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A direct cross-talk between interferon-γ and sonic hedgehog signaling that leads to the proliferation of neuronal precursor cells

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ABSTRACT

Interferon-γ (IFN-γ) is a pleiotropic cytokine that is critical for innate and adaptive immunity. Recent evidence suggests a connection between IFN-γ signaling and the sonic hedgehog (Shh) pathway in the developing brain with CNS-targeted expression of IFN-γ transgene in mice. To determine the relationship between these distinct pathways, we have found that IFN-γ induces a rapid *Shh* transcription in cultured primary granular neuron precursor (GNP) cells. The transcriptional induction of *Shh* by IFN-γ is resistant to protein synthesis inhibition. Chromatin immunoprecipitation (ChIP) analysis reveals a direct binding of signal transducer and activator of transcription (STAT) 1 to the Shh promoter. Functional analyses, including dual immunofluorescent labeling with 5-bromodeoxyuridine (BrdU) incorporation indicate that IFN-γ treatment leads to significant GNP proliferation. This mitogenic effect of IFN-γ is blocked by inhibition of Shh signaling. Therefore, Shh is an IFN-γ target gene and is responsible for IFN-γ-induced GNP proliferation. This previously unrecognized cross-talk between IFN-γ and Shh highlights a potential importance of this immune mediator in the pathogenesis of human developmental and psychiatric disorders.

1. Introduction

IFN-γ is involved in many aspects of cell biology (Billiau, 1996). While natural killer cells and T lymphocytes represent the most important sources for IFN-γ production, cells other than those in the immune system including neurons also express IFN-γ (Neumann et al., 1997b). Signaling through the Janus kinase (JAK)/STAT pathway, binding of IFN-γ to its receptor on the cytoplasmic membrane of target cells triggers the activation of the receptor, which results in phosphorylation of its transcription factor STAT1 (pSTAT1). Finally, the nuclear-translocated pSTAT1 homodimer interacts with defined DNA sequences (TTNCNNNAA) called IFN-gamma-activated site (GAS) to modulate the transcription of genes regulated by IFN-γ (Stark et al., 1998). Gene profiling has identified a long list of IFN-regulated gene (IRGs) or IFN-stimulated gene (ISGs) (de Veer et al., 2001). However, the biological functions of most IRGs remain to be characterized. The best well-known function for IFN-γ is its contribution to adaptive immunity including the critical role of clearing viral particles from CNS neurons (Binder and Griffin, 2001).

Discovered originally in *Drosophila*, Shh controls the development of a variety of organ systems, including the brain (Marti and Bovolenta, 2002). The cellular response to the Shh signal depends on Patched (Ptch) and Smoothened (Smo). Acting as a receptor for the Shh molecule, Ptch is a negative regulator of Shh signaling. Binding of Shh to the Ptch disinhibits the access of Smo to the transcription factors Gli proteins, and subsequently activates the expression of target genes including Ptch and Gli-1. As a morphogen, Shh promotes proliferation and facilitates differentiation of GNP cells, migration, and formation of the internal granular layer (IGL) in cerebellar development (Marti and Bovolenta, 2002). In addition to normal neurodevelopment, Shh signaling pathway is also implicated in the formation of medulloblastoma (MB) (Wechsler-Reya and Scott, 2001). A direct demonstration of this causal link between the Shh pathway and MB is revealed in animal studies including induction of MB by increases of Shh pathway activity and inhibition of tumor growth by cyclopamine (a Shh pathway inhibitor), respectively (Berman et al., 2002).

In addition to its beneficial effects in host defense, IFN-γ has also been demonstrated in association with developmental psychiatric disorders in humans such as schizophrenia (Kim et al., 2004) and autism (Sweeten et al., 2004). However, the molecular mechanisms for IFN-γ in pathogenesis of these neurodevelopmental disorders are unknown. Because of the complexity of the CNS, the neurobiology of IFN-γ in the brain remains poorly understood (Stoll et al., 2000). Nevertheless, association of IFN-γ with neurode-

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velopment was found while exploring the cellular and molecular basis of neurodegeneration and neurogenesis in the brain with chronic production of interferons (Corbin et al., 1996; LaFerla et al., 2000; Wang et al., 2003). A direct connection between IFN-γ and cerebellar development is further supported by conditional expression of IFN-γ transgene in the mouse brain (Lin et al., 2004; Wang et al., 2004). With increased expression and activation of Shh in the cerebellum, histological examination of the brains in these transgenic mice revealed a cerebellar dysplasia with profound proliferation of the granular neuron precursor (GNP) cells and persistence of the external granular layer (EGL), which strongly suggests a relationship between IFN-γ expression and Shh signaling.

To determine the relationship between IFN-γ and the Shh pathway and its functional consequences, GNP cells prepared from the cerebella of neonatal mice were treated with IFN-γ. We found that IFN-γ directly activates the transcription of the Shh gene and increases activity of the Shh pathway. Resultant induction of Shh mediates the mitogenic effect of IFN-γ on these GNP cells. Chromatin immunoprecipitation (ChIP) assay reveals STAT1 recruitment to the Shh promoter upon IFN-γ stimulation, thus demonstrating Shh as a novel IFN-γ-regulated gene.

2. Materials and methods

2.1. Animals

C57BL/6J mice from the Jackson Laboratory were bred and maintained in the animal facility at University of Missouri-Kansas City (UMKC). All animal handling and procedures were approved by the institutional animal care and use committee at UMKC.

2.2. Granule neuron culture

Cerebellar GNP cells were isolated and cultured as described previously (Kenney and Rowitch, 2000) except no serum was used in the culture. Briefly, cerebella from postnatal day 5–6 mice were dissected. The meninges were stripped, and pooled cerebella were digested in 0.25% trypsin (Invitrogen) and dissociated in Hanks buffered saline solution (HBSS) with 0.09% trypsin inhibitor (Invitrogen) and 0.006% DNase I (Sigma) by trituration. After washing with HBSS, dispersed cells were recovered in Neurobasal A medium supplemented with 2% B27, 0.5 mM L-glutamine and 0.01% Penicillin/streptomycin (all from Invitrogen). The final cell suspension was passed through a 70 μm nylon cell strainer (BD Biosciences), aliquot counted following trypan blue staining, and then seeded on poly-D-lysine (Sigma) coated culture dishes, plates or chamber slides at the desired density (see below for details). Cycloheximide (CHX) was obtained from Sigma. Immunocytochemical characterization showed that the cultures contained around 90% granule neuron precursor cells (Math1-positive), similar to a previous report (Kenney and Rowitch, 2000).

2.3. MTT cell proliferation assay

According to previous studies for non-radioactive colorimetric assay of granule cell proliferation (Keller et al., 2004), prepared GNP cells were seeded at the density of 2.5×10^5 cells/well in 24-well microplates. IFN-γ (specific activity: 1.02×10^7 IU/mg protein; PBL biomedical laboratories) or Shh (R&D Systems Inc.) was added to the culture following overnight incubation after the plating and incubated at 37 °C in 5% CO_2 for the desired time. Four hours before the end of culture, MTT substrate thiazolyl blue tetrazolium bromide (Sigma–Aldrich) was added to the culture. After color development, the MTT formazan was dissolved in acidic isopropanol and measured spectrophotometrically at 570 nm. For Shh signal blocking studies, cultured cells were treated with cyclopamine (Toronto Research Chemicals), anti-Shh antibody 5E1 or IgG1 isotype control anti-Yan antibody (both from Developmental Studies Hybridoma Bank, University of Iowa) 30 min before the addition of IFN-γ. For each condition, all assays were carried out in triplicate.

2.4. BrdU proliferation labeling and quantification

BrdU incorporation for cell proliferation assay was detected by an immunocytochemical staining kit (Roche Diagnostics) according to the protocol provided by the manufacturer. Forty-six hours after IFN-γ treatment of the culture on 2-well chamber slides (1.2×10^6 cells/well), cells were pulsed with BrdU (10 μM) for 2 h. After fixation, cells were treated with anti-BrdU. Bound anti-BrdU antibody was detected using Vectastain ABC Kit (Vector Laboratories) followed by hematoxylin staining. The slides were visualized with microscopy (Nikon eclipse E800). For quantification of the proliferation, four fields per condition were randomly selected on individual slides under a 20 × objective for image capture. Two hundred cells in each field were counted and the percentages of BrdU-labeled cells among hematoxylin-counterstained cells were calculated as a measure of cell proliferation. All staining and counting were performed in triplicate.

For dual BrdU/Math1 immunofluorescent labeling, bound anti-BrdU and anti-Math1 (Chemicon) were detected by different fluorescent-labeled secondary antibodies (Molecular Probes). The slides were examined and the images were acquired using a Zeiss Axio Imager MOT Z1 fluorescent microscope.

2.5. Protein preparation and Western blotting

GNP cells were plated on poly-D-lysine-coated 60 mm dishes at the density of 3.0×10^6 cells. After initial overnight incubation, cells were treated with IFN-γ or vehicle for 48 h. Preparation of total protein lysates and Western blot were carried out as previously described (Wang et al., 2003). Anti-PCNA was purchased from Santa Cruz Biotechnology.

2.6. RNA extraction and RNase protection assay

Total RNA was isolated from GNP cultures using the TRIZOL reagent (Invitrogen). RNase protection assays (RPAs) were performed and RNA levels were quantified from autoradiographs by densitometry using NIH Image software (version 1.62) as described previously (Wang et al., 2003). RPA probes for Shh, Gli-1, Ptch1 and IFN-γ regulated genes were kindly provided by Dr. Iain L. Campbell at Scripps Research Institute as previously described (Wang et al., 2003).

2.7. Chromatin immunoprecipitation assay (ChIP)

ChIP was performed according to published methods (Bowie et al., 2004; Nelson et al., 2006). In brief, cultured GNP cells (~10 million) were harvested for chromatin preparation 40 min after IFN-γ treatment. To precleared chromatin, 2-μg anti-STAT1 or control IgG (both from Santa Cruz) was used. DNA was eluted from protein A-Sepharose beads. After de-crosslinking and Proteinase K digestion, DNA fragments were cleaned up with the Qiaquick PCR purification kit (Qiagen Inc.). Primers designed for the detection of potential STAT1 binding sites in mouse *Shh* promoter (SHHP) region include: SHHP1, forward 5′-TGCGGGGTATTTTTCCAATA-3′, and reverse 5′-GGCTGCTGTGGC TTTATTTT-3′; SHHP2, forward 5′-AGAGTGTTGGACACCCCAAG-3′, and reverse 5′-ATGTGGAATTCAGGCTCCTT-3′ and SHHP3, forward 5′-CGCCCATCCTAGAGGAATCT-3′, and reverse 5′-CTCTTGGGGTGTC

CAACACT-3′. Immunoprecipitated or diluted input DNA was used for PCR reaction in a thermocycler (Eppendorf). The conditions were: initial denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 40 s, 56 °C for 40 s, and 72 °C for 30 s, and a final extension at 72 °C for 7 min. PCR products were resolved on 2% agarose.

2.8. Statistics

Data were expressed as mean ± SEM (standard error of the mean). Two-group data were analyzed using unpaired Student's *t*-test. One-way analysis of variance (ANOVA) was employed for multiple analyses among groups with Dunnett's *t*-test as the post hoc test. Significance is set at a p value $\leqslant 0.05$ using two-tailed tests.

3. Results

3.1. IFN-γ activates Shh and Gli-1 expression in GNP cells

To assess a possible link between IFN-γ and the Shh signaling pathway suggested from recent studies *in vivo* (Wang et al., 2003, 2004), we examined the activity of IFN-γ in regulating the genes in Shh signaling components in primary cultured cerebellar GNP cells. Both dose response and time course studies for IFN-γ treatment were performed, and total RNAs was extracted and analyzed for the expression of Shh pathway genes. Without IFN-γ treatment, no detectable *Shh* expression was found by RNase protection assay (Fig. 1). In the presence of IFN-γ, GNP cells exhibited a dramatic increase in transcription of the *Shh* in a dose dependent manner from 10 to 1000 IU/ml (Fig. 1A). While stimulation of *Shh* transcription peaked at a concentration of IFN-γ between 250 and 500 IU/ml, a significant increase in *Shh* expression was detected at IFN-γ concentration as low as 20 IU/ml, indicating that Shh gene expression is sensitive to even a small dose of IFN-γ.

To characterize temporal profiles for the genes that responded to IFN-γ, mRNA levels of three major genes in the Shh pathway, Shh, Gli-1 and Ptch1, were tracked at 2-, 4-, 8- and 24-h following treatment of IFN-γ. As shown in Fig. 1B and C, *Shh* transcription was activated as early as 2-h after addition of IFN-γ into the culture (100 IU/ml), and the expression continued to increase until it reached a maximum at 24-h after treatment. Meanwhile, *Gli-1*, a downstream target gene in Shh signaling, was also remarkably upregulated at 24-h only but not at an earlier time. Increased transcription was also detected for another Shh target *Ptch1* following IFN-γ treatment. Whereas the expression of Ptch1 mRNA was readily detectable in the absence of IFN-γ, IFN-γ challenge led to a significant enhancement on *Ptch1* expression at 24-h after treatment. In summary, our results indicate that IFN-γ can directly trigger Shh expression and subsequently regulates the expression of Shh target genes in neuronal precursor cells.

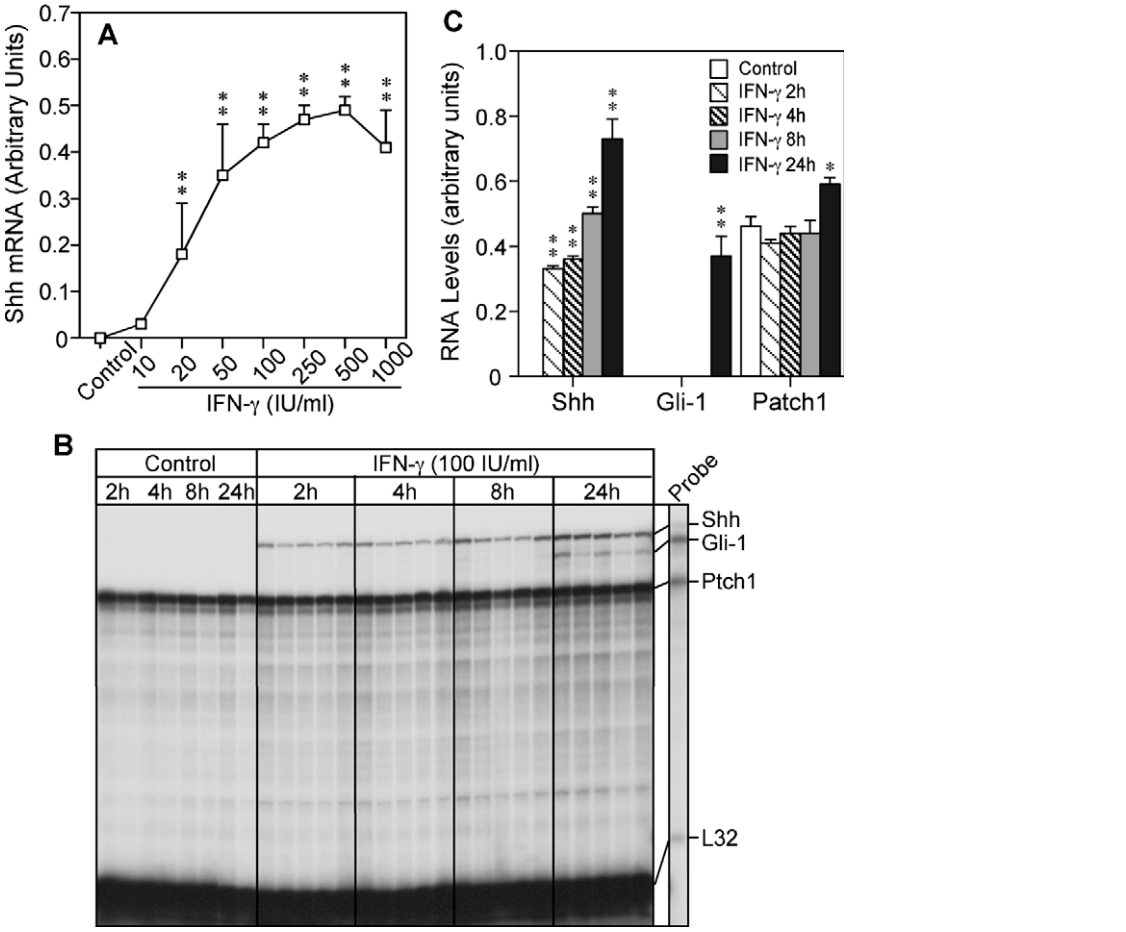

Fig. 1. Transcriptional activation of the Shh pathway genes by IFN-γ. (A) GNP cells were treated with different concentrations of IFN-γ for 4 h before total RNA extraction. RPA analysis for Shh gene expression was performed, and autoradiographs quantified by densitometry ($n = 4$–6). (B and C) GNP cultures were treated with IFN-γ (100 IU/ml) or PBS, and collected at different times following treatment for RNA extraction. For the analysis of Shh, Gli-1 and Ptch1 RNA expression, 5.0 μg of total RNA was analyzed by RPA. (B) A representation of a scanned RPA film; (C) Quantified RPA image from (B) ($n = 5$–8). The densitometric values for each transcript were expressed as a ratio to the L32 RNA. ∗∗: $p < 0.01$ and ∗: $p < 0.05$ (one-way ANOVA followed by Dunnett's *t*-test).

Furthermore, Western blot revealed that STAT1 was constitutively expressed in the GNP cells and was substantially phosphorylated following IFN-γ stimulation (Fig. 2A). Activated IFN-γ signaling was evidenced also by the overall upregulation of several prototypic IFN-γ-regulated genes: CTIIA (MHC class II trans-activator gene), TGTP (T-lymphocyte GTPase), OAS (2′,5′-oligoadenylated synthase), IRF1 (IFN-regulatory factor 1) and IRF2, but not IDO (indoleamine 2,3-dioxygenase) in IFN-γ-treated GNP culture (Fig. 2B). Overall, both RNA and protein analyses showed an IFN-γ-triggered JAK/STAT activation in neuronal precursor cells.

3.2. IFN-γ promotes GNP proliferation

Shh determines neuronal proliferation and patterning in cerebellum development (Marti and Bovolenta, 2002), and is a mitogen in cultured GNP cells as well (Wechsler-Reya and Scott, 1999). Since IFN-γ stimulated *Shh* expression and activated the pathway (Fig. 1), we next asked whether IFN-γ could exert any impact on GNP proliferation *in vitro*. A MTT assay of cell proliferation showed a gradual increase in GNP cell numbers as IFN-γ concentration increased (Fig. 3A). To ensure a direct comparison of our findings to others on induced GNP proliferation *in vitro* (Dahmane et al., 2001; Kenney and Rowitch, 2000), the optical density values were transformed into percentage change over the control. As indicated in Fig. 3B, the maximal stimulation on the GNP proliferation by IFN-γ was attained at and above 250 IU/ml that is about 55% increase compared with control. Such stimulatory activity of IFN-γ on GNP proliferation was similar to those of direct Shh challenge, which was included as a positive control for GNP proliferation (Fig. 3B), demonstrating a potent neurotrophic effect of IFN-γ for GNP *in vitro*. To assess the kinetics of the proliferative effects exerted by IFN-γ on GNP cells, we next explored 3 time points in a time-course study. As shown in Fig. 3C, IFN-γ (100 IU/ml) induced only a slight increase in GNP cells at 24-h (5.4%, $p = 0.4$), while the proliferation was remarkably enhanced at 48-h (23.6%, $p = 0.006$) and reached a peak at 72-h (40.3%, $p = 0.001$), respectively. In addition, we also measured the levels of proliferating cell nuclear antigen (PCNA), a key protein involved in DNA polymerase function in cell proliferation (Lee and Hurwitz, 1990), in IFN-γ stimulated GNP cells at 48-h after treatment. Although a low PCNA expression was detected in control by Western blot, the PCNA level was significantly enhanced after IFN-γ treatment (Fig. 3D), which supports the proliferative action of IFN-γ on GNP cells.

To verify IFN-γ-induced GNP proliferation observed in the MTT assay, a BrdU (5-bromodeoxyuridine) incorporation assay was performed to display the cells in S phase by immunocytochemical staining. The percentages of BrdU-labeled cells (arrow heads) among hematoxylin-counterstained cells (to label cell nuclei) were calculated as a quantitative measure of cell proliferation (Fig. 4). As illustrated in Fig. 4 (left panel), there were only 1–2% BrdU-labeled cells in control. Nevertheless, IFN-γ treatment resulted in a 12.5-fold increase in BrdU-positive cells that account for 19–24% of the total cell population ($p < 0.001$), similar to the induction upon direct Shh stimulation described previously by others (Dahmane et al., 2001; Kenney and Rowitch, 2000). Importantly, dual immunofluorescent staining revealed that labeling of the BrdU-positive cells (red) was exclusively colocalized with the staining of the GNP marker Math1 (green) (Ben-Arie et al., 1997; Kenney and Rowitch, 2000) (Fig. 4 right panel, arrow heads).

Together, the results showed that IFN-γ stimulates GNP proliferation. Notably, the effect of different IFN-γ concentrations on GNP proliferation was remarkably in agreement with that on Shh

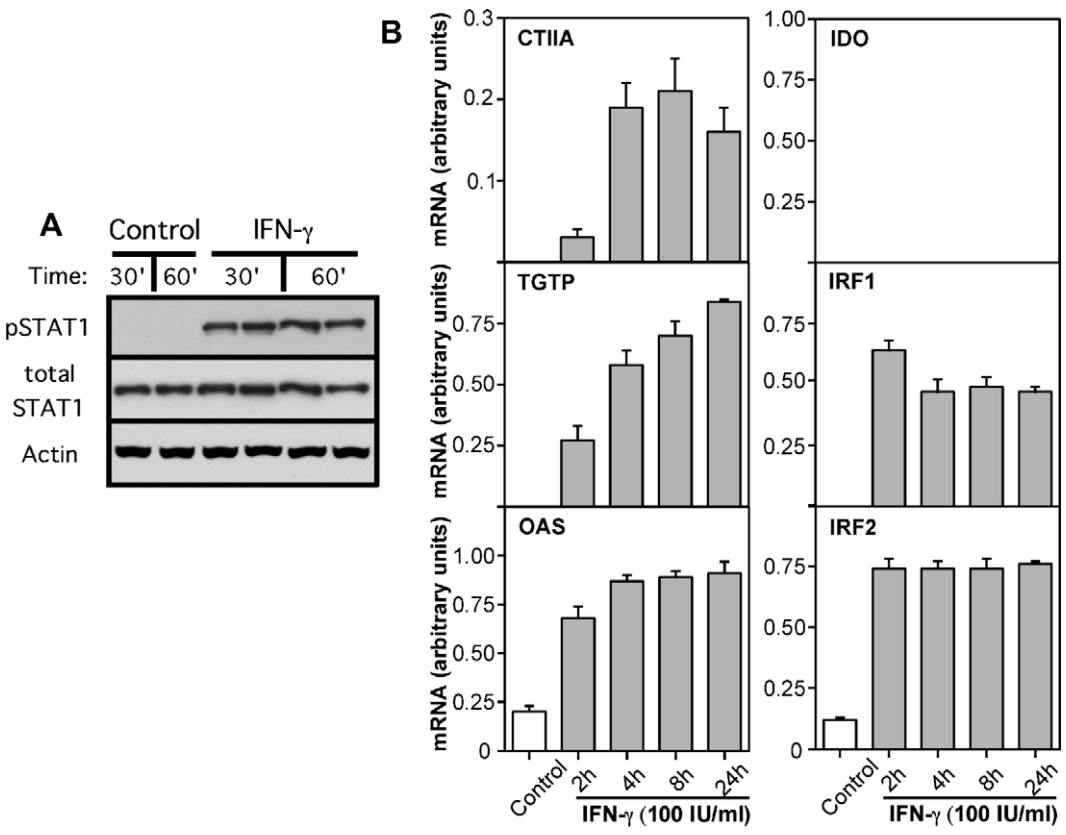

Fig. 2. Activation of STAT1 phosphorylation and IFN-γ-regulated gene expression in the GNP cells by IFN-γ. GNP cultures were incubated in the presence of IFN-γ (100 IU/ml) and extracted for protein lysate and total RNA preparation at different time points following treatment. (A) The GNP cultures were treated with IFN-γ and analyzed by Western blot; (B) Temporal expression of IFN-γ-regulated genes in the GNP cells after IFN-γ treatment ($n = 5–8$). For the analysis of IFN-γ-regulated gene expression, 5.0 μg of total RNA per sample was analyzed by RPA.

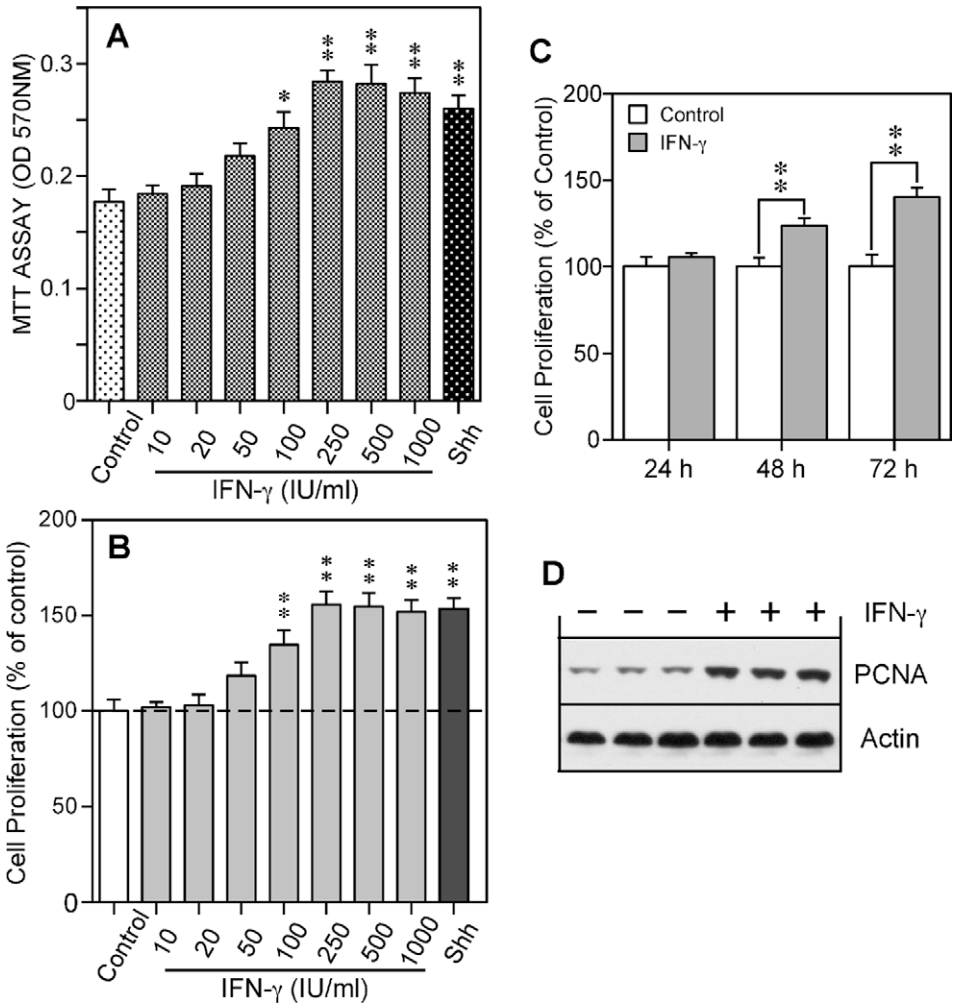

Fig. 3. Stimulation of granular precursor proliferation by IFN-γ. The primary GNP cells were treated with IFN-γ or Shh and the proliferative effect was analyzed by MTT assay (A–C) and PCNA protein expression (D), respectively (n = 3–4; ∗: $p < 0.05$; ∗∗: $p < 0.01$). For MTT measurement, a dose–response curve of IFN-γ-stimulated GNP proliferation expressed by actual measurement at OD570 nm (A) and by percentages over the control converted from the values at OD570 nm (B) was depicted respectively. (C) Represents time-course study of IFN-γ treatment (100 IU/ml) on GNP proliferation by MTT assay. (D) The GNP cells were treated with IFN-γ (100 IU/ml) for 48 h before protein preparation and total protein lysates (12 μg per lane) were blotted with anti-PCNA antibody.

gene expression (Fig. 1), thereby raising the possibility that Shh signaling might act as an intermediate candidate leading to GNP proliferation in the presence of IFN-γ.

3.3. Blockade of Shh signaling significantly attenuates IFN-γ-induced GNP proliferation

Given the action of IFN-γ on Shh transcription, and especially a direct stimulatory activity of IFN-γ on GNP proliferation that is the same as exhibited by Shh, we then investigated the role of Shh and its signaling in IFN-γ-induced GNP proliferation. In these experiments, GNP cells were treated with a small molecule Shh pathway inhibitor cyclopamine (CPM) (Berman et al., 2002) and the antibody against Shh (5E1) (Wechsler-Reya and Scott, 1999) prior to addition of IFN-γ, respectively. As expected, CPM significantly inhibited GNP proliferation stimulated by IFN-γ dose-dependently. At concentrations of 1, 3 and 6 μM, CPM decreased IFN-γ-triggered GNP proliferation by 40%, 45% and 80%, respectively (Fig. 5A). The inhibitory effect on GNP proliferation was also observed by anti-Shh, but not control IgG. A widely used Shh-neutralizing antibody 5E1 at 0.5 and 1.0 μg/ml significantly suppressed the IFN-γ-induced GNP proliferation by 60 and close to 70%, respectively, compared with IFN-γ treatment control (Fig. 5B). Thus, the results indicate that Shh and Shh signaling pathway is largely responsible for IFN-γ-induced GNP proliferation.

3.4. Shh is a target gene of IFN-γ that is directly modulated by STAT1

The mRNA profiling study above indicated coactivation of the IFN-γ and Shh signaling pathways in IFN-γ-treated GNP cells. To determine whether the rapid upregulation of *Shh* is a direct response to IFN-γ or not, the protein synthesis inhibitor cycloheximide (CHX) was employed. Both CHX (10 μg/ml) and IFN-γ (100 IU/ml) were added simultaneously to GNP culture and RNA was prepared at 4 h following treatment. Interestingly, blocking *de novo* protein synthesis did not attenuate the IFN-γ-activated *Shh* transcription (Fig. 6A). In contrast, addition of CHX enhanced the stimulatory activity of IFN-γ on Shh transcription. Thus, the fast upregulation of the Shh gene following IFN-γ treatment does not involve any synthesis of new proteins, implying that Shh is a target gene of direct IFN-γ signaling.

As the transcription factor for IFN-γ signaling, activated STAT1 (pSTAT1) recognizes the GAS element and then regulates the transcription of its target genes (Stark et al., 1998). To assess a possible binding of IFN-γ-activated STAT1 to the Shh gene, four potential STAT1 binding sites (TTNCNNNAA) were identified in the promoter

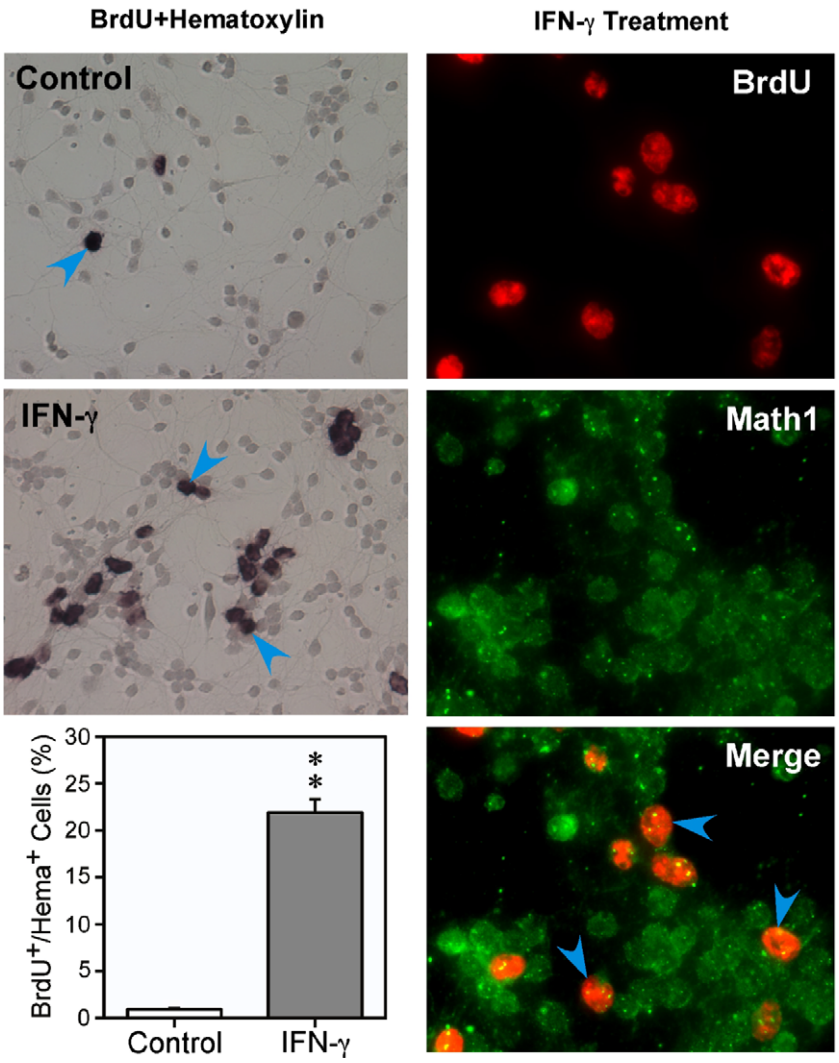

Fig. 4. IFN-γ-induced GNP proliferation detected by BrdU incorporation. The primary GNP cultures were treated with IFN-γ (200 IU/ml) or vehicle for 48 h including pulsing with BrdU for 2 h before fixation. Left Panel: Immunocytochemistry was performed using anti-BrdU (arrow heads) for the cells followed by Hematoxylin counterstaining. Percentages of the BrdU-positive ($BrdU^+$) cells in hematoxylin-positive ($Hema^+$) population were calculated respectively ($n = 3$). $**$: $p < 0.01$ (Student's t-test). Right Panel: A dual immunofluorescent staining was performed with anti-BrdU (red) and anti-Math1 (green). Dual-labeled cells were designated by arrowheads. Original magnifications: x200 (peroxidase staining) and x600 (immunofluorescence). (For interpretation of color mentioned in this figure, the reader is referred to the web version of this article.)

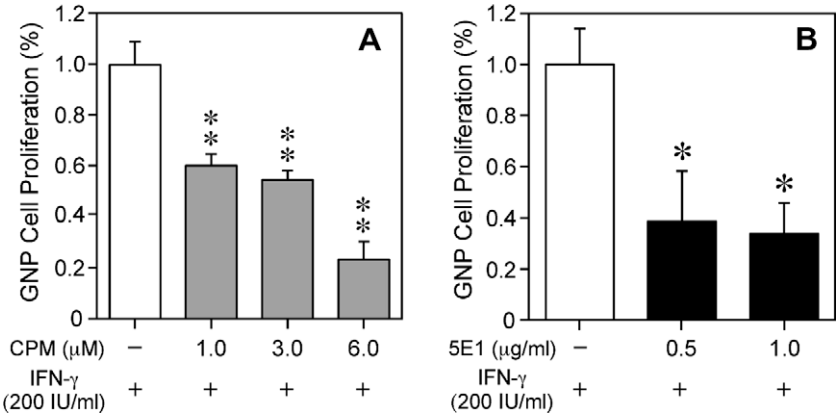

Fig. 5. Attenuation of IFN-γ-induced GNP proliferation by inhibition of Shh or Shh signaling pathway. The primary GNP cultures were treated with cyclopamine (CPM) (A) or anti-Shh antibodies (B) at the indicated concentrations before the addition of IFN-γ (200 IU/ml), and MTT proliferation assay was performed after 72-h treatment. ($n = 3$–4; $*$: $p < 0.05$; $**$: $p < 0.01$ (one-way ANOVA followed by Dunnett's t-test)).

region of mouse *Shh* (www.genome.ucsc.edu), and their locations and sequences are shown in detail in Fig. 6B. To determine the site(s) for direct protein-DNA interactions, GNP cells were harvested for chromatin preparation after IFN-γ or vehicle treatment

and ChIP assay with anti-STAT1 antibody was performed. Specific primers were designed to amplify the immunoprecipitated STAT1 binding regions including site 1 (SHHP1), 2 (SHHP2), and 3 and 4 (SHHP3), respectively. PCR product analysis revealed a significant abundance of amplicons encompassing suspected GAS1, 2, 3 and 4 elements in chromatin from IFN-γ-stimulated, but not control cells (Fig. 6C), indicating enriched occupancy of STAT1 in GAS containing regions of Shh gene upon IFN-γ treatment. Taken together, our results on CHX and ChIP analysis strongly indicate a direct event of IFN-γ-induced Shh transcription in GNP cells through a direct binding of activated STAT1 with *Shh* promoter, thus adding *Shh* as a novel member to the catalog of IFN-γ-stimulated genes.

4. Discussion

Beyond host defense against infections, immune molecules such as cytokines and MHC molecules have recently been identified to affect the development and plasticity of neuronal connections (Boulanger and Shatz, 2004) and adapted immunity to be implicated in cognition (Kipnis et al., 2004). However, the neurobiology of IFN-γ is unclear and conflicting results on the CNS impact of IFN-γ have been reported because of its proinflammatory properties (Billiau, 1996) and diverse effects on different brain parenchymal cells (Jonakait et al., 1994; Popko and Baerwald, 1999; Turnley et al., 2001; Yong et al., 1992). Emerging evidence supports a protective, rather than deleterious effect to the brain by IFN-γ (Willenborg et al., 2007). Given the neurotrophic effect of IFN-γ *in vitro* (Butovsky et al., 2006; Jonakait et al., 1994; Turnley et al., 2001) as well as the association of IFN-γ with neurogenesis and neuroproliferation in *vivo* (Baron et al., 2008; Lin et al., 2004; Wang et al., 2004), demonstration of Shh as a novel GAS-regulated gene reveals a hitherto unrecognized molecular mechanism in neuroimmune interactions. In addition to ChIP assay, direct regulation of Shh transcription by IFN-γ is also supported by studies using the protein synthesis inhibitor cycloheximide. Not surprisingly, cycloheximide increased the effect of IFN-γ on Shh transcription. Such superinduction of *Shh* expression in the presence of cycloheximide and IFN-γ is consistent with previous reports in the expression of many other genes in cultured cells following exposure to other growth factors together with cycloheximide (Kenney et al., 2003).

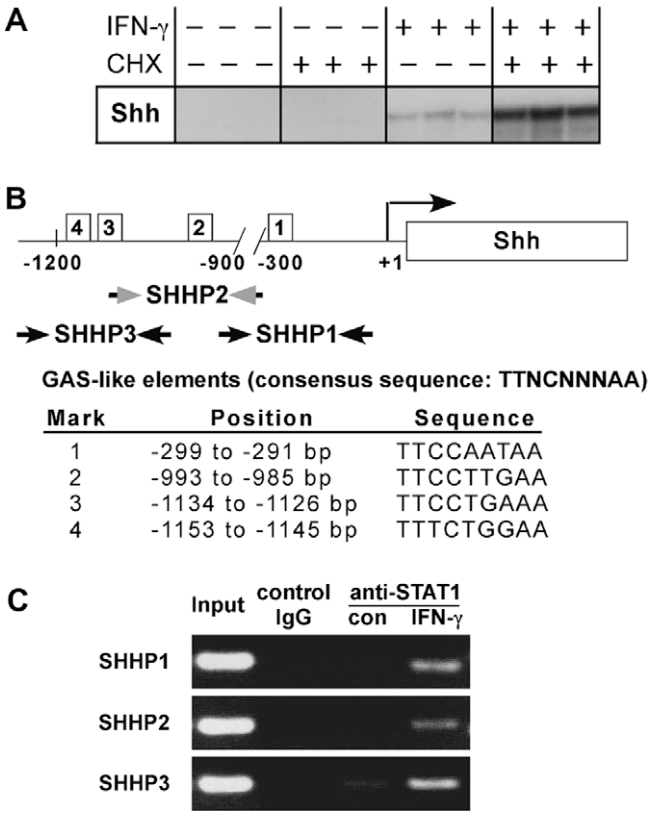

Fig. 6. Direct regulation of Shh gene by IFN-γ. (A) Inhibition of *de novo* protein synthesis shows no attenuation of IFN-γ-stimulated Shh expression. Protein synthesis inhibitor cycloheximide (CHX) (10 μg/ml) and IFN-γ (100 IU/ml) were added simultaneously to GNP culture and RNA was prepared at 4 h after treatment. (B) A schematic of GAS sites in the mouse Shh promoter sequence (not proportional to scale). The boxes 1–4 represent the location of four potential STAT1 binding sites (GAS). SHHP1, SHHP2 and SHHP3 are PCR primers designed to amplify the potential STAT1 binding sites for ChIP assay. (C) Enriched binding of STAT1 to the Shh promoter upon IFN-γ stimulation by ChIP assay. Cultured GNP cells were treated with IFN-γ (200 IU/ml) for 40 min before chromatin preparation.

Responses to IFN-γ are dependent on cell type (i.e., neuronal or non-neuronal) and stage of cellular differentiation. For example, human fetal astrocytes proliferate in response to IFN-γ stimulation *in vitro* (Yong et al., 1992). In contrast, IFN-γ is toxic to cultured oligodendrocytes (Chew et al., 2005). However, compared with the developing oligodendrocyte progenitor cells, mature oligodendrocytes are less susceptible to the cytotoxic action of IFN-γ (Chew et al., 2005; Popko and Baerwald, 1999). Similar to glia cells, neurons are also a very responsive target cell population for interferons. Interestingly, neurons isolated from embryonic (Neumann et al., 1997a), but not adult (Turnley et al., 2002), nervous tissue respond to IFN-γ treatment with a robust upregulation of MHC antigen expression. Present study demonstrates that Shh is an IFN-γ-regulated gene and mediates IFN-γ-triggered proliferation of neuronal precursor cells. As a result of IFN-γ receptor activation, IFN-γ treatment also induces the expression of several prototypic IFN-γ-regulated genes including CTIIA, TGTP, OAS, IRF1 and IRF2, but not IDO in the GNP cells. The unchanged IDO expression by IFN-γ in neuronal cells observed in our study is consistent with previous observations (Kwidzinski et al., 2005), and support a distinct cell-type-specific and cell-type-non-specific expressions of IFN-regulated genes in response to IFN stimulation in different cells (Rani et al., 2007). Such differences are likely responsible for different biological activities of IFN-γ in different cells. Nevertheless, further investigation of various brain parenchymal cells in their response to IFN-γ including potential neuron-glia interactions is necessary in order to define the neurobiological action of IFN-γ in the brain.

The mitogenic effect by IFN-γ through its Shh induction may contribute to the pathogenesis of neurodevelopmental disorders in humans. In this regard, viral infection has long been suspected in the development of psychiatric disorders including schizophrenia, autism and bipolar disorder (Fatemi et al., 2002; Hornig et al., 2001; Yolken and Torrey, 1995). In addition to epidemiological evidence, recent animal studies from different laboratories have demonstrated changes in behaviors, relevant to the behavioral deficits in human schizophrenia and autism, in the adult offspring of pregnant mice treated with either virus directly (Shi et al., 2003) or synthetic double stranded RNA (poly (I:C)) to mimic viral infection (Meyer et al., 2008). Because no virus is detectable in the fetal tissues including the brain after maternal viral injection, an indirect action is suggested (Shi et al., 2005). As a prime innate immune mediator released upon viral infection or poly (I:C) injection, IFN-γ represents a pathogenic factor candidate. Interestingly, a recent study found histological abnormalities in the cerebellum of offspring mice after maternal viral challenge during pregnancy (Shi et al., 2009). The results provide a support for the importance of the cerebellum in the regulation of cognition/emotion (Schutter and van Honk, 2005) and development of psychiatric disorders such as autism and schizophrenia (Andreasen and Pierson, 2008; Fatemi et al., 2008).

Nevertheless, the details of neuropathological and neurochemical disturbances in cerebellum in specific relationship with autism or schizophrenia in humans are still poorly understood. Surprisingly, the histological phenotype especially the persistence and proliferation of the external granular layer identified in the cerebellum of young offspring mice following maternal viral inoculation during pregnancy (Shi et al., 2009), although less severe, is strikingly similar to the one developed in the CNS-targeted IFN-γ transgenic mice (Lin et al., 2004; Wang et al., 2004). More importantly, a high sensitivity of the GNP cells to IFN-γ was demonstrated in the present study with dramatic *Shh* upregulation at concentrations of IFN-γ that are attainable *in vivo* following viral infection (Pien et al., 2000). Taken together, our findings strongly suggest a potential role for IFN-γ in regulating Shh signaling in the brains under conditions in which expression of this cytokine is induced. Furthermore, earlier studies have shown that CNS-targeted expression of IFN-γ (Corbin et al., 1996; LaFerla et al., 2000), but not IFN-α (Akwa et al., 1998), IL-6 (Campbell et al., 1993) or TNF-α (Stalder et al., 1998) transgene in mice leads to a developmental abnormality in brain with increased expression and activity of Shh and hyperplasia of the external granular layer in cerebellum (Lin et al., 2004; Wang et al., 2004). Such observation indicates a specific effect on Shh regulation and neurodevelopment by IFN-γ, but not other cytokines. Therefore, demonstration of a direct cross communication between IFN-γ signaling and Shh may provide the molecular basis that bridges the knowledge gap of the events/processes that occur between the onset of viral infection and the development of neuropathology that may lead to developmental abnormalities and mental illnesses in humans. However, further studies are warranted to determine the role of this immune mediator for its direct contribution in developmental psychiatric disorders in humans.

The physiology of IFN-γ and its signaling for CNS development and brain function is unclear. Despite constitutive expression of IFN-γ receptor and its transcription factor STAT1 in the CNS and brain parenchymal cells (Bentivoglio and Kristensson, 2007; Cattaneo et al., 1999), expression of IFN-γ is undetectable at both RNA or protein levels unless viral infection or other immune stimulation takes place in the CNS (Billiau, 1996). The mice with depletion of IFN-γ, IFN-γ receptor or STAT1 gene are viable and fertile with no overt CNS abnormalities except increased susceptibility to infection (Huang et al., 1993), suggesting that IFN-γ is dispensable for normal brain development. Nonetheless, a newly published study demonstrates that the susceptibility to seizure subsequent to viral infection in mature adult mice requires the presence of IFN-γ during the development stage (Getts et al., 2007). The results imply an importance of IFN-γ for normal brain development and CNS function.

In addition to a critical role in the developing brain, Shh also contributes to the neurogenesis in the adult brain (Ahn and Joyner, 2005). In a commonly used mouse model for human Parkinson's disease, pretreatment with Shh protein before injection of neurotoxin 6-hydroxydopamine resulted in a significant neuroprotective effect due to the enhanced neurogenesis by Shh (Dass et al., 2005). On the other hand, expression of low levels of IFN-γ in the adult brain enhances neurogenesis both in wild-type mice and in a mouse model of Alzheimer's disease (Baron et al., 2008). Given a critical role of Shh for the development of neuronal precursor (Marti and Bovolenta, 2002) and oligodendrocyte progenitor cells (Miller, 2002), future study of the mitogenic action of IFN-γ on different types of neural precursor cells may not only increase our understanding of this cross communication in the brain physiology and pathophysiology, but will also provide the knowledge base for efforts towards stimulation of cell regeneration, in the search for potential therapy for degenerative brain disorders.

In conclusion, we have demonstrated that IFN-γ directly regulates the gene coding for the morphogen Shh, which leads to proliferation of neuronal precursor cells. This novel direct cross-talk between IFN-γ signaling and a signaling pathway that is instrumental in brain development highlights the potential importance of this immune mediator in the pathogenesis of developmental CNS disorders in humans.

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