**Introduction**

The central nervous system (CNS) was thought to be a terminally differentiated organ. This was partly because the majority of cells in adult mammalian brain emerge at prenatal period which has limited ability to grow and replace lost cells or restore function. During embryonic development these cells originate from neural progenitor cells (NPCs) [1–3]. But recent studies have localized a small population of resident neural stem cells (NSCs) in the subventricular zone of adult brain [4]. It is well recognized that NSCs can proliferate, migrate and differentiate into neurons and glia in normal brain [5–8]. Under optimum conditions, NSCs grow and differentiate into neuro-glial cells in culture [9,10]. Adoptively transferred NSCs migrate, grow and differentiate into neuro-glial cells in the brain of experimental models [11], suggesting their use in the treatment of human neurodegenerative diseases such as multiple sclerosis (MS), Alzheimer’s disease (AD), Parkinson’s disease (PD), spinal cord injury, trauma, and stroke [12–15]. Unfortunately, the behavior of cells in normal brain or in tissue culture does not adequately predict how these cells will behave in the CNS of patients with neurodegenerative diseases. This is more significant in compromised CNS niche with neuroinflammation where multiple factors converge on to influence the normal physiology. Thus an effective therapy for neurodegenerative diseases hinges on novel strategies to improve the ability of NSCs to thrive, integrate, and function in a physiologically meaningful manner without causing adverse side effects.

Peroxisome proliferator-activated receptors (PPAR) are a family of ligand-dependent nuclear receptor transcriptional factors that regulate lipid metabolism and glucose homeostasis [16–18]. PPARζ, PPARγ and PPARδ are three known subtypes of the PPAR family [19]. Several fatty acids, leukotrienes and 15-Deoxy-

**Abstract**

Neural stem cells (NSCs) are a small population of resident cells that can grow, migrate and differentiate into neuro-glial cells in the central nervous system (CNS). Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear receptor transcription factor that regulates cell growth and differentiation. In this study we analyzed the influence of PPARγ agonists on neural stem cell growth and differentiation in culture. We found that in vitro culture of mouse NSCs in neurobasal medium with B27 in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) induced their growth and expansion as neurospheres. Addition of all-trans retinoic acid (ATRA) and PPARγ agonist ciglitazone or 15-Deoxy-A1,4-Prostaglandin J2 (15d-PGJ2) resulted in a dose-dependent inhibition of cell viability and proliferation of NSCs in culture. Interestingly, NSCs cultured with PPARγ agonists, but not ATRA, showed significant increase in oligodendrocyte precursor-specific O4 and NG2 reactivity with a reduction in NSC marker nestin, in 3–7 days. In vitro treatment with PPARγ agonists and ATRA also induced modest increase in the expression of neuronal β-III tubulin and astrocyte-specific GFAP in NSCs in 3–7 days. Further analyses showed that PPARγ agonists and ATRA induced significant alterations in the expression of many stemness and differentiation genes associated with neuro-glial differentiation in NSCs. These findings highlight the influence of PPARγ agonists in promoting neuro-glial differentiation of NSCs and its significance in the treatment of neurodegenerative diseases.

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allergic encephalomyelitis (EAE) model of MS by blocking inflammatory signaling networks [39], suggesting a physiological role for the PPARγ in the regulation of inflammation and CNS repair in neurodegenerative diseases.

However, recent studies examining the influence of PPARγ agonists on cultured NSCs generated conflicting results. While many reports showed reduced growth with increased differentiation, others demonstrated increased growth with reduced differentiation of NSCs in culture [40–42]. In this study we examined the mechanisms by which PPARγ agonists regulate growth and differentiation of NSCs in culture. Our results demonstrate that PPARγ agonists promote oligodendrocyte differentiation of mouse NSCs by modulating the expression of stemness and differentiation genes, suggesting its use in the treatment of demyelinating diseases.

Results

PPARγ agonists inhibit proliferation of NSCs

To study the effect of PPARγ agonists on NSCs, we first established conditions to grow and expand mouse NSCs in culture. As shown in Figure 1, in vitro culture of brain cells from newborn mice in NBM with EGF+bFGF induced neurosphere formation in 3 to 5 days that increased in size by 10 days (Fig 1A, B). The NSCs dissociated from neurospheres (Fig 1C) showed proliferation in NBM and that increased significantly following addition of EGF and bFGF alone or in combination (Fig 1D). 3H Thymidine uptake assay showed that NSCs cultured in medium displayed a background count of 2970 cpm that increased to 8168±297 and 9556±223 cpm following the addition of 10 ng/ml bFGF and EGF, respectively (Fig 1D). Moreover, addition of EGF and bFGF in combination resulted in further increase in proliferation reaching 13960±520 cpm, suggesting a potentiating effect for these growth factors on NSCs in culture.

We then examined the effect of PPARγ agonists on the proliferation of NSCs in culture. As shown in Figure 2, in vitro culture of NSCs in the presence of EGF+bFGF showed a dose-dependent increase in proliferation or viable cell count as determined by WST-1 assay. Viable NSCs in the absence of growth factor was 3% that increased to 56, 68, 69 and 100 percent by the addition of 1, 2.5, 5 and 10 ng/ml EGF+bFGF, respectively in culture (Fig 2A). Interestingly, addition of PPARγ agonists resulted in a dose-dependent decrease in viable cell count in culture (Fig 2B, C). While NSCs cultured in NBM with EGF+bFGF in the absence of 15d-PGJ2 showed 100% viability, which decreased to 98, 62, 51, 44, 20, and 10 percent following addition of 1, 2.5, 5, 10, 20 and 25 μM 15d-PGJ2, respectively (Fig 2B). Similarly, NSCs cultured in the absence of cigitazone showed 100% viability, which decreased to 98, 66, 48, 27, 18 and 1 percent following addition of 1, 2.5, 5, 10, 20 and 25 μM cigitazone, respectively (Fig 2C). Moreover, NSCs cultured with ATRA also showed a dose-dependent decrease in EGF+bFGF-induced proliferation/viability. While NSCs cultured with EGF+bFGF in the absence of ATRA showed 100% viability that decreased to 98, 65, 44, 42, 22 and 13 percent following addition of 1, 2.5, 5, 10, 20 and 25 μM ATRA, respectively in culture (Fig 2D). These results suggest that PPARγ agonists inhibit EGF+bFGF-induced proliferation and survival of NSCs in culture.

PPARγ agonists induce oligodendrocyte differentiation of NSCs

To study the effect of PPARγ agonists on NSC differentiation we examined the expression of neuro-glial markers by Western blot analysis. As shown in Figure 3A, NSCs cultured in NBM+B27 in the absence of EGF+bFGF expressed elevated levels of neuron-specific β-III tubulin that decreased significantly following addition of 10 ng/ml EGF+bFGF. Interestingly, NSCs cultured with EGF+bFGF in the presence of 5 μM cigitazone, 15d-PGJ2, or ATRA showed a significant increase in the expression of β-III tubulin compared to EGF+bFGF treated cells. Similarly, NSCs cultured in the absence of EGF+bFGF expressed elevated levels of astrocyte-specific GFAP that decreased significantly following the addition of 10 ng/ml EGF+bFGF. Treatment with 1 or 5 μM cigitazone, 15d-PGJ2, or ATRA resulted in a partial increase in GFAP expression, reaching statistical significance at 5 μM 15d-PGJ2 and 1 μM ATRA compared to EGF+bFGF control (Fig 3A). Moreover, NSCs cultured in NBM with EGF+bFGF expressed detectable levels of oligodendrocyte progenitor-specific NG2 proteoglycan that increased significantly after the addition of 5 μM cigitazone or 15d-PGJ2 but not with ATRA (Fig 3B). In addition, NSCs cultured with EGF+bFGF showed elevated expression of stem cell marker Nestin that decrease significantly after the addition of 5 μM cigitazone or 15d-PGJ2 but increased after treatment with ATRA (Fig 3B). However, NSCs cultured in NBM with EGF+bFGF in the presence of PPARγ agonists failed to express myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG), as detected in adult mouse brain (Fig 3C).

To further define the effect of PPARγ agonists on NSC differentiation, we examined the expression of neuro-glial markers by immunocytochemical techniques. As shown in Figure 4 and 5, NSCs cultured in NBM+B27 in the presence of EGF+bFGF showed detectable expression of astrocyte-specific GFAP and neuronal β-III tubulin that increased after treatment with cigitazone, 15d-PGJ2 or ATRA for three and seven days, respectively. Quantitative analysis showed a trend towards increase in GFAP and β-III tubulin expression in NSCs cultured with cigitazone, 15d-PGJ2 or ATRA compared to DMSO control. In addition, NSCs cultured with EGF+bFGF in the presence of cigitazone or 15d-PGJ2 showed considerable increase in the expression of pre-oligodendrocyte specific O4 reactivity with characteristic morphology and migration pattern in 3 days that further increased with 15d-PGJ2 by day 7 (Fig 4, 5). However, NSCs cultured with EGF+bFGF in the absence of PPARγ agonists or in the presence of ATRA showed only minimal O4 reactivity on day 3 with a marginal increase by day 7 (Fig 4, 5). These findings suggest that PPARγ agonists induce the differentiation of oligodendrocyte progenitor cells from NSCs and may require additional signals to promote their maturation to myelinating oligodendrocytes.

PPARγ agonists modulate the expression of stemness genes in NSCs

To define the mechanisms by which PPARγ agonists regulate neuro-glial differentiation of NSCs, we analyzed stemness gene profile using TaqMan low density gene array. This array includes a panel of 40 stemness and 50 differentiation genes. As shown in Table 1 and 2, in vitro culture of NSCs in NBM with EGF+bFGF in the presence of PPARγ agonists resulted in significant changes in the expression of many stemness and differentiation genes in three days. Treatment with cigitazone induced ≥100-fold increase in 22, 1–100 fold increase in 4 and 4 fold decrease in one stemness gene. Moreover, 9 stemness genes expressed in NSCs were undetectable after treatment with cigitazone (D-ND). 4 stemness genes were undetected in NSCs cultured in the absence or presence of cigitazone (ND-ND). Similarly, in vitro treatment of NSCs with 15d-PGJ2 induced ≥100-fold increase in 14, 1–100 fold increase in 5 and ≥100 fold decrease in 2 stemness genes. Moreover, 6 stemness genes expressed in NSCs were undetectable.
after treatment with 15d-PGJ2 (D-ND), 5 stemness genes undetectable in NSCs were detected after treatment with 15d-PGJ2 (ND-D) and 10 stemness genes remained undetectable following treatment with 15d-PGJ2 (ND-ND). In addition, NSCs treated with ATRA showed 100-fold increase in 12, 1–100 fold increase in 5 and 1–1000 fold decrease in 7 stemness genes. Furthermore, 7 stemness genes expressed in NSCs were undetectable after treatment with ATRA (D-ND), 3 stemness genes not detected in NSCs were detected after treatment with ATRA (ND-D), and 5 stemness genes remained undetectable after treatment with ATRA (ND-ND) (Table 1). The NSCs cultured with any of the three agonists exhibited elevated expression of 10 stemness factors (Nog, Crabp2, Dmnt3b, Srfp2, Gal, Bxdc2, Podxl, Kit, Lefty1, and Nodal) along with the suppression of 7 other stemness factors (Ssc2, Lifr, CD9,Nr6A1, Nanog, Gabrb3 and Iifim1) (Table 1).

Further analyses revealed significant alterations in the stemness gene expression profile of NSCs that were common or distinct among treatment groups. As shown in Figure 6A, the heat map demonstrates altered expression of many stemness genes following treatment of NSCs with PPARγ agonists when compared to controls. The Ct values presented as box plots (Fig 6B) also demonstrates changes in the expression of stemness genes in NSCs by PPARγ agonists. The horizontal line represents the median while the numbers represent the mean. We found that ciglitazone induced a mild suppression of stemness genes, while 15d-PGJ2 increased overall expression of stemness genes. Treatment with ATRA showed no marked difference in the expression of stemness genes. Scatter plots of ΔCt values (Fig. 6C) further confirmed the altered expression of stemness genes in NSCs following treatment with PPARγ agonists in culture. Moreover, as shown in Figure 6D, Venn diagram demonstrates elevated expression of 10 stemness genes by all three agonists, 7 by ciglitazone and 15d-PGJ2, 3 by 15d-PGJ2 and ATRA and 5 by ciglitazone and ATRA in NSCs. We have also found a decrease in the expression of 7 stemness genes by all three agonists, 2 by ciglitazone and 15d-PGJ2, 4 by 15d-PGJ2 and ATRA and 2 by ciglitazone and ATRA in NSCs. While 4 stemness genes were elevated only by ciglitazone, 2 by 15d-PGJ2 and 2 by ATRA, 3 stemness genes were inhibited only by ciglitazone, 5 by 15d-PGJ2 and 7 by ATRA in NSCs (Fig. 6D). These findings suggest that PPARγ agonists regulate self-renewal and differentiation by modulating stemness gene expression profile in NSCs.

PPARγ agonists modulate the expression of differentiation genes in NSCs

We then examined the expression of 50 differentiation factors in NSCs cultured with PPARγ agonists. As shown in Table 2, NSCs cultured with ciglitazone showed ≥100-fold increase in 21, 1–100 fold increase in 6, and 1–100 fold decrease in 2 differentiation genes. Meanwhile, 6 differentiation genes not expressed in NSCs were detected after treatment with ciglitazone (ND-D), 8 differentiation genes expressed in NSCs were not detected after treatment with ciglitazone (D-ND) and 7 differentiation genes were not detected (ND-ND) in NSCs cultured in the absence or presence of ciglitazone. Similarly, in vitro treatment of NSCs with...
15d-PGJ2 induced ≥100 fold increase in 21, 1–100 fold increase in 4 and 1–1000 fold decrease in 8 differentiation genes. Moreover, 3 differentiation genes expressed in NSCs were undetectable after treatment with 15d-PGJ2 (D-ND), 4 differentiation genes not detected in NSCs were detected after treatment with 15d-PGJ2 (ND-D) and 9 differentiation genes remained undetected in NSCs cultured in the absence or presence of 15d-PGJ2 (ND-ND). In addition, in vitro treatment of NSCs with ATRA induced 100-fold increase in 21, 1–100 fold increase in 4 and 1–1000 fold decrease in 6 differentiation genes. Moreover, 7 differentiation genes expressed in NSCs were undetectable after treatment with ATRA (D-ND), 3 differentiation genes not detected in NSCs were detected after treatment with ATRA (ND-D) and 9 differentiation genes remained undetected in NSCs after treatment with ATRA (ND-ND) (Table 2). Among the differentiation genes altered by ciglitazone or 15d-PGJ2 in NSCs, the expression of 6 differentiation factors increased, whereas the expression of 3 differentiation factors decreased (Table 2). NSCs treated with any of the three drugs in this study exhibited elevated expression of 14 differentiation factors (Pecam1, Hbb, Pax6, Sat, Grg, Krt1, Neurod1, Hlb29, Flt1, Eras, T, Actc1, Tat, and Olig2) with the suppression of 5 differentiation factors (Gata6, Isd1, Foxa2, Gcm1 and Gata4) (Table 2).

We have also found significant alterations in the differentiation gene expression profile of NSCs that were common or distinct among treatments. As shown in Figure 6A, heat map demonstrates altered expression of many differentiation genes in NSCs following treatment with PPARγ agonists when compared to controls. The Ct values presented as box plots (Fig 6B) demonstrates changes in the expression of differentiation genes induced by PPARγ agonists in NSCs. We found that ciglitazone induced a mild suppression of differentiation genes, while 15d-PGJ2 increased the overall expression of differentiation genes, while ATRA showed no effect. Scatter plots of ΔCt values (Fig. 6C) further confirmed the altered expression of differentiation genes by PPARγ agonists in NSCs. Moreover, as shown in Figure 6D, Venn diagram demonstrates an elevated expression of 14 differentiation genes by all three agonists, 6 by ciglitazone and 15d-PGJ2, 5 by 15d-PGJ2 and ATRA and 6 by ciglitazone and ATRA in NSCs. We also found a decrease in the expression of 5 differentiation genes by all three agonists, 3 by ciglitazone and 15d-PGJ2, 7 by 15d-PGJ2 and ATRA and 4 by ciglitazone and ATRA in NSCs. While 7 differentiation genes were elevated only by ciglitazone, 4 by 15d-PGJ2 and 3 by ATRA, 5 differentiation genes were inhibited only by ciglitazone, 6 by 15d-PGJ2 and 6 by ATRA in NSCs (Fig. 6D). These results suggest that PPARγ agonists promote neuro-glial differentiation by modulating distinct stemness and differentiation gene expression profile in NSCs.

Discussion

The past decade has seen tremendous progress in understanding the molecular mechanisms in the regulation of growth, self-renewal and differentiation of stem cells along specific lineages. This progress was made possible by the discovery of myriad

Fig. 2. Inhibition of NSC proliferation by PPARγ agonists. NSCs dissociated from 7–10 day old neurospheres were cultured in 96 well tissue culture plates (1×10^3/0.2 ml/well) in NBM+B27 with different doses of EGF+bFGF (A) or 10 ng/ml EGF+bFGF in the presence of different doses of 15d-PGJ2 (B), ciglitazone (C) and ATRA (D). The cell proliferation/viability was measured by WST-1 assay. The values are means of triplicates ± SD and the p values are expressed as *(p<0.05), **(p<0.01), and ****(p<0.001). The figure is a representative of three independent experiments.

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PPARγ Promotes Oligodendrocyte Differentiation

A  EGF+bFGF (ng/ml)  0  10 10 10 10 10 10 10
Ciglitazone (μM)  - - 1 5 - - - -
15d-PGJ2 (μM)  - - - 1 5 - - -
ATRA (μM)  - - - - - 1 5

βIII Tubulin

GFAP

β Actin

B  EGF+bFGF (ng/ml)  10 10 10 10 10 10 10 10
Ciglitazone (μM)  1 5 - - - - - -
15d-PGJ2 (μM)  - - 1 5 - - - -
ATRA (μM)  - - - - 1 5 - -

NG2

Nestin

β Actin

C  EGF+bFGF (ng/ml)  0  10 10 10 10 10 10 10
Ciglitazone (μM)  - - 1 5 - - - -
15d-PGJ2 (μM)  - - - 1 5 - - -
ATRA (μM)  - - - - - 1 5

MBP

MOG

β Actin

Brain Extract  5 μg  10 μg

MBP

MOG

β Actin
growth factors including EGF and bFGF and their signaling pathways responsible for maintaining self-renewal and pluripotency of stem cells in culture. PPARγ is an important regulator of growth and differentiation of many cell types during pre and postnatal development. In this study we found that NSCs cultured in NBM+B27 with EGF+bFGF grow and expand as neurospheres, but PPARγ agonists decrease growth and enhance neuro-glial differentiation in culture. Among the many neuro-glial markers

Figure 3. Modulation of stem cell and differentiation markers by PPARγ agonists in NSCs. NSCs were cultured in NBM+B27 with EGF+bFGF in the presence of 0, 1 and 5 µM ciglitazone, 15d-PGJ2 or ATRA at 37 °C for 72 h. The expression of βIII tubulin, GFAP (A), NG2, Nestin (B), MBP, MOG (C) and β-Actin was analyzed by Western blot and ECL detection system. Mouse brain extract was used as positive control (C). The relative quantities of protein bands normalized to β-Actin in the blots were determined by densitometry and presented as histograms. The values are mean±SD and the p values are expressed as *(p<0.05), **(p<0.01), and ****(p<0.001). The figure is a representative of five independent experiments. doi:10.1371/journal.pone.0050500.g003

Figure 4. PPARγ agonists induce the expression of oligodendrocyte markers in three days in NSCs. Neurospheres were cultured in poly-D-lysine coated 8 well chamber slides in NBM+B27 with 10 ng/ml EGF+bFGF in the presence of 0 (DMSO) or 1 µM ciglitazone, 15d-PGJ2 or ATRA. After 3 days the cells were stained with GFAP, βIII tubulin, and O4 antibodies along with DAPI and photographed (200 ×) under fluorescence microscope. The figure is a representative of three independent experiments. The values are mean±SEM and the p values are expressed as *(p<0.05), **(p<0.01), and ****(p<0.001). The figure is a representative of three independent experiments. doi:10.1371/journal.pone.0050500.g004
analyzed, we observed a significant increase in the expression of NG2 in PPARγ agonist treated NSCs, suggesting oligodendrocyte progenitor differentiation of NSCs in culture. Moreover, an increase in the expression of antigens detected by O4 antibody, suggests pre-oligodendrocyte differentiation of NSCs following treatment with PPARγ agonists. These findings are consistent with previous reports showing the detection of NG2 and O4 as markers of NSC differentiation to oligodendrocyte progenitor cells in culture. Stabenfeldt et al., used O4 as a marker for oligodendrocyte differentiation of mouse NSCs in 7 day old cultures [43]. A study by SyPECka et al., using human cord blood derived NSCs also demonstrated oligodendrogial differentiation by O4 staining [44]. Sher et al., used several markers to identify various stages of oligodendrocyte differentiation (PDGFRα precursor, progenitor, NG2 - progenitor, RIP - preoligodendrocyte and MBP - myelinating mature oligodendrocyte) in NSCs derived from C57BL/6 mice [45]. However, we found that NSCs cultured in the presence of PPARγ agonists failed to express MBP or MOG as detected in mouse brain extracts, suggesting that additional signals are required to induce maturation of oligodendrocytes.

Growth and differentiation signals are integrated by key transcription factors which regulate specific gene clusters to allow...
proliferation or differentiation to acquire specialized functions in NSCs. To define the mechanisms by which PPARγ agonists regulate growth and self-renewal of NSCs, we analyzed the expression of 40 stemness genes. Among the many altered stemness genes, Sox2 was one of the important genes inhibited by PPARγ agonists and ATRA in NSCs. Sox2 is a key member of the Sox (SRY-like HMG box) family transcription factors expressed in embryonic stem cells (ES), NSCs and trophoblast stem cells, but not in differentiated cells and is essential for maintaining pluripotency [46–49]. CD9 is a surface protein expressed in neural progenitor cells [50] that was also suppressed by PPARγ agonists. The suppression of Sox2, CD9 and other

Table 1. Regulation of stemness gene profile by PPARγ agonists in neural stem cells.

| Symbol | Gene Name            | Ciglitazone   | 15d-PGJ2   | Retinoic acid |
|--------|----------------------|---------------|------------|--------------|
| Nog    | Noggin               | 9.2E18↑       | 1.6E15↑    | 4 E12↑       |
| Crabp2 | Cellular retinoic acid binding protein 2 | 9.2E18↑       | ND-D↑      | 4.8E14↑      |
| CommD3 | COMM domain containing 3 | 9.2E18↑       | ND-ND↓     | 8.1E12↑      |
| Dnmt3b | DNA cytosine-5-methyltransferase 3b | 1.4E17↑       | 1.8E12↑    | 1.6E12↑      |
| Srfp2  | Frizzled-related protein | 7.1E13↑       | 2.5E7↑     | 4082↑        |
| Gdf3   | Growth differentiation factor 3 | 1.9E12↑       | 577↑       | ND-ND↓       |
| Rest   | RE1-silencing transcription factor | 3.3E11↑       | ND-ND↓     | 0.001↓       |
| Igfip2 | Insulin-like growth factor binding protein 2 | 1.3E11↑       | ND-ND↓     | 1.7↑         |
| Nes    | Nestin               | 3.0E10↑       | ND-ND↓     | 1439↑        |
| Gal    | Galanin prepropeptide | 5.8E8↑        | 1.2E9↑     | 24.5↑        |
| Pou5f1 | POU class 5 homeobox 1 | 2.3E8↑        | 3.8E14↑    | 0.022↓       |
| Xist   | X (inactive)-specific transcript | 8.05E7↑      | 9.17E9↑    | ND-ND↓       |
| Zfp42  | Zinc finger protein 42 homolog | 8.8E6↑       | 18.8↑      | ND-ND↓       |
| Bdxc2  | BRX1, biogenesis of ribosomes, homolog | 1.1E6↑       | 7.6E9↑     | 9.56E↑       |
| Podxl  | Podocalyxin-like      | 2.8E5↑        | 7.8E14↑    | 16.1↑        |
| Tdgf1  | Teratocarcinoma-derived growth factor 1 | 4.3E4↑       | 14.7↑      | 0.003↓       |
| Lin28  | Lin-28 homolog A     | 2.1E4↑        | ND-ND↓     | ND-ND↓       |
| Fgf5   | Fibroblast growth factor 5 | 1.8E4↑       | D-ND↓      | 0.11↓        |
| Utf1   | Undifferentiated embryonic cell transcription factor | 1.4E4↑       | 2.9↑       | D-ND↓        |
| Pten   | Phosphatase and tensin homolog | 6799↑        | ND-ND↓     | ND-ND↓       |
| Kit    | Feline sarcoma viral oncogene | 757.2↑       | 191.9↑     | 8.1E11↑      |
| Lefty1 | Left-right determination factor 1 | 112.4↑       | 5.9E11↑    | 7.2E11↑      |
| Nrf2   | Nuclear receptor subfamily 5 | 12.1↑        | 0.003↓     | ND-D↑        |
| Ifitm2 | Interferon induced transmembrane protein 2 | 11.8↑        | 1.4E7↑     | D-ND↓        |
| Nodal  | Nodal homolog        | 9.8↑          | 0.002↓     | 1.5E10↑      |
| Lefty2 | Left-right determination factor 2 | 1.2↑         | ND-D↑      | 1.6↑         |
| Grb7   | Growth factor receptor-bound protein 7 | 0.4↓         | ND-D↓      | 2.5E7↑       |
| Fgf4   | Fibroblast growth factor 4 | D-ND↓        | 4.3E16↑    | 2.6E14↑      |
| Gbx2   | Gastrulation brain homeobox 2 | D-ND↓        | 6.3E10↑    | 2.5↑         |
| Tert   | Telomerase reverse transcriptase | D-ND↓        | 16.4↑      | 0.002↓       |
| Tfp2l1 | Transcription factor CP2-like 1 | D-ND↓        | 8.9↑       | 0.003↓       |
| Sox2   | SRY-box 2            | D-ND↓         | D-ND↓      | 0.011↓       |
| Lifr   | Leukemia inhibitory factor receptor alpha | D-ND↓        | D-ND↓      | D-ND↓        |
| CD9    | CD9 molecule         | D-ND↓         | D-ND↓      | D-ND↓        |
| Nrd1a  | Nuclear receptor subfamily 6, group A, member 1 | D-ND↓        | D-ND↓      | D-ND↓        |
| Nanog  | Nanog homeobox       | D-ND↓         | ND-ND↓     | D-ND↓        |
| Gabrb3 | GABA A receptor, beta 3 | D-ND↓        | ND-ND↓     | 0.5↓         |
| Iil6t  | Interleukin 6 signal transducer | ND-ND↓       | ND-ND↓     | ND-D↑        |
| Sema3a | Sema domain, immunoglobulin domain 8g | ND-ND↓       | ND-ND↓     | ND-D↑        |
| Ifitm1 | Interferon induced transmembrane protein 1 | ND-ND↓       | ND-ND↓     | ND-ND↓       |

NSCs were cultured in NBM with EGF+bFGF in the presence of DMSO, ciglitazone, 15d-PGJ2 or ATRA for 3 days. The stemness gene expression was analyzed by qRT-PCR using a 384 gene card array. The fold change was calculated using automatic threshold setting and is based on expression levels in DMSO treated cells as1 after normalizing to 18S or GAPDH. The genes in the table are arranged from high to low expression in ciglitazone treated cells. ND, not detected and D, detected. Arrows indicate up or down regulated genes. This data is a representative of two independent experiments.

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Table 2. Regulation of differentiation gene profile by PPARγ agonists in neural stem cells.

| Symbol | Gene Name                                      | Ciglitazone | 15d-PGJ2 | Retinoic acid |
|--------|-----------------------------------------------|-------------|-----------|--------------|
| Col1a1 | Collagen, type I, alpha 1                     | ND-D        | 0.6 ↓     | 1.1 ↑        |
| Ptf1a  | Pancreas specific transcription factor 1a      | ND-D        | ND-ND     | 1.9E14 ↑     |
| Col2a1 | Collagen, type II, alpha 1                    | ND-D        | ND-ND     | ND-D ↑       |
| Pecam  | Platelet/endothelial cell adhesion molecule    | ND-D        | ND-ND     | ND-D ↑       |
| Hbb    | Hemoglobin, beta                              | ND-D        | 443.2 ↑   | 2.2E13 ↑     |
| Foxd3  | Forkhead box D3                               | ND-D        | ND-ND     | ND-ND ↓      |
| Pax6   | Paired box 6                                  | 9.2E18 ↑    | 6.3E4 ↑   | 3.4E10 ↑     |
| Sst    | Somatostatin                                  | 2.5E18 ↑    | 1.1E15 ↑  | 3.628 ↑      |
| Gcg    | Glucagon                                      | 1.6E18 ↑    | 1.4E15 ↑  | 7.4E13 ↑     |
| Lamb1  | Laminin, beta 1                               | 3.8E16 ↑    | 4.8E14 ↑  | D-ND ↓       |
| Krt1   | Keratin 1                                     | 5.3E15 ↑    | ND-D ↑    | 1.5E11 ↑     |
| Des    | Desmin                                        | 9.6E14 ↑    | 7.0 ↑    | 0.002 ↓      |
| Cd34   | CD34 molecule                                 | 3.6E12 ↑    | ND-ND     | ND-ND ↓      |
| Lama1  | Laminin, alpha 1                              | 2.8E12 ↑    | 0.002 ↓  | 9.482.2 ↑    |
| Neurod1| Neurogenic differentiation 1                  | 3.8E11 ↑    | 4.8E13 ↑  | 5.0E10 ↑     |
| Gfap   | Gial fibrillary acidic protein                 | 2.9E11 ↑    | ND-D ↑    | 0.2 ↓        |
| Hlxb9  | Motor neuron and pancreas homeobox 1          | 8.8E10 ↑    | 1.3E9 ↑   | 1.1E5 ↑      |
| Wt1    | Wilms tumor 1                                 | 2.5E10 ↑    | 0.001 ↓  | 0.022 ↓      |
| Fli1   | Fms-related tyrosine kinase 1                 | 1.2E10 ↑    | 143.4 ↑   | 1.6E8 ↑      |
| Eras   | ES cell expressed Ras                         | 4.7E7 ↑     | 4.6E14 ↑  | 3.2E11 ↑     |
| T      | T, brachyury homolog                          | 1.2E7 ↑     | 5.3E14 ↑  | 8.0E13 ↑     |
| Fn1    | Fibronectin 1                                 | 1.7E8 ↑     | 159.3 ↓   | D-ND ↓       |
| Pax4   | Paired box 4                                  | 1.3E6 ↑     | 3.7 ↑     | 0.005 ↓      |
| Sycp3  | Synaptonemal complex protein 3                | 6.1E5 ↑     | 2.1E8 ↑   | ND-ND ↓      |
| Th     | Tyrosine hydroxylase                          | 8.7E5 ↑     | 0.3 ↓     | 0.003 ↓      |
| Eomes  | Eomesodermin                                  | 3.9E4 ↑     | ND-ND     | 1.1E7 ↑      |
| Actc1  | Actin, alpha, cardiac muscle 1                | 12335 ↑     | 14.6 ↑    | 55.8 ↑       |
| Tat    | Tyrosine aminotransferase                     | 58.9 ↑      | 2052 ↑    | 4.6E9 ↑      |
| Iapp   | Islet amyloid polypeptide                     | 47.2 ↑      | 0.5 ↓     | 0.001 ↓      |
| Serpina1| Serpin peptide inhibitor, clade A member 1    | 33.2 ↑      | 0.579 ↓   | 6.248 ↑      |
| Olig2  | Oligodendrocyte transcription factor 2        | 20.1 ↑      | 6.3E11 ↑  | 8.2E9 ↑      |
| Nppa   | Natriuretic peptide A                         | 19.7 ↑      | 0.005 ↓   | ND-ND ↓      |
| Ins2   | Insulin                                       | 15.9 ↑      | D-ND ↓    | D-ND ↓       |
| Ipf1   | Pancreatic and duodenal homeobox 1            | 0.1 ↓       | 1.1E4 ↑   | 4.8E8 ↑      |
| Gapa   | GATA binding protein 6                        | 0.051 ↓     | 0.008 ↓   | D-ND ↓       |
| Runx2  | Runx-related transcription factor 2           | D-ND ↓      | 1.3E15 ↑  | 7.5E4 ↑      |
| Cdh5   | Cadherin 5, type 2                            | D-ND ↓      | 1.4E9 ↑   | 6.3E14 ↑     |
| Sox17  | SRY-box 17                                    | D-ND ↓      | 5182 ↓    | ND-ND ↓      |
| Myod1  | Myogenic differentiation 1                   | D-ND ↓      | 28.58 ↓   | D-ND ↓       |
| Ddx4   | DEAD (Asp-Glu-Ala-Asp) box polypeptide 4      | D-ND ↓      | ND-D ↑    | ND-ND ↓      |
| Isl1   | ISL LIM homeobox 1                            | D-ND ↓      | 0.043 ↓   | D-ND ↓       |
| Afp    | Alphaefoto protein                            | D-ND ↓      | 202.5 ↑   | 94 ↑         |
| Hb2    | Hemoglobin, zeta                              | D-ND ↓      | D-ND ↓    | 3.2E5 ↑      |
| Myf5   | Myogenic factor 5                             | ND-ND ↓     | 7.27E12 ↑ | 3.86E12 ↑    |
| Foxa2  | Forkhead box A2                               | ND-ND ↓     | D-ND ↓    | D-ND ↓       |
| Gcm1   | Glial cells missing homolog 1                 | ND-ND ↓     | ND-ND ↓   | ND-ND ↓      |
| Gata4  | GATA binding protein 4                        | ND-ND ↓     | ND-ND ↓   | ND-ND ↓      |
| Lamc 1 | Laminin, gamma 1                              | ND-ND ↓     | ND-ND ↓   | ND-D ↑       |
| Syp    | Synaptophysin                                 | ND-ND ↓     | ND-ND ↓   | ND-D ↑       |
| Cdx2   | Caudal type homeobox 2                        | ND-ND ↓     | ND-D ↑    | ND-ND ↓      |
Stemness factors in NSCs suggest the inhibition of self-renewal and stemness by PPARγ agonists. We have also observed the upregulation of many stemness genes by PPARγ agonists in NSCs. Among them Noggin, a stemness gene implicated in neurogenesis and the formation of anterior neural patterning [51], was elevated by PPARγ agonists and ATRA. PPARγ agonists and ATRA also increased the expression of cellular retinoic acid binding protein (Crabp2), known to mediate retinoic acid induced motor neuron differentiation [52]. Moreover, PPARγ agonists and ATRA increased the expression of Galanin, a protein essential for the development and survival of a subset of dorsal root ganglia cells [53] and basal forebrain cholinergic neurons [54]. This is consistent with earlier studies showing the upregulation of Galanin mRNA and protein levels after sciatic [55], facial [56] or vagal [57] nerve injury, suggesting its involvement in nerve repair. Our findings suggest that the down-regulation of a subset of stemness genes is sufficient to inhibit growth and self-renewal of NSCs.

To further determine the mechanism in the promotion of neuro-glial differentiation of NSCs by PPARγ agonists, we analyzed the expression of 50 differentiation genes. Oligodendrocyte differentiation factor 2 (Olig2) is one of the many differentiation factors elevated following treatment with PPARγ agonists that is critical in maintaining oligodendrocyte phenotype [58]. Therefore, elevated Olig2 and O4 expression as demonstrated in this study could be a mechanism by which PPARγ agonists promote oligodendrocyte differentiation of NSCs. We have also found that PPARγ agonists increase the expression of glial fibrillary acidic protein (GFAP) in NSCs, indicating astrocyte differentiation in culture. PPARγ agonists and ATRA also induced the expression of Pecam1 in NSCs. Earlier studies have shown that PPARγ ligands induce gastro-protective and ulcer healing properties by increasing the expression of Pecam-1 [59]. Pecam-1 expressed in NSCs residing in specialized niches closely associated with blood vessels in adult brain [60,61] may mediate cross-talk with endothelial cells (ECs) to regulate neurogenesis and angiogenesis [62]. PPARγ agonists and ATRA also induced the expression of Neurogenic differentiation factor (Neurod1), a member of the basic helix-loop-helix (bHLH) transcription factor that plays a role in the development of nervous and endocrine systems [63]. Neurod1-null mice exhibit behavioral abnormalities due to a reduction in sensory neurons and Neurod1 regulates insulin gene expression by binding to a critical E-box motif on insulin promoter [64]. Pancreatic specific transcription factor 1a (Ptf1a), involved in the maturation of pancreatic β cells, regulates insulin production and glucose homeostasis [65,66] is also induced by ciglitazone and ATRA in NSCs. Alphafeto protein (Afp) expression was enhanced in NSCs following treatment with 15d-PGJ2 or ATRA. Afp is expressed in early embryos, hematopoietic progenitor cells and in adult brain [67] and PPARγ agonists upregulate Afp expression and differentiation of hepatic oval cells [68]. Thus the upregulation of selective differentiation factors could be a mechanism by which PPARγ agonists promote neuro-glial differentiation of NSCs. Other studies have demonstrated that in vitro differentiation and maturation of oligodendrocytes depends on many factors, including T3 function, ECM interactions and modulation of signaling pathways [69,70]. We have also found an increase in the expression of Olig2 and other genes relevant to oligodendrocyte differentiation of NSCs following exposure to PPARγ agonists. Our future studies will further explore the role of specific stemness and differentiation genes altered by PPARγ agonists in promoting neuro-glial differentiation of NSCs. Our findings suggest that PPARγ agonists could prove beneficial in the treatment of neurodegenerative diseases.

Earlier studies have reported conflicting results on the influence of PPARγ agonists on NSCs. Wada et al., reported elevated expression of PPARγ in NSCs and PPARγ agonists induce proliferation and inhibited neuronal differentiation by activating EGFR/ERK pathway, which are attenuated in PPARγ−/− and PPARγ-silenced NSCs in culture [40]. Morales-Garcia et al., showed that PPARγ agonists increase the number of proliferating NSCs in the subventricular zone and rostral migration in adult rats and neurosphere formation and differentiation of NSCs that are blocked by PPARγ antagonists in culture [41]. On the other hand, Katura et al., reported a novel biphasic effect of 15d-PGJ2 on EGFR-induced proliferation of NSCs with an increase at lower doses (≤0.3 μM) and suppression at higher doses (0.5–10 μM) in culture [42]. In this study we demonstrated that PPARγ agonists inhibit EGF+bFGF-induced proliferation of NSCs at 1 to 25 μM doses in culture. We have also found that PPARγ agonists promote neuro-glial differentiation by modulating stemness and differentiation genes in NSCs. We believe that the discrepancy between earlier reports and our results on the effect of PPARγ agonists on NSCs could be due to difference in culture conditions used. In particular, we performed all our proliferation and differentiation assays by culturing NSCs in NBM+B27 with EGF+bFGF in the absence or presence of PPARγ agonists, while in other studies NSCs were cultured with PPARγ agonists in the absence of EGF+bFGF [40]. Earlier studies have used serum containing medium in the absence of EGF and bFGF that promote spontaneous differentiation of NSCs into different types of neuro-glial cells in culture. However, our objective was to promote selective lineage specific differentiation of NSCs. We have shown earlier that PPARγ agonists regulate leukemia inhibitory factor (LIF) induced growth and self-renewal of mouse embryonic stem cells by modulating Jak-Stat signaling pathway [71,72]. We reasoned that PPARγ agonists would regulate selected signaling pathways induced by EGF+bFGF in stem cell culture condition. We also believe that the discrepancy between earlier reports and our results could be due to difference in the PPARγ agonists and the dose-ranges used. In particular, in earlier studies the growth inducing effect on NSCs was observed only at lower doses of PPARγ agonists, while higher doses caused an anti-proliferative effect, which is consistent with our findings [40]. Thus further studies on

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**Table 2.** Cont.

NSCs were cultured in NBM with EGF+bFGF in the presence of DMSO, ciglitazone, 15d-PGJ2 or ATRA for 3 days. The differentiation gene expression profile was analyzed by qRT-PCR using a 384 gene card array. The fold change was calculated using automatic threshold setting and is based on expression levels in DMSO treated cells as 1 after normalizing to 18S or GAPDH. The genes are arranged in the table from high to low expression in ciglitazone treated cells. ND, not detected and D, detected. Arrows indicate up or down regulated genes. This data is a representative of two independent experiments.

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Figure 6. Regulation of stemness and differentiation genes by PPARγ agonists in NSCs. NSCs were cultured in NBM+B27 with 10 ng/ml EGF+bFGF in the presence of 0 or 1 μM ciglitazone, 15d-PGJ2 or ATRA for 3 days and the stem cell gene expression analyzed by qRT-PCR. (A) Heat map showing the expression levels of stemness and differentiation genes in NSCs treated with agonists compared to control. (B) Box plots showing the Ct values of differentiation (Red) and stemness (Green) genes in NSCs treated with agonists compared to control. (C) Scatter plots showing ΔCT.
the regulation of NSCs by PPARγ agonists would help to determine their use in the treatment of neurodegenerative diseases.

Materials and Methods

Reagents

The murine recombinant epidermal growth factor (EGF) was purchased from PeproTech (Rocky Hill, NJ) and basic fibroblast growth factor (bFGF) was purchased from R&D Systems (Minneapolis, MN). Cigitazone was purchased from CalBiochem (La Jolla, CA), while 15-Deoxy-A12,14-Prostaglandin J2 (15d-PGJ2) came from Sigma Chemicals (St Louis, MO). Anti-β-Actin antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The HRP conjugated secondary Abs, all-trans retinoic acid (ATRA) and other chemicals were purchased from Sigma Chemicals Co. (St Louis, MO). WST-1 reagent (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulfonate) was purchased from Roche (Indianapolis, IN). Primary antibodies specific to glial fibrillary acidic protein (GFAP, anti-goat polyclonal IgG, sc-6170), beta III tubulin (ßIII tubulin, anti-mouse mAb IgG, sc-51670), Nestin (goat polyclonal IgG, sc-21248), myelin basic protein (MBP, anti-goat polyclonal IgG, sc-13912), myelin oligodendrocyte glycoprotein (MOG, anti-mouse mAb IgG, sc-376130), Neural/Glial Antigen 2 (NG2, anti-rabbit polyclonal IgG, sc-01415), and fluorochrome conjugated secondary antibodies (Goat anti-rabbit IgG, sc-2004; Donkey anti-goat IgG, sc-2031) came from Jackson ImmunoResearch Laboratories (West Grove, PA). The mouse monoclonal oligodendrocyte progenitor marker O4 antibody (Clone 81-IgM); GFAP (rabbit polyclonal IgG, 01415) and fluorochrome conjugated secondary antibodies (AMCA conjugated goat anti-rabbit polyclonal IgG, 10214; Texas red conjugated goat-anti-mouse mAb IgG, 10213); FITC conjugated goat anti-mouse IgM, 10211) were purchased from Stem Cell Technologies (Vancouver, Canada). The 384 well TaqMan low density mouse stem cell array and other PCR reagents were obtained from Applied Biosystems (Foster City, CA).

Cell culture

C57BL/6 mice were obtained from Harlan (Indianapolis, IN) and the breeding colonies were maintained in the animal care facility at Methodist Research Institute. All animal protocols used in the experiments were approved by the Institutional Animal Care and Use Committee. Primary mouse NSCs were generated by culturing dissociated brain cells from new born (post natal 0–3 day) C57BL/6 mice in neurobasal medium (NBM) supplemented with B27 in the presence of 10 ng/ml bFGF and EGF. The cells were cultured in 12 well tissue culture plates in 5% CO2 incubator at 37°C with a medium change on every 2–3 days. The neurospheres generated in 7–10 days were photographed under phase contrast microscope (AX70, Olympus Optical, Japan).

Proliferation assay

Proliferation of NSCs was measured by 3H thymidine uptake and WST-1 assays. Briefly, NSCs obtained by dissociating neurospheres using accutase (Invitrogen) were cultured in 96-well tissue culture plates (1x10^4/200 µl/well) in NBM with B27 in the absence or presence of 10 ng/ml EGF, bFGF or EGF+bFGF. 3H thymidine (0.5 µCi/well) was added at 48 h and the cells were harvested after 72 h using a Tomtech harvester 96 (Hamden, CT, USA). The amount of 3H thymidine uptake was counted on Wallac Microbeta liquid scintillation counter (Perkin Elmer, Fremont, CA) as a measure of proliferation. For WST-1 assay, NSCs were cultured in 96-well tissue culture plates (1x10^4/200 µl/well) in NBM with B27 and 10 ng/ml EGF+bFGF in the presence of 0, 1, 2.5, 5, 10, 20 and 25 µM cigitazone, 15d-PGJ2 or ATRA. After 72 h, 10 µl of WST-1 reagent was added to each well and the absorbance determined at 450 nm using a tier-plate reader (Alpha Diagnostics, San Antonio, TX).

Immunocytochemistry

Neurospheres generated by culturing brain cells from newborn mice were transferred to poly-D-lysine coated 8 well chamber slides (BD Biosciences, San Jose, CA) with NBM+B27 and 10 ng/ml of EGF+bFGF. We have added DMSO vehicle in the absence or presence of 1.0 µM cigitazone, 15d-PGJ2 or ATRA in two identical wells. After 3 and 7 days the cells were fixed with 1% paraformaldehyde in PBS for 15 min and stained with a combination of primary and secondary antibodies by indirect immunofluorescence technique. The cells in one set were stained with rabbit polyclonal IgG specific to GFAP followed by AMCA conjugated polyclonal anti-rabbit IgG and mouse monoclonal IgG specific to ßIII-tubulin followed by Texas red conjugated mouse monoclonal IgG (Fig 4A and Fig 5A). Other set of cells were stained with mouse monoclonal IgM (O4) followed by FITC conjugated anti-mouse IgM and DAPI (Fig 4B and Fig 5B). The images were photographed using Leica Leitz DMRB fluorescent microscope (Leica Microsystems, Buffalo Grove, IL, USA) and presented as individual or merged pictures. Quantitative analysis of immunofluorescence was performed using ImageJ software (NIH, http://rsbweb.nih.gov/ij/) and presented as histograms.

Western Blot Analysis

To examine the effect of PPARγ agonists on neuro-glial differentiation, NSCs were cultured in NBM+B27 with 10 ng/ml EGF+bFGF in the presence of 0, 1, and 5 µM cigitazone, 15d-PGJ2 or ATRA at 37°C. The cells were harvested after 72 h and whole cell lysates prepared using lysis buffer (0.2 M Tris-HCl pH 6.8, 0.8% SDS, 4% Glycerol, 0.588 M β-mercaptoethanol, 0.05 M EDTA, 8 µg/ml bromophenol blue) for 5 min. Adult C57BL/6 mouse brain was homogenized in lysis buffer and used as positive control. The total protein samples were resolved on 8% (Nestin and NG-2) or 12% (β-III Tubulin, GFAP, MBP and MOG) SDS-PAGE, transferred to nylon (PVD) membrane (BioRad, Hercules, CA), and the residual binding sites blocked by incubation with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) containing 3% BSA for 1 h. Membranes were incubated with anti-ßIII tubulin, anti-GFAP, anti-NG2, anti-Nestin, anti-MBP, anti-MOG or anti-β-Actin antibody (1:200–500) in TBST containing 1% BSA at 4°C overnight. The blots were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies in TBST (1:2500–5000) for 1 h and developed using enhanced chemiluminescence (ECL) detection system and film (Amersham Life Science, Arlington Heights, IL) according to manufacturer’s instructions. Quantitative analyses of Western blots were performed using FluorChem HD2 software (Alpha Innotech/Quansys Biosciences, West Logan, Utah).
Quantitative reverse transcription polymerase chain reaction
To determine the effect of PPARγ agonists on the expression of stemness and differentiation genes, NSCs were cultured in NBM+B27 with 10 ng/ml EGF+FGF in the presence of 0 or 1 μM ciglitazone, 15d-PGJ2 or ATRA at 37 °C for 3 days. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and equal amount of RNA was then reverse transcribed into cDNA using TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using 384-well TaqMan Low Density Mouse Stem Cell Gene Array Card with 90 primer sets in 7900 HT fast Real time PCR system (Applied Biosystems, Foster City, CA) and the gene expression levels were normalized to 18S or GAPDH and presented as relative fold change (RQ) compared to control. Heat map was constructed using the DataAssist software (Applied Biosystems, Foster City, CA, USA). Box plot, scatter plot, and Venn diagram were generated using GraphPad Prism 5.0 software (GraphPad, La Jolla, CA, USA).

Statistical analysis
The experiments were repeated three or more times and the values are expressed as mean±SD/SEM. The differences between groups were analyzed by one way ANOVA using GraphPad Prism 5.0 software and the values * (p<0.05), ** (p<0.01), and *** (p<0.001) were considered significant.

Author Contributions
Conceived and designed the experiments: SK EP WC SG SA JB. Performed the experiments: SK EP WC SG SA. Analyzed the data: SK EP WC SG SA JB. Contributed reagents/materials/analysis tools: JB. Wrote the paper: SK EP WC SG JB.

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