Peroxisome Proliferator-activated Receptor γ-mediated Regulation of Neural Stem Cell Proliferation and Differentiation*

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Peroxisome proliferator-activated receptor γ (PPARγ) plays an important role in insulin sensitivity, tissue homeostasis, and regulating cellular functions. We found high-level expression of PPARγ in embryo mouse brain and neural stem cells (NSCs), in contrast to extremely low levels in adult mouse brain. Here, we show that PPARγ mediates the proliferation and differentiation of murine NSCs via up-regulation of the epidermal growth factor receptor and activation of the ERK pathway. Cell growth rates of NSCs prepared from heterozygous PPARγ-deficient mouse brains, PPARγ-RNA-silenced NSCs, and PPARγ dominant-negative NSCs were significantly decreased compared with those of wild-type NSCs. Physiological concentrations of PPARγ agonists, rosiglitazone and pioglitazone, stimulated NSC growth, whereas antagonists caused cell death in a concentration-dependent manner via activation of the caspase cascade. The stimulation of cell growth by PPARγ was associated with a rapid activation of the ERK pathway by phosphorylation and up-regulation of epidermal growth factor receptor and cyclin B protein levels. In contrast, activation of PPARγ by agonists inhibited the differentiation of NSCs into neurons. The inhibition of differentiation was associated with an activation of STAT3. These data indicate that PPARγ regulates the development of the central nervous system during early embryogenesis via control of NSC proliferation.

It has recently been reported that peroxisome proliferator-activated receptor γ (PPARγ) and its related pathways play an important role in not only insulin sensitivity, but also in regulating other cellular functions and tissue homeostasis (1–3). We have previously reported that endogenous PPARγ provides anti-inflammatory activity against inflammatory bowel disease, ischemia-reperfusion injury, and rheumatoid arthritis (4, 5). We also reported an important role of PPARγ in the suppression of colon carcinogenesis and hepatocellular carcinoma (6). These reports suggest that the PPARγ pathway plays important roles in regulating cellular function and tissue homeostasis.

The effects of PPARγ on cellular proliferation and differentiation are potentially important to tissues such as the central nervous system that are critically dependent upon an ability to exhibit regeneration post-injury. In the central nervous system, most self-renewal is dependent upon neural stem cells (NSCs) that can be isolated from embryonic brains. NSCs are multipotent and self-renewing progenitor cells that can differentiate into neurons and glial cells (7–9). As such, NSCs are expected to be of utility in the treatment of neurodegenerative disorders, such as Parkinson disease, Huntington disease, nerve injury, stroke, and multiple sclerosis. Likewise, NSCs are not only considered the main source of neurons but are also useful for investigating central nervous system development in vitro. Therefore, many studies have been performed regarding the mechanisms or factors involved in the proliferation and differentiation of NSCs (10–12). However, no studies have been reported about the role of PPARγ in NSC functions. Therefore, we tried to investigate the novel hypothesis that PPARγ might play a role in regulating the proliferation and differentiation of murine NSCs.

We found high-level expression of PPARγ in embryo mouse brain and NSCs. In contrast, extremely low levels were observed in adult mouse brain (Fig. 1, B and C). In this study, we investigated the role of PPARγ in regulating the proliferation and differentiation of murine NSCs into neurons. Whereas optimal activation of the PPARγ pathway stimulated NSC proliferation and inhibited differentiation of NSCs into neurons, inhibition or deficiency of PPARγ was associated with an inhibition of NSC proliferation and subsequent apoptosis via activation of the caspase cascade. Because excessive activation of the PPARγ pathway also caused death of NSCs, these results indicate that NSC expansion pre-differentiation to neurons is highly dependent upon PPARγ at a critical threshold level of signaling. These studies have important implications for understanding central nervous system development and the therapy of neurodegenerative disorders that require nerve cell replenishment.


**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—The PPARγ-specific ligands, rosiglitazone and pioglitazone, were kind gifts from Glaxo SmithKline (Tokyo, Japan) and Takeda Pharmaceuticals (Osaka, Japan), respectively. 15-Deoxy-

**Preparation of Neural Stem Cells**—All mice were treated humanely according to the National Institutes of Health and AERI-BBRI Animal Care and Use Committee guidelines. All animal experiments were approved by the institutional animal care and use committee of Osaka University.

Preparation of NSCs was performed according to the method described previously with some modifications (11–14). An ordinal pregnant BALB/c mouse was anesthetized and euthanatized on days 13–14 of gestation. The brain was removed from the embryo and placed in culture medium that consisted of the following: Dulbecco’s modified Eagle's/F-12 medium (Invitrogen) containing 0.6% glucose, 0.1% NaHCO3, 5 mM HEPES, 100 ng/ml bovine insulin (Invitrogen), 25 μg/ml bovine insulin (Sigma), 10 μg/ml putrescine (Sigma), 30 mM sodium selenite (Sigma), 20 mM progesterone (Sigma), 20 ng/ml human EGF (PeproTech EC), 20 ng/ml human fibroblast growth factor (PeproTech E), 100 μg/ml penicillin, 100 units/ml streptomycin (Invitrogen). The cells were dissociated by mechanical dispersion with a fire-narrowed Pasteur pipette. After centrifugation, cells were re-suspended in culture medium. Primary stem cell proliferation was observed after 7–8 days. The neurospheres were collected, gently triturated with a fire-narrowed Pasteur pipette, and subcultured at a density <5 × 104 cells/ml in a culture bottle (Nalge Nunc, Naperville, IL). Confirmation of NSCs was performed by the detection of nestin expression and neurosphere formation (12–14).

A wild-type pregnant BALB/c female mated with a PPARγ+/- male mouse was also used to prepare heterogenous PPARγ knockout (PPARγγ−/−) NSCs. Confirmation of heterozygous PPARγ knock-out (PPARγγ−/-) clones was performed by PCR of genomic DNA from each clone. The maintenance of the undifferentiated state of NSCs was performed in non-coated culture bottles (Nalge Nunc).

**Cell Culture of Neural Stem Cells for Differentiation and Treatment with Agonists and Antagonists**—NSCs formed neurospheres in non-coated culture bottles, representing a state of undifferentiation. When cultured on poly-L-ornithine/laminin-coated plates (Biocoat, Becton Dickinson Labware, Bedford, MA), NSCs adhered to the plates, proliferated, extended their neurites, and differentiated into neurons (12–14).

Treatment with PPARγ agonists, antagonists, or vehicle control (dimethyl sulfoxide) was started when NSCs were dissociated and scattered on the ornithine/laminin-coated plates or in non-coated culture bottles. Three days after treatment, morphological changes and the rate of cell growth were investigated.

**Evaluation of Cell Growth**—After NSCs were treated with PPARγ agonists, antagonist, or vehicle for 3 days, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution (Sigma) was added to the culture medium and the cells were incubated. SDS (20%) was then added, and absorbance at a wavelength of 595 nm was measured. The cell growth rate was expressed as a percentage compared with the vehicle control. In another set of experiments, NSCs were cultured on non-coated bottles after treatment with reagents. Various neuroblastoma-like cell lines, such as NB1 (human neuroblastoma), NB2a (mouse neuroblastoma), C1300N18 (mouse neuroblastoma), and SH-SY5Y (human neuroblastoma), were also used to evaluate cell growth in comparison to the NSCs.

**Chromatin Staining for Detection of Apoptosis**—NSCs treated with PPARγ agonists or antagonists were cultured on poly-L-ornithine/laminin-coated Lab-Tek II Chamber Slides (Nalge Nunc International) for 24 h. The cells were then fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min and rinsed. Chromatin staining was performed with Hoechst 33342 (Sigma) to detect nuclear condensation (a morphological change associated with apoptosis) and the cells were observed using a fluorescence microscope (IX 70, Olympus, Tokyo, Japan).

**Western Blotting Analysis for Detection of Activated Caspase**—The cells were collected and homogenized in lysis buffer containing a mixture of protease inhibitors. Cell extracts, prepared by centrifugation at 16,000 × g, were resolved by SDS-PAGE and Western blot analysis was performed. The detection was performed by using an ECL Plus kit (Amersham Biosciences).

**Evaluation of Differentiation**—NSCs were treated with PPARγ agonists or antagonist, and cultured on poly-L-ornithine/laminin-coated Lab-Tek II Chamber Slides for 3 days. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min, permeabilized in phosphate-buffered saline containing 0.1% Triton X-100 for 30 min, and rinsed with phosphate-buffered saline. The fixed cells were incubated with anti-MAP2 or anti-nestin antibody for 16 h at 4 °C, and then visualized with Alexa Fluor 488 anti-rabbit IgG antibody (Molecular Probes, Inc., Eugene, OR). Nuclear staining was performed by incubation with SYTOX™ Orange (Molecular Probes, Inc.) and NSCs were observed using a confocal laser-scanning microscope (MRC1024, Nippon Bio-Rad).

**Preparation of Genomic DNA and PCR Amplification**—Genomic DNA was prepared by standard procedures with SDS/proteinase K digestion overnight, extraction with phenol/chloroform, and precipitation with ethanol. To detect wild-type and knock-out alleles in DNA derived from tail tips, PCR was performed with Tag DNA polymerase (Sigma) using standard PCR mixtures containing purified genomic DNA and 10 pmol each of following three primers, 5’-TCCACTTCA-GAAATTACC-3’, 5’-ACTTTGATTCTTGGAG-3’, and 5’-GCC-AACAAAAGACGGGAGCCG-3’, in 20 μl of mixture. The mixture was heated at 95 °C for 3 min, subjected to 40 cycles of 45 s at 95 °C, 30 s at 52 °C, and 45 s at 72 °C, with an extra 3-min incubation at 72 °C after the last cycle. The PCR products were determined by 2% agarose gel electrophoresis in a 0.5 × Tris borate-EDTA (TBE) buffer.

**Preparation of Lentiviral Vector Expressing Short Hairpin RNA (shRNA) against PPARγ and Transduction into NSCs**—Vectors were constructed by using standard cloning procedures. pHM5-H1 was designed to express shRNA when an appropriate sequence was inserted into the BglII/XbaI sites. Oligonucleotides encoding both strands of the targeting sequence were annealed and ligated into BglII/XbaI sites in pHM5-H1 (17). The sequence was verified on a DNA sequencer (ABI PRISM 310, Applied Biosystems) and the cassette containing the H1 promoter plus the shRNA was transferred to the self-inactivating lentiviral vector construct generating CS-H1-shRNA-EG.

Because it is difficult to maintain the constant expression or deficiency of target genes in bone marrow cells or stem cells using Lipofec-
Deficiency Causes Disorder of Embryo and NSC Growth—A 1.4-kb fragment of murine PPARγ1 cDNA (wild-type PPARγ, WT-PPARγ) was isolated from an intestinal cDNA library and conserved cysteine residues in the first zinc finger of the DNA-binding domain (Cys-126 and Cys-129) were mutated to serine residues (MT-PPARγ), which is unable to form a functional DNA-binding complex with RXR-α (18) and could be a dominant-negative. Lentiviral vectors pseudotyped with vesicular stomatitis virus-G glycoprotein were prepared by the same methods described in the section of shRNA, except for the self-inactivating vector construct (CSII-EF-WT-PPARγ-IRES-hrGFP or CSII-EF-MT-PPARγ-IRES-hrGFP), which could express the PPARγ gene and hrGFP under control of the human elongation factor 1-α subunit promoter. Dissociated NSCs were then infected with viral stocks at a m.o.i. of 0.44 to 12 for 48 h, followed by FACs analysis to determine transduction efficiency. Constant and viral concentration-dependent expression of the target genes was confirmed by expression of GFP proteins in infected NSCs. Approximately 80% of NSCs were GFP-positive at the m.o.i. of 12. The cell growth rate in non-coated culture bottles for 4 days was measured by MTT assay and expressed as a percentage compared with that of non-infected NSCs.

We have previously reported two effective shRNA sequences, shRNA-P15 (GTTTGCTGTGAAG) and -P17 (GGTTGCTGTGAAGG), to suppress PPARγ RNA, and one ineffective sequence, shRNA-P18 (ATGAGCCTTCACCCCCCTG). We, therefore, used shRNA-P15 and -P17 as effective sequences to inhibit PPARγ RNA, and shRNA-P18 and -Lu (shRNA against firefly luciferase) as negative controls (17).

NSCs were dissociated by mechanical dispersion with a fire-narrowed Pasteur pipette, the remaining neurospheres were removed by filtration through filters of 70-μm pore size (Cell Strainer, FALCON) and scattered at 5 × 10^4 cells/ml into 12-well non-coated plates. NSCs were then infected with viral stocks at a multiplicity of infection (m.o.i.) of 2.5 to 40, followed by FACs analysis to determine the transduction efficiency. Increases in EGFP expression in NSCs were observed in a viral concentration-dependent manner. Under the conditions used, transduction efficiency was almost 80% and >95% at m.o.i. of 10 and 20, respectively. The cell growth rate was measured by MTT assay after 5 days of infection and expressed as a percentage compared with that of non-infected NSCs.

RESULTS

PPARγ Deficiency Causes Disorder of Embryo and NSC Growth—As it has been reported that homozygous PPARγ knock-out (PPARγ−/−) mouse embryos are lethal at days 10–11 of gestation because of placental dysfunction and disordered development of embryo (Ref. 2 and Fig. 1A), we prepared clones of NSCs from heterozygous PPARγ-
deficient (PPARY<sup>+/−</sup>) mice. Definition of PPARY<sup>+/−</sup> or wild-type NSC clones was determined by PCR of genomic DNA (Fig. 1D, upper panel) and confirmed by Western blot analysis (Fig. 1D, lower panel). The PPARY<sup>+/−</sup>-NSCs exhibited low levels of PPARY protein compared with those of wild-type NSCs. The rate of cell growth of PPARY<sup>+/−</sup>-NSCs was observed to be much lower than that of wild-type NSCs in non-coated culture bottles (Fig. 1F). Morphologically, a disorder in the formation of neurospheres was observed in PPARY<sup>+/−</sup>-NSCs compared with that observed with the wild-type clones (Fig. 1F). These results suggest that in vivo deficiency of PPARY is associated with diminished growth and disordered formation of neurospheres.

**Analysis of Cell Growth on PPARY-RNA-silenced or Dominant-negative PPARY-NSCs**—To confirm the effect of PPARY deficiency, we prepared PPARY-RNA-silenced NSCs by shRNA and dominant-negative PPARY-NSCs (17). We used two effective shRNAs (shRNA-P15 and shRNA-P17), one ineffective shRNA (shRNA-P18), and shRNA against luciferase (shRNA-Lu) as a control (17). PPARY-RNA-silenced NSCs (shRNA-P15 and -P17) exhibited a significant decrease in their cell growth rates compared with that of the ineffective shRNA (shRNA-P18) or shRNA-Lu-transfected NSCs (Fig. 2B) in a viral concentration-dependent manner (m.o.i.). Morphologically, PPARY-RNA-silenced NSCs also failed to form neurospheres in comparison to that observed with the ineffective shRNA or shRNA-Lu-transfected NSCs (Fig. 2A). Similar results were also observed when mutant PPARY-transfected NSCs expressing a dominant-negative form of PPARY was investigated. Namely, mutant PPARY-transfected NSCs that are dominant-negative exhibited a significant decrease in their cell growth rates compared with that of wild-type PPARY-transfected NSCs in a viral concentration-dependent manner (Fig. 2D).

Dominant-negative PPARY-NSCs also failed to form neurospheres in comparison to that observed with wild-type PPARY-NSCs (Fig. 2C). Furthermore, we examined the recovery of the impaired cell growth observed with PPARY-deficient (PPARY<sup>+/−</sup>) NSCs (clone number 2) by increasing the expression of PPARY using adenosivirus-mediated PPARY-gene transfer as previously established by us (19). Adenosivirus-mediated PPARY-gene transfer successfully increased PPARY protein levels in both wild-type (clone number 7) and PPARY<sup>+/−</sup>-NSCs (clone number 2) in a viral concentration-dependent manner (data not shown). This increase in PPARY protein levels in PPARY<sup>+/−</sup>-NSCs resulted in reversal of the impaired cell growth (Fig. 2E). These results strongly suggest that PPARY regulates the proliferation and the absence of PPARY directly inhibits the self-renewal functions of NSCs.

**Biphasic Effects of PPARY Agonists on Cell Growth of NSCs**—We also used PPARY-specific agonists and antagonists to confirm the effect of the PPARY pathway on cell growth of NSCs prepared from ordinal BALB/c mouse embryo brains. NSCs treated with PPARY-specific agonists, rosiglitazone or pioglitazone, at concentrations of 100 nM to 3 μM exhibited a significant stimulation of cell growth in non-coated culture bottles (Fig. 3, A and B). However, inhibition of cell growth was conversely observed at a concentration of PPARY agonists above 30 μM. Similar results were observed when NSCs were treated with PPARY agonists on poly-L-ornithine/laminin-coated plates that initiate differentiation of NSCs into neurons accompanied with outgrowth of neurites (Fig. 3, E and F). Interestingly, NSCs treated with the PPARY agonists expanded while maintaining neurosphere morphology consistent with an undifferentiated state (Fig. 3E). The biphasic action of PPARY agonists on cell growth was not observed in various neuroblastoma-like cell lines (Fig. 3D). These results suggest that the effect of PPARY agonists on NSC growth is specific for NSCs supporting a role for PPARY function in NSCs but not transformed neuroblastomas.
To confirm that PPARγ was involved in stimulating NSC proliferation, we also examined PPARγ antagonists, BADGE and GW9662. BADGE and GW9662 inhibited cell growth in a concentration-dependent manner (Fig. 3C).

Cell Death of NSCs Induced by the PPARγ Antagonist Is Due to Apoptosis Mediated by the Activation of the Caspase Cascade—We next investigated whether the decrease in NSC growth by PPARγ antagonists, BADGE and GW9662, was because of apoptosis using nuclear condensation, a morphological change associated with apoptosis. Treatment of NSCs with either vehicle or the PPARγ agonist, rosiglitazone (3 μM), did not cause any evidence of nuclear condensation in the fields observed (Fig. 4A). However, treatment with BADGE or GW9662 caused nuclear condensation in NSCs. Using Western blot analysis, we observed that treatment with BADGE or GW9662 caused an increase in cleaved caspase-3, a marker of caspase cascade activation (Fig. 4B). In contrast, bisphenol A did not induce activation of the caspase cascade. These results indicate that the PPARγ pathway protects NSCs from apoptosis and promotes cellular proliferation and expansion.

Activation of PPARγ Pathway Inhibits the Differentiation of NSCs into Neurons—NSCs treated with the PPARγ agonists showed the maintaining of neurosphere morphology consistent with an undifferentiated state, although vehicle-treated NSCs differentiated into neurons when cultured on poly-L-ornithine/laminin-coated plates (see Fig. 3E). Therefore, we next investigated the role of PPARγ in regulating the differentiation of NSCs into neurons. To do so, we used confocal microscopy to detect the expression of MAP2 protein, which is a marker for neurons. As shown in Fig. 4C, NSCs treated with a vehicle control showed MAP2 expression in most differentiated cells at day 3. However, treatment with PPARγ agonist, rosiglitazone (3 μM), inhibited the expression of MAP2. In contrast, the expression of nestin, a marker of undifferentiated NSCs, was strongly maintained in the rosiglitazone-treated group compared with that observed in the vehicle control group (Fig. 4D). These results were also confirmed by Western blot analysis in that the expression of MAP2 and nestin were inversely regulated in a concentration- and time-dependent manner (Fig. 4E). Taken together, these data indicate that activation of the PPARγ pathway by specific agonists inhibits the differentiation of NSCs into neurons while at the same time maintaining NSCs in an undifferentiated state as neurospheres.

PPARγ Agonists Activate ERK Pathway as a Mechanism of Increased Proliferation—To investigate the mechanisms by which the PPARγ pathway controls the proliferation of NSCs, we focused on activation of the ERK pathway that is known to be important for growth in many cell types (20, 21). We investigated activation of the ERK pathway in NSCs treated with a PPARγ agonist and antagonist by Western blot analysis using an antibody capable of detecting phosphorylated ERK. Strong activation of ERK was observed in NSCs treated with rosiglitazone at concentrations of 0.3–3 μM (Fig. 5A). However, excessive concentrations of PPARγ ligand inhibited the activation of ERK. These data sug-
suggest that rapid activation of ERK by PPARγ is important for the increase in NSC proliferation.

**PPARγ Agonists Activate Tyr-705 STAT3 in the STAT Family as a Mechanism of Inhibited Differentiation**—To investigate the mechanisms by which the PPARγ pathway controls the differentiation of NSCs, we initially focused on activation of the STAT family. Preliminary experiments used the Cell Cycle Antibody Array System (Hypromatrix, Inc., Millbury, MA), an alteration of STAT3 phosphorylation was observed in NSCs that were treated with a PPARγ agonist (Rosiglitten, 3 μM), or antagonist (BADGE, 3 μM or GW9662, 10 μM) for 24 h, then cells were stained with Hoechst 33342. Nuclear condensations were detected as shown (white arrows). NSCs were treated with vehicle, BADGE (1–10 μM), bisphenol A (10 μM), or GW9662 (1–10 μM) for 6 h. Western blot analysis was performed using anti-cleaved caspase-3 antibody to investigate the caspase-3 activation of NSCs. Typical photographs from three to four independent experiments are shown. The expression of MAP2 (C) and nestin (D) in NSCs treated with PPARγ agonist is shown using confocal laser microscopy or Western blot analysis (D). NSCs were treated with vehicle (Control) or rosiglitazone (Rosi, 3 μM) on poly-L-ornithine/laminin-coated plates for 3 days, then, the cells were fixed. Staining of MAP2 or nestin was performed by anti-MAP2 or anti-nestin antibody, followed by Alexa Fluor 488 secondary antibody. Nuclear staining was performed with SYTOX Orange. The confocal laser microscopic images indicate MAP2 and nestin expression by green and nuclear localization by red. Western blot analysis of the expression of MAP2 and nestin in NSCs treated with vehicle (Control, 0 μM) or rosiglitazone (0.3–3 μM) was for 1 or 3 days. MAP2 in the upper panel, nestin in the lower panel. NS is undifferentiated neural stem cells without treatment.

of other STAT family members was not observed. In fact, rosiglitazone caused a slight decrease in the phosphorylation of STAT6. This increase in STAT3 phosphorylation by treatment of NSCs with rosiglitazone was associated with neither a change in the total amount of STAT3 within 30 min (data not shown), Ser-727 phosphorylation of STAT3, nor Tyr-701 phosphorylation of STAT1. These studies suggest that the maintenance of NSCs in an undifferentiated state may be caused by PPARγ induction of STAT3 phosphorylation.

**PPARγ Agonist Up-regulates Cyclin B and EGF Receptor**—We performed a Gene Chip analysis to define the molecules regulated by PPARγ. Several molecules involved in cell growth were up-regulated in NSCs treated with the PPARγ ligand. Among these, we observed up-regulation of cyclin B. The cyclin family plays an important role in cell cycle control (23–25). We, therefore, investigated the effects of the PPARγ pathway on cyclin family members as a potential mechanism to explain the control of NSC proliferation by PPARγ. Western blot analysis revealed an increase in cyclin B protein levels in NSCs after treatment with rosiglitazone in a concentration-dependent manner (Fig. 6A). Consistent with a role of PPARγ in regulating cyclin B, the levels of cyclin B were observed to decrease in association with blockade of PPARγ by treatment with the antagonist BADGE. PPARγ activation also increased slightly the levels of cyclin A (data not shown). On the other hand, the levels of other cyclin family members such as cyclin D and cyclin E were not altered by treatment with a PPARγ agonist (data not shown). These results suggest that PPARγ stimulates NSC proliferation by up-regulating cyclin B.

We also observed strong up-regulation of the EGF receptor that plays an important role in cell growth via activation of the ERK pathway (26). Protein levels of the EGF receptor were up-regulated by treatment of NSCs with rosiglitazone in a concentration-dependent manner (Fig. 6B). Interestingly, protein levels of the EGF receptor in wild-type NSCs were dramatically higher than those observed in PPARγ-deficient NSCs (Fig. 6C). These data strongly indicate that PPARγ directly controls the
level of the EGF receptor in NSCs. PPARγ-mediated stimulation of cell growth may be dependent on EGF signaling, because the stimulation by PPARγ ligands was not observed on NSCs cultured in EGF-fibroblast growth factor-free medium (data not shown). Furthermore, PPARγ agonist-mediated up-regulation of EGF receptor (EGFR) on NSCs. NSCs treated with rosiglitazone for 24 h were analyzed by Western blot using an anti-EGF receptor antibody. C, EGF receptor protein levels on NSCs prepared from wild-type (Wild) and knock-out (KO) mouse. Clone numbers 3, 7, and 8 are wild-type and 2, 4, and 6 are knock-out NSCs. D, cancellation of the PPARγ agonist-mediated up-regulation of EGF receptor by antagonist. NSCs treated with antagonist (rosiglitazone, 3 µM) in the presence of antagonist (BADGE, 1–10 µM) for 24 h were analyzed by Western blotting. E, cancellation of PPARγ agonist-mediated stimulation of cell growth by antagonist. NSCs were treated with antagonist (rosiglitazone, 3 µM) in the presence of antagonist (BADGE, 1–10 µM) for 3 days, then cell growth was measured by the MTT assay. Data represent mean ± S.E. from three independent experiments.

Comparison of Effect of PPARγ Activation between NSCs and Other Cells—To clarify whether the effect of PPARγ observed in the present study are NSC specific or not, we investigated the comparison of cell growth between NSCs and fibroblasts by the treatment with PPARγ agonist. The effects of agonist or antagonist on the cell growth of fibroblast were very weak (Fig. 7B). In addition, we applied PPARγ agonist to well differentiated cells (almost neurons, because they differentiated for 3–5 days and strongly expressed MAP2) to investigate cell growth. However, a significant increase in cell growth mediated by PPARγ was not observed (Fig. 7A). These results indicate that the effects of PPARγ may be specific for NSCs.

To clarify mechanisms of the specificity of PPARγ effects on NSCs, we compared the expression of the PPARγ protein levels between NSCs and various cells. As shown in Fig. 7D, the expression of the PPARγ protein level in NSCs was extremely higher than those in other cells, such as neuroblastosmas and fibroblasts. Interestingly, the high level expression of PPARγ protein in immature NSCs was gradually decreased during the differentiation in a time-dependent manner (Fig. 7C).

Discussion
In the present study, we show that PPARγ plays an important role in controlling the proliferation and differentiation of murine NSCs into neurons. NSCs, which are present in the embryonic brain, are multipotent and self-renewing progenitor cells. NSCs are not only useful in investigating the proliferation, likely important in brain development and repairs but also differentiation, and development of the central nervous system in vitro.

In the current study, moderate activation of the PPARγ pathway by specific agonists stimulated proliferation and concurrently inhibited the differentiation of NSCs into neurons. However, excessive activation of PPARγ with higher concentrations of agonists resulted in the death of NSCs. This biphasic action of PPARγ suggests that PPARγ tightly regulates the physiological expansion and contraction of NSC mass in a concentration-dependent fashion and indicates that optimal concentrations of PPARγ agonists exist for NSC survival and proliferation in vivo. In fact, such a relationship between the potency of PPARγ agonist and functional outcome has been previously described in insulin-independent diabetes mellitus (2, 27, 28). Consistent with this role of PPARγ in regulating NSC proliferation, the PPARγ antagonists, BADGE and GW9662, caused apoptosis of NSCs via activation of the caspase cascade in a concentration-dependent manner. The inhibition of NSC growth by the antagonists was more dramatic than the stimulation of NSC growth by agonists. These results suggest that PPARγ signaling activated by endogenous ligands in vivo normally regulates NSC growth and in their absence, causes programmed cell death.

Two previous reports have described a relationship between PPARγ expression and apoptosis using PPARγ agonists on neuroblastoma and glioma cells (29, 30). However, these studies did not describe the bipha-
sic action of PPARγ agonists on promotion of cell growth and the simultaneous inhibition of NSC differentiation into neurons observed in the current study. The differences observed between the current and previously reported studies may be because of differences in the concentrations of agonists used. The prior studies used comparatively higher concentrations of agonists (10–100 μM) consistent with our observations that high PPARγ agonist concentrations induced death of NSCs. Alternatively, the results reported here may reflect the nature of NSCs that, unlike neuroblastoma-like cell lines, are non-transformed. Consistent with previous results, we did not detect significant effects of PPARγ agonists on either the cell growth or death of several neuroblastoma cell lines. We also investigated PPARγ-mediated stimulation of cell growth on human fibroblast prepared from a normal infant. However, the effects of both agonist and antagonist on fibroblasts were much weaker than those on NSCs. Furthermore, we applied PPARγ agonist to well differentiated cells almost like neurons to investigate cell growth. However, an increase in cell growth mediated by PPARγ was not observed. These results indicate that the effects of PPARγ may be specific for NSCs. Why is the specificity of PPARγ on NSCs derived? One reason may be the difference in PPARγ protein levels between NSCs and various cells. In fact, the level of PPARγ on NSCs is extremely higher than that on fibroblasts and neuroblastoma cell lines.

To clarify the mechanisms by which the PPARγ pathway controls the proliferation of NSCs, we investigated the activation of the ERK pathway that is known to be important for growth in many cell types (20, 21). Moderate concentrations of PPARγ agonists strongly activated the ERK. However, excessive concentrations of PPARγ agonists inhibited the activation of ERK. These data suggest that rapid activation of ERK by PPARγ is important for the increase in NSC proliferation. Although it is unclear how PPARγ rapidly activates ERK in NSCs, it has been reported that PPARγ ligands activate the ERK pathway via a PPAR-dependent manner (31). Therefore, similar activation mechanisms of the ERK pathway by PPARγ may exist in NSCs. It is reported that higher doses of thiazolidinediones, such as pioglitazone and troglitazone, can activate AMP-activated kinase in a PPARγ-independent manner (32, 33). The biphasic effect of PPARγ agonists on cell proliferation and ERK activation might therefore be due to both a PPARγ-dependent and -independent process.

According to the results of the Gene Chip analysis, we observed strong up-regulation of the EGF receptor that plays an important role in cell growth via activation of the ERK pathway (26). These data strongly indicate that PPARγ directly controls the level of the EGF receptor in NSCs. PPARγ-mediated stimulation of cell growth may be dependent on EGF signaling, because the stimulation by PPARγ ligands was not observed on NSCs cultured in EGF-fibroblast growth factor-free medium (data not shown). Furthermore, PPARγ agonist-mediated up-regulation of the EGF receptor inhibited by the presence of antagonist resulted in the cancellation of agonist-mediated stimulation of cell growth. These results clearly indicated that the PPARγ action on the stimulation of cell growth is due to the up-regulation of EGF receptor via the PPARγ pathway. Taken together, we show here that PPARγ mediates the proliferation of NSCs via up-regulation of the EGF receptor and activation of the ERK pathway.

Furthermore, PPARγ may simultaneously increase cyclin B levels resulting in the stimulation of proliferation. PPARγ stimulation of cell growth was associated with up-regulation of cyclin family members, the main controllers of the cell cycle (23–25). Although several studies have reported that PPARγ activation causes apoptosis in pulmonary, hepatic, and pancreatic carcinomas via cell-cycle arrest because of a reduction in cyclin levels (34–36), we observed an increase in cyclin B levels when NSCs were treated with PPARγ agonists. These observations suggest that either PPARγ has differential effects on stem cell progenitors in comparison to transformed tumor cell lines or the results reflect the differences in concentrations of agonists used.

The detailed mechanisms are still unclear how PPARγ increases the proliferation of NSCs. However, at least two possible mechanisms are considered, one is activation of the ERK pathway via up-regulation of the EGF receptor, the other is acceleration of the cell cycle via up-regulation of the cyclin B level. Further investigations are required to clarify the details.

Interestingly, PPARγ activation maintained NSCs as undifferentiated neurospheres and inhibited the morphologic differentiation of NSCs into neurons. Such observations were confirmed by the expression of MAP2 and nestin using two independent methods, confocal laser microscopy and Western blot analysis. Specifically, treatment with PPARγ agonists inhibited the expression of MAP2 and maintained nestin expression. This maintenance of NSCs in an undifferentiated state may involve PPARγ-mediated activation of the STAT pathway. This is supported by the observation that PPARγ specifically induced STAT3 (Y705) phosphorylation but not phosphorylation of other members of the STAT family, STAT1, STAT5, and STAT6. This is consistent with the role of STAT3 activation in maintaining mouse embryonic stem cells in an undifferentiated state (22). Taken together, these data support the hypothesis that moderate activation of the PPARγ pathway causes activation of STAT3, which maintains the undifferentiated state of NSCs.

The physiological importance of those proposed pathways was shown by our evaluation of PPARγ-deficient mice. The rate of cell growth of PPARγ+/−-NSCs was observed to be much lower than that of wild-type NSCs. Morphologically, a disorder in the formation of neurospheres was observed in PPARγ+/−-NSCs compared with that observed with the wild-type clones. These results suggest that in vivo deficiency of PPARγ is associated with diminished growth and disordered formation of neurospheres.

To confirm the effect of PPARγ deficiency, we prepared PPARγ-RNA-silenced NSCs by shRNA and dominant-negative PPARγ-NSCs. PPARγ-RNA-silenced NSCs exhibited a significant decrease in their cell growth rates and morphologically failed to form neurospheres. Similarly, dominant-negative PPARγ-NSCs exhibited a significant decrease in their cell growth rates and also failed to form neurospheres in comparison to that observed with wild-type PPARγ-NSCs. These results strongly suggest that PPARγ regulates the proliferation and the absence of PPARγ directly inhibits the self-renewal functions of NSCs. Furthermore, we examined the recovery of the impaired cell growth observed with PPARγ-deficient (PPARγ+/−) NSCs by increasing the expression of PPARγ using adenovirus-mediated PPARγ-gene transfer. Adenovirus-mediated PPARγ-gene transfer successfully increased PPARγ protein levels and the increase in PPARγ protein levels in PPARγ+/−-NSCs resulted in reversal of the impaired cell growth. These results strongly suggest that PPARγ regulates the proliferation and differentiation state of NSCs in vivo.

Taken together, these PPARγ-mediated mechanisms to control the proliferation and differentiation may be important for self-renewal of NSCs, because a lack of PPARγ directly inhibits the formation of neurospheres and cell growth both of which are important characteristics of NSCs. Such a role of NSCs is likely to be important to early stages of brain development in that visible disorders of development were observed in whole embryos of PPARγ−/− mice (see Fig. 1A). Consistent with this, the expression level of nestin, a marker of undifferentiated NSCs, and the number of nestin-positive cells that are considered as NSCs in PPARγ+/− and PPARγ−/− embryo brains was much lower than
that of wild-type embryo brains (data not shown). Furthermore, PPARγ and nestin expression was observed in the area of the future lateral ventricle that has been reported as the main area of NSC localization in embryos (7, 10, 13), further emphasizing the potential importance of PPARγ in the proliferation of NSCs in embryos. Interestingly, the expression of PPARγ and nestin was not observed in the brains of newborn and adult wild-type mice. This is consistent with the fact that PPARγ expression has been reported to mainly occur in adipose tissue, colon, and small intestine, but not in the brains of adult animals such as mice or rats (37, 38). However, in our present study, we showed expression of PPARγ during the embryonic development in rats (37). These results indicate that the endogenous PPARγ has been expressed in the brains of embryos at days E13–E15 with disappearance at day E18 during the embryonic development in rats (37). These results indicate the endogenous PPARγ pathway in NSCs is likely to be important for the early development of the central nervous system. The findings confirmed in the present study show that pathways associated with PPARγ control NSC proliferation with important implications for re-generation therapies in neurodegenerative disorders.

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