites. Infection of the cells with a retrovirus encoding the Cre recombinase (pMX-Cre-GFP) resulted in an 80% decrease in the abundance of STAT3 within 3 days compared with that apparent in cells infected with a control retrovirus (Fig. 3B). The FGF2-dependent formation of primary neurospheres by the STAT3-depleted cells was inhibited by 47% (±8% s.e.m.) compared with that observed with the control cells (Fig. 3C). Introduction of Cre recombinase did not markedly reduce the number of secondary neurospheres, most probably because of a selective expansion of STAT3-remaining neurospheres. Expression of a dominant negative form of mouse STAT3 also reduced by 47% (±14% s.e.m.) the number of primary neurospheres formed by wild-type neuroepithelial cells expressing control vector. Moreover, expression of a constitutively active form of mouse STAT3 (STAT3-C, which harbors cysteine mutations at Ala^661^ and Asn^663^) (Bromberg et al., 1999) increased in the number of secondary neurospheres formed by wild-type neuroepithelial cells (Fig. 3D). The proliferation and survival rates of these cells, however, were not much affected by the manipulation of STAT3 (see Fig. 3E,F,H,I). These results suggest that STAT3, in addition to JAK2, is important for the maintenance of NPCs in vitro.

**STAT3 inhibits neurogenesis in vitro and in vivo**

We next investigated whether STAT3 influences the neuronal fate of NPCs. We prepared neuroepithelial cell cultures from E12.5 STAT3^floxed/floxed^ mice and infected the cells with a retrovirus encoding GFP (pMX-GFP). More than 90% of the GFP-positive cells were found to express nestin in the presence of FGF2. Infection of the neuroepithelial cells with a retrovirus encoding both Cre recombinase and GFP (pMX-C-GFP) at a high titer resulted in a marked increase in the proportion of GFP-positive cells that expressed the neuronal marker βIII-tubulin (TuJ1), compared with cells infected with the control retrovirus encoding GFP alone (pMX-GFP) (Fig. 3K,L). Moreover, infection of wild-type neuroepithelial cell cultures with a retrovirus encoding STAT3-C and GFP (pMX-STAT3-C-GFP) at a high titer resulted in a significant decrease in the proportion of TuJ1-positive cells among GFP-positive (infected) cells compared with cells infected with pMX-GFP (Fig. 3M). STAT3-C expression and STAT3 deletion had little effects on the expression of the astrocyte marker glial fibrillary acidic protein (GFAP) at this early stage (Fig. 3G,J), consistent with the previous studies (Molne et al., 2000; Takizawa et al., 2001). These results indicated that STAT3 inhibits neuronal differentiation of NPCs in this culture system.

**Fig. 4.** (A) Requirement of STAT3 for inhibition of neurogenesis in vivo. Vectors encoding GFP alone (Control) or Cre recombinase plus GFP (Cre) were injected into the lateral ventricle of STAT3^floxed/floxed^ mice at E14.5. After 2 days, the fate of the GFP-positive cells was examined by immunohistochemistry with anti-GFP, anti-βIII-tubulin (TuJ1), anti-SOX2, anti-nestin, anti-Musashi and anti-MAP2, as indicated. The percentages of TuJ1-positive cells and SOX2-positive cells among total cells in the transfected VZ region (60 μm width from the ventricular surface) are shown. The boxed regions in A are shown enlarged in B. Arrows in B indicate TuJ1-positive cells and MAP2-positive cells co-expressed without GFP. Scale bars: 20 μm in A; 40 μm in B.
The in-vivo function of STAT3 in the developing neocortex was then investigated by injection of expression vectors for GFP alone (pMX-GFP) or for both GFP and Cre recombinase (pMX-Cre-GFP) into the telencephalic ventricle of STAT3\textsuperscript{flox/flox} mouse embryos at E14.5 in utero, and introduction of the vectors into NPCs in the VZ of the neocortex by electroporation. Two days after electroporation (E16.5), the fate of the GFP-positive (transfected) cells was determined by immunostaining (see Fig. S2 in the supplementary material). In the control embryos transfected with pMX-GFP (see Fig. S4B in the supplementary material), most cells in the region of GFP expression comprised NPCs (nestin, SOX2, Musashi positive and TuJ1, MAP2 negative) in the VZ [the percentages of cells positive for TuJ1 and SOX2 were 3.6% (±1.6% s.e.m.) and 65% (±5.9% s.e.m.), respectively, in the GFP-positive area of VZ; Fig. 4A; see Fig. S4A in the supplementary material]. However, in embryos transfected with pMX-Cre-GFP, many of the cells in the region of GFP expression, even those in the VZ, were nestin, SOX2, Musashi negative and TuJ1, MAP2 positive (the percentages of cells positive for TuJ1 and SOX2 were 25% (±4.1% s.e.m.) and 30% (±4.5% s.e.m.), respectively, in the GFP-positive area of VZ; Fig. 4A; see S4A in the supplementary material). Moreover, the percentages of SOX2-positive cells among GFP-positive cells at the VZ were 60% (±12% s.e.m.) in control and 29% (±3.4% s.e.m.) in Stat3-deleted experiments. These results suggest that deletion of Stat3 induces premature neurogenesis. STAT3 thus appeared to be required for suppression of neurogenesis and maintenance of NPCs in the developing brain.

**STAT3 inhibits neurogenesis in a non-cell-autonomous manner**

Reduced SOX2 expression in GFP-positive cells at the VZ in Stat3-deleted experiments suggests that STAT3 is necessary for the maintenance of NPC in a cell-autonomous manner. Detailed examination of the results of the in-vivo electroporation experiment, however, revealed that deletion of Stat3 in NPCs (as reflected by GFP expression) also resulted in ectopic neuronal differentiation in surrounding GFP-negative cells (Fig. 4B, arrows). Indeed, a large proportion [94% (±1.9% s.e.m.)] of the TuJ1-positive cells in the STAT3-deleted VZ area were GFP-negative. This observation suggests that STAT3 is required for inhibition of neurogenesis in surrounding cells.

To verify whether STAT3 indeed inhibits neurogenesis in a non-cell-autonomous manner, we performed a series of co-culture experiments. A neuroepithelial cell culture prepared from the neocortex of E12.5 STAT3\textsuperscript{flox/flox} mice was infected with a retrovirus encoding CD8 (pMX-CD8) or one encoding both CD8 and Cre recombinase (pMX-Cre-CD8). The infection efficiency was relatively high (>80% of NPCs were CD8-positive) in these experiments. These cells were then co-cultured with a small number (1% of the number of CD8-expressing cells) of cells from an NPC culture that had been infected with a retrovirus encoding GFP (pMX-GFP) (Fig. 5A). After incubation for 4 days, the proportion of GFP-positive NPC clones that expressed TuJ1 was markedly greater for co-cultures with neuroepithelial cells infected with pMX-Cre-CD8 (STAT3 depleted) than for those with neuroepithelial cells infected with pMX-CD8 (Fig. 5B). This result again suggests that STAT3 is required for inhibition of neuronal differentiation in surrounding cells.

We then co-cultured GFP-expressing NPCs with either control neuroepithelial cells or those expressing STAT3-C. A neuroepithelial cell culture prepared from wild-type mouse embryos was thus infected with pMX-CD8 or the corresponding vector encoding both STAT3-C and CD8 (pMX-STAT3-C-CD8). These cells were then co-cultured with a small number of GFP-expressing NPCs for 3 days. The proportion of neuron-only clones among GFP-positive clones was markedly smaller for co-cultures with neuroepithelial cells infected with pMX-STAT3-C-CD8 than for those with neuroepithelial cells infected with pMX-CD8 (Fig. 5C), further supporting the notion that STAT3 can inhibit neuronal differentiation of NPCs in a non-cell-autonomous manner.

**STAT3 directly regulates expression of the Notch ligand DLL1**

A potential target of STAT3 in its non-cell-autonomous inhibition of neurogenesis was a Notch ligand, given that activation of the Notch receptor in neighboring cells would be expected to block

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**Fig. 5. Inhibition of neurogenesis by STAT3 in a non-cell-autonomous manner.** (A, B) NPC cultures were prepared from the neocortex of E12.5 STAT3\textsuperscript{flox/flox} mice and infected with a retrovirus encoding GFP (pMX-GFP). Neuroepithelial cell cultures were prepared from STAT3\textsuperscript{flox/flox} embryos and infected with retroviruses encoding CD8 alone (Control) or both CD8 and Cre recombinase (Cre). The infected NPCs (1x10^3 cells) were co-cultured for 4 days in the presence of low dose FGF2 (2 ng/ml) with the infected neuroepithelial cells (1x10^5 cells) and stained with anti-GFP and anti-CD8 (A), or anti-GFP and TuJ1 antibody (B). (A) GFP and TuJ1 fluorescence merged image are shown for typical field of co-culture experiments. Scale bar: 80 μm. (B) The percentage of clones containing only TuJ1-positive cells among GFP-positive clones was then determined by immunocytofluorescence analysis. *P<0.01. (C) NPC cultures prepared from the neocortex of E12.5 wild-type mice were infected with pMX-GFP and co-cultured with neuroepithelial cell cultures infected with pMX-CD8 (Control) or a retrovirus encoding both CD8 and STAT3-C (STAT3-C) as in B. *P<0.05.
their neuronal differentiation. A previous study has shown that the Notch ligand DLL1 is expressed in the VZ of the developing mouse brain (Campos et al., 2001). Immunohistofluorescence analysis indeed showed that the expression of DLL1 overlapped with that of STAT3 in the mouse embryonic neocortex (Fig. 6A-D), which prompted us to examine the functional relation between STAT3 and DLL1.

We examined whether Stat3 deletion affected DLL1 expression in NPC cultures. RT-PCR analysis revealed that the amounts of DLL1 and GFAP mRNAs in NPC cultures prepared from the neocortex of E12.5 Stat3flox/flox mice were markedly reduced by infection with a retrovirus encoding Cre recombinase (Fig. 6E). The abundance of NOTCH1 mRNA was not affected by Stat3 deletion, even though leukemia inhibitory factor (LIF), a potent activator of STAT3, was not expressed.

**Fig. 6. Regulation of DLL1 expression by STAT3.** (A–D) Brain sections of E16.5 mice were immunostained with anti-STAT3 and anti-DLL1, as indicated. DLL1 was detected in STAT3-expressing cells. Dorsal side is up in all panels. Scale bars: 400 μm in A; 80 μm in B. (E) NPC cultures prepared from the neocortex of E12.5 Stat3flox/flox mice were infected with retroviruses encoding GFP alone (Control) or both GFP and Cre-recombinase (Cre). After culture for 2 days, the cells were subjected to RT-PCR analysis for the indicated mRNAs. Data are expressed relative to the corresponding normalized value for control cells. *P < 0.01. (F) NPC cultures infected and cultured for 2 days as in E were subjected to immunoblot analysis with the indicated antibodies. (G) Promoter regions of the mouse DLL1 genes that contain consensus sequences for STAT3 binding (white arrowheads and the nucleotides in boldface type). NPC cultures prepared from the neocortex of E12.5 mice were subjected to ChIP analysis with anti-STAT3 or control immunoglobulin G (IgG). The immunoprecipitates were analyzed by PCR with primers spanning the indicated regions (underlined) of mouse Dll1.
STAT3 in NPCs, or FGF2 has been shown to result in an increase in NOTCH1 expression (Chojnacki et al., 2003; Faux et al., 2001). Immunoblot analysis showed that the amount of DLL1 protein in NPC cultures was also reduced as a result of Stat3 deletion (Fig. 6F). These results thus suggest that STAT3 is required for DLL1 expression in the NPC culture, which contains both NPCs and newborn neurons.

We next examined whether STAT3 directly associates with the promoter ofDll1 in NPC cultures by ChIP analysis. Five putative consensus sequences for STAT3 binding (Ehret et al., 2001) were found to be located at nucleotide positions –665 to –657, –1315 to –1307, –1393 to –1385, –2622 to –2614, and –2717 to –2709 relative to the transcription start site in the promoter region of the mouse DLL1 gene (Fig. 6G), including two sequences conserved among mouse and human (–1393 to –1385 and –665 to –657). The region between nucleotides –1 to –4265 of the mouse DLL1 gene has been shown to be sufficient to reproduce the normal pattern of Dll1 expression during early organogenesis (Beckers et al., 2000). Chromatin immunoprecipitation assay prepared from NPC lysates with anti-STAT3 revealed the presence of genomic DNA containing the three regions (–2819 to –2639, –1486 to –1323, –760 to –644), which include the putative STAT3-binding sequences within the Dll1 promoter region (Fig. 6G). By contrast, PCR analysis with primers targeted to other regions of Dll1 did not yield a signal with anti-STAT3 immunoprecipitates greater than that with control immunoprecipitates (Fig. 6G). These results thus suggest that STAT3 might directly regulate the transcription of Dll1.

Suppression of Notch signaling inhibits NPC maintenance by STAT3

Our observations indicated that STAT3 contributes to NPC maintenance and promotes expression of DLL1. We therefore next examined whether DLL1 expression is necessary for STAT3-mediated NPC maintenance with the use of DLL1-directed RNAi. Transfection of neuroepithelial cell cultures with a vector for an siRNA specific for DLL1 mRNA resulted in an ~80% decrease in the abundance of DLL1 protein (Fig. 7A). The potentiation of secondary neurosphere formation induced by expression of STAT3-C in NPCs was prevented by RNAi-mediated depletion of DLL1 (Fig. 7B). We also found that treatment of neuroepithelial cells with the γ-secretase inhibitor L685,458, which inhibits Notch receptor activation, blocked the stimulatory effect of STAT3-C on secondary neurosphere formation (Fig. 7C). Together, these results suggest that DLL1 is an essential mediator of the action of STAT3 in NPC maintenance.

DISCUSSION

Our results have shown that STAT3 plays an essential role in the maintenance of mouse neocortical NPCs during development: (1) STAT3 was found to be expressed in NPCs during neocortical development; (2) conditional deletion of Stat3 resulted in premature neurogenesis, both in the developing neocortex and in neocortical cell cultures; (3) deletion of Stat3 reduced the capacity of neocortical cell cultures to generate neurospheres, which are thought to reflect the abundance of NPCs; (4) overexpression of an active form of STAT3 in neocortical cell cultures increased their capacity to generate neurospheres and inhibited neuronal differentiation; (5) treatment of neocortical cell cultures with the JAK2 inhibitor AG490, which inhibited the phosphorylation of endogenous STAT3, also reduced the capacity for neurosphere formation; and (6) STAT3 was shown to be necessary for expression of DLL1, which also plays an important role in the maintenance of NPCs.

Although FGF2 has been known to mediate the maintenance of NPCs both in vitro and in vivo, the underlying mechanism of this action has remained unclear. Our data now indicate that STAT3 is a downstream mediator of this effect of FGF2. It is not likely, however, that STAT3 is a direct downstream effector of FGF2 in NPCs, as FGF2 treatment did not induce immediate phosphorylation of STAT3 (data not shown). It is possible that FGF2 activates STAT3 indirectly through induction of upstream regulator(s) of STAT3. In addition, STAT3 may not be the only mediator of FGF2 function in NPC maintenance, given that LIF was not able to mimic the effect of FGF2 on neurosphere formation and that overexpression of an active form of STAT3 (STAT3-C) was not able to induce neurosphere formation in the absence of FGF2 (T.Y. and Y.G., unpublished). STAT3 is thus necessary, but not sufficient, for maintenance of NPCs by FGF2. PI3K may contribute to an
alternative pathway for NPC maintenance by FGF2, given that the PI3K inhibitor LY294002 partially inhibited the effect of FGF2 on primary neurosphere formation in neocortical cell cultures. The extracellular signals responsible for the activation of STAT3 in the developing neocortex remain to be identified, although we used FGF2 to promote maintenance of NPCs in vitro. Characterization of FGF2 knockout mice has indicated that FGF2 partially contributes to the maintenance of neocortical NPCs in vivo (Raballo et al., 2000). EGF also promotes the expansion of NPCs, especially at the late stage of neocortical development (Reynolds and Weiss, 1996). Activation of the EGF receptor results in an increase in the amount of STAT3 mRNA, which may account in part for NPC maintenance at this late stage (Viti et al., 2003). In addition to FGF2 and EGF, gp130-coupled signaling molecules such as LIF, ciliary neurotrophic factor and interleukin-6 activate STAT3 (Cattaneo et al., 1999). LIF and its receptor might also contribute to NPC maintenance by activating STAT3, especially in the embryonic ganglionic eminences and in the adult SVZ, given that deletion of the gene for LIFRβ, the β subunit of the LIF receptor, reduced the capacity of cells from these regions to form neurosurfaces (Gregg and Weiss, 2005; Shimazaki et al., 2001), that the addition of LIF increased the number of passageable neurosurfaces generated from ganglionic eminences (Gregg and Weiss, 2005), and that injection of LIF into the lateral ventricle induced progenitor cells to re-enter the cell cycle (Hatta et al., 2002). However, the addition of LIF did not increase the capacity of cells from other brain regions, including the embryonic neocortex, to form neurosurfaces (Gregg and Weiss, 2005) (T.Y. and Y.G., unpublished), suggesting that LIF may not be the major regulator of STAT3 in the developing neocortex. Regardless, endogenous ligands that induce STAT3 activation may be essential determinants of the pool size of NPCs.

We found that STAT3 is expressed in the VZ, SVZ and the apical side of the IMZ, and that its expression is downregulated around the time of neuronal differentiation in the developing neocortex. The level of STAT3, as well as that of the Tyr705-phosphorylated form of the protein, were also high in NPC cultures in the presence of FGF2 but were reduced in association with the induction of neuronal differentiation by removal of FGF2 (Fig. 3A). The high level of STAT3 mRNA in the VZ, SVZ and the apical side of the IMZ could be ascribed to the high level of STAT3 activity in this area, as it has been shown that STAT3 consists of a positive feedback loop between its activity and expression levels (He et al., 2005; Ichiba et al., 1998). Given that STAT3 inhibits neuronal differentiation, the downregulation of STAT3 expression and activity probably contributes to the mechanism of neuronal differentiation.

The interaction between the Delta-Notch and JAK-STAT pathways has previously been reported (Chojnacki et al., 2003; Kamakura et al., 2004). In this study, we have shown that Dll1 is a key target of STAT3 in both inhibition of neurogenesis and maintenance of NPCs, which may underlie the non-cell-autonomous action of STAT3 in inhibiting neuronal differentiation. These observations reveal a previously unrecognized mechanism of interaction between the Delta-Notch and JAK-STAT pathways, and they may explain, at least in part, previous findings obtained with γ-secretase inhibitors or PS1, NOTCH1 or RBP-J knockout cells suggesting that Notch signaling is required for FGF-induced maintenance of NPCs (Chojnacki et al., 2003; Hitoshi et al., 2002). It will be interesting to investigate the regulation of other Notch ligands by STAT3 as well, especially because acute knockdown ofDLL1 by RNAi results in bigger effects than DLL1 gene deletion on NPC maintenance (Grandbarbe et al., 2003) (this study), implicating a compensatory mechanism among the Notch ligands.

STAT genes regulate maintenance of stem cells in several systems, including that of germline stem cells in male Drosophila as well as that of embryonic and hematopoietic stem cells in mammals (Kiger et al., 2001; Tulina and Matunis, 2001). The Notch pathway also plays an important role in inhibition of differentiation in many systems, including the hematopoietic system (Maillard et al., 2005). It remains to be determined whether the regulation of Dll1 expression and maintenance of the undifferentiated state by STAT3 demonstrated here are also operative in stem cells of other tissues. We thank Drs Toshio Kitamura, Jacqueline F. Bromberg and Shigeaki Kato for reagents, as well as members of the Gotoh laboratory for discussions. This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, as well as by SORST of the Japan Science and Technology Corporation.

Supplementary material
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