Interferon-γ suppresses activin A/NF-E2 induction of erythroid gene expression through the NF-κB/c-Jun pathway

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Submitted 3 October 2013; accepted in final form 10 December 2013

Lee WH, Chung MH, Tsai YH, Chang JL, Huang HM. Interferon-γ suppresses activin A/NF-E2 induction of erythroid gene expression through the NF-κB/c-Jun pathway. Am J Physiol Cell Physiol 306:C407–C414, 2014. First published December 11, 2013; doi:10.1152/ajpcell.00312.2013.—Interferon (IFN)-γ is a proinflammatory cytokine that is linked to erythropoiesis inhibition and may contribute to anemia. However, the mechanism of IFN-γ-inhibited erythropoiesis is unknown. Activin A, a member of the transforming growth factor (TGF)-β superfamily, induces the erythropoiesis of hematopoietic progenitor cells. In this study, a luciferase reporter assay showed that IFN-γ suppressed activin A-induced ζ-globin promoter activation in K562 erythroblast cells in a dose-dependent manner. Activin A reversed the suppressive effect of IFN-γ on the luciferase activity of ζ-globin promoter in a dose-dependent manner. IFN-γ also suppressed the activation of activin A-induced α-globin promoter. IFN-γ reduced the mRNA expression of α-globin, ζ-globin, NF-E2p45, and GATA-1 induced by activin A. The results also showed that IFN-γ induced c-Jun expression when NF-κBp65 and c-Jun bound to two AP-1-binding sites on the c-Jun promoter. The luciferase activity of α-globin and ζ-globin promoters were enhanced by wild-type c-Jun and eliminated by dominant-negative (DN) c-Jun. The suppressive effects of IFN-γ on the mRNA expression of α-globin and ζ-globin were absent in cells expressing DN c-Jun. The ability of NF-κB to enhance activin A-induced ζ-globin promoter activation decreased when c-Jun was present, and IFN-γ treatment further enhanced the decreasing effect of c-Jun. Chromatin immunoprecipitation revealed that NF-E2p45 bound to the upstream regulatory element (HS-40) of the α-globin gene cluster in response to activin A, whereas c-Jun eliminated this binding. These results suggest that IFN-γ modulates NF-κB/c-Jun to antagonize activin A-mediated NF-E2 transcriptional activity on globin gene expression.

interferon-γ; activin A; erythroid gene; c-Jun; NF-E2

ANEMIA, A DISORDER CHARACTERIZED by abnormally low levels of healthy red blood cells or hemoglobin, reduces the oxygen-carrying capacity of the blood. Anemia may be caused by a malfunction or decrease in erythropoiesis. Erythropoiesis is the process of hematopoietic progenitor cells differentiating into mature erythrocytes and is regulated by various cytokines. Clinical studies have shown that patients with different types of anemia exhibit high levels of proinflammatory cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α. These anemias include hematologic diseases, such as aplastic anemia (16) and Fanconi anemia (17), and anemia of chronic diseases (ACDs), such as renal anemia (2) and cancer-related anemia (6, 8). Studies have reported the suppressive effects of IFN-γ (43) and TNF-α (9) on erythroid differentiation. The erythroid differentiation process of hematopoietic progenitor cells depends on numerous transcription factors, such as NF-E2 (3) and GATA-1 (18). NF-E2 is an erythroid-specific transcription factor that induces globin gene expression. NF-E2 is a heterodimeric protein complex composed of two bZIP proteins: widely expressed NF-E2p18 (also called ManK) and hematopoietic-specific NF-E2p45 (3). Liu et al. (32) reported that TNF-α suppresses the transcription activity directed by α-globin or ζ-globin promoter in K562 erythroblast cells. They also showed that the overexpression of NF-κBp65 suppresses the expression of α-globin, ζ-globin, and NF-E2p45 genes (32). In addition, TNF-α inhibits the erythroid differentiation of K562 and HEL cells by modulating erythroid-specific transcription factors, including GATA-1, GATA-2, friend of GATA1 (FOG1), and PU.1 (8). A recent study showed that IFN-γ induces the interferon regulatory factor (IRF)-1-PU.1 axis, which suppresses the erythroid differentiation of erythroid precursor cells (31). However, the molecular mechanism by which IFN-γ-inhibited erythroid differentiation remains unknown.

Members of the transcription factor AP-1 family, such as c-Jun and c-Fos, regulate numerous cellular processes. Similar to NF-κB, c-Jun was identified as participating in the inhibition of erythroid differentiation (37). NF-κB interacts directly with c-Jun and enhances the binding of AP-1 to κB- and AP-1-binding sites on the 5′-long terminal repeat (LTR) of human immunodeficiency virus (HIV) type 1 (40).

Activin A, a member of the transforming growth factor (TGF)-β superfamily, performs various functions in a broad range of cells, including proliferation and differentiation (15, 38). Activin A induces the erythroid differentiation of hematopoietic progenitor cells in vitro and in vivo; therefore, activin A is also called the erythroid differentiation factor (23, 38). Activin A can act as a paracrine regulator that modulates erythropoiesis in the bone marrow microenvironment (49). Activin A also stimulates the expression of α-globin and ζ-globin genes in K562 cells (24).

In this study, we used K562 cells to determine whether IFN-γ can act on activin A-induced globin gene expression and investigated their mechanism of action at a cellular level. Our results revealed that IFN-γ can suppress the activin A-induced expression of erythroid genes. IFN-γ also triggered NF-κBp65 and c-Jun binding to two AP-1 sites on the c-Jun promoter to induce c-Jun expression, reducing activin A-induced α-globin
and ζ-globin expression by reducing the transcriptional activity of NF-E2.

MATERIALS AND METHODS

Cell line and reagents. Human erythroblast cells of the cell line K562 were purchased from the Bioresource Collection and Research Center (Taiwan). The cells were cultured in an RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in a 5% CO2 incubator at 37 °C. Recombinant human activin A, IFN-γ, IL-1α, IL-10, and IL-13 were purchased from R&D Systems (Minneapolis, MN). MTT was purchased from Sigma (St. Louis, MO). Antibodies specific to c-Jun, NF-κBp65, and NF-E2p45 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody for the HA tag was obtained from Abcam (Cambridge, MA), and the anti-α-tubulin antibody was purchased from Sigma.

Luciferase reporter assay. Promoter activity was determined using a reporter assay, as described previously (25). The transient transfection of 10⁶ K562 cells was performed using lipofection. Two micrograms of plasmids, which included 0.05 μg of the pRL-TK internal control vector, were used for each transfection experiment. The indicated cDNAs were added to the culture medium at 5 h post-transfection. The cells were incubated for 24 h and then harvested. The luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, Madison, WI). The luciferase activity was adjusted for transfection efficiency by normalizing firefly luciferase activity to the Renilla luciferase activity generated by pRL-TK (Promega). Dr Che-Kun James Shen provided reporter plasmids pH540-oα590 Luc and pH540-597 Luc (32). Human c-Jun mutant cDNA was generated by deleting transactivation domain (TAM-67) (22). Dr Michael J. Birrer provided pCMV-c-Jun and pCMV-TAM-67 (7, 22).

RT, PCR, and real-time PCR. Total RNA was extracted from K562 cells by using the Trizol reagent (GIBCO, Life Technology, Grand Island, NY) according to the manufacturer’s instructions. After purification, 1 μg of RNA was reverse transcribed at 42°C for 60 min with primer oligo dT₁₈, followed by enzyme inactivation at 70°C for 15 min.

The resulting cDNA samples were amplified and quantified by performing a PCR and a real-time PCR, respectively, using the primer oligo dT₁₈, followed by enzyme inactivation at 70°C for 15 min. The resulting cDNA samples were amplified and quantified by performing a PCR and a real-time PCR, respectively, using the following primers: α-globin sense strand 5'-GGGCAAGACCCACATG-CAAGGGCCGC3' and antisense strand 3'-CAGGAACTTGCTCGAGGAGG3' to generate a 372-bp product; ζ-globin sense strand 5'-GGCCACCGCGACACCCAG3' and antisense strand 5'-TAGGGCTGACGCTGCCTG3' to generate a 163-bp product; NF-E2p45 sense strand 5'-TCGACAGGAGGTTGAGAG3' and antisense strand 5'-GCTCCGGTGGACCTGAG3' to generate a 177-bp product; GATA-1 sense strand 5'-GGCTTTGGGATCC-ACAGCG3' and antisense strand 5'-GGGTGGGGACACACAGGT- GG3' to generate a 220-bp product; and β-actin sense strand 5'-GCATCCCAAAAGTTCAAC3' and antisense strand 5'-GAGCCTGGCCATCTCTT3' to generate a 150-bp product as the internal control. The PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and photographed under ultraviolet light. The real-time PCR was performed using a QuantiT Fast SYBR Green PCR Kit (Qiagen, Valencia, CA) on a Roter-GenQ real-time PCR machine (Qiagen). The normalized gene expression was calculated relative to β-actin for all samples.

Western blot analysis. Cells were lysed at 4°C in a lysis buffer (1% Triton X-100, 20 mM Tris at pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM PMSF, 1 μg/ml of leupeptin, and 1 mM Na3VO4). SDS-PAGE was used to resolve protein lysates (30 μg), and the protein bands were transferred to PVDF membranes (Millipore, Bedford, MA), and the membranes were then probed with primary antibodies. After binding with horseradish peroxidase-conjugated secondary antibodies, the blots were visualized using an enhanced chemiluminescence detection system (Perkin Elmer Life and Analytical Sciences, Waltham, MA).

Chromatin immunoprecipitation assay. The cells were cross-linked with 1% formaldehyde for 10 min at room temperature. Glycine was then added to a final concentration of 0.125 M and incubated for 5 min at room temperature. The cells were lysed with an SDS buffer (0.5% SDS, 100 mM NaCl, 50 mM Tris·HCl at pH 8.1, and 5 mM EDTA). After centrifugation, the cell pellets were lysed through sonication on ice with an IP buffer (0.1% SDS, 100 mM NaCl, 50 mM Tris·HCl at pH 8.1, 5 mM EDTA, 2% Triton X-100, and a protease inhibitor cocktail (Roche)). The supernatants were precleared with salmon sperm DNA/protein G-agarose, followed by immunoprecipitation overnight at 4°C with an antibody specific to c-Jun, NF-κBp65, NF-E2p45, or mouse IgG as a negative control. The input consisted of total chromatin samples that were used as a positive control in the PCRs. The immune complexes were then precipitated with protein G-agarose for 2 h at 4°C. The precipitates were sequentially washed with TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris·HCl at pH 8.1, and 500 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris·HCl at pH 8.1, and 200 mM NaCl), and TSE III (0.25 mM LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris·HCl at pH 8.1) and washed twice with a TE buffer. Beads were eluted with an elution buffer (1% SDS and 0.1 M NaHCO₃) and incubated overnight at 65°C to reverse the formaldehyde cross-linking and were subsequently incubated with proteinase K to digest the remaining proteins. DNA was purified by conducting phenol/chloroform/isoamyl alcohol extraction. The immunoprecipitated DNA was used as a template for the PCR. The PCR was performed by using the following primer pairs (see Fig. 4C): for the pAP-1-containing region of the c-Jun promoter (nucleotides −152 to +110), sense strand 5'-ATCTTCTAGGGTGTGCCTCA-TGG-3' and antisense strand 5'-CAGTTCGGACTATCTGCGACCTG-3' were used to generate a 263-bp product (F1 fragment); and for the pAP-1-containing region of the c-Jun promoter (nucleotides −330 to −126), sense strand 5'-CAGGACGTCAGCCCAACTGACCG3' and antisense strand 5'-CACCAGTGAGACTCTCACCTAAG3' were used to generate a 205-bp fragment (F2 fragment). The primers H540–26 (5'-CCACAGTCGAGGACTCTCACCTAAG3') and H590–215 (5'-CAGTACCATCCACCCCTTTCTCCCTG-3') were used to amplify the HS-40 enhancer.

Plasmid construction. Human NF-E2p45 and NF-E2p18 cDNAs were amplified using the RT-PCR and were subcloned into the pGEM-T Easy Plasmid (Promega, Madison, WI). The forward and reverse primers for NF-E2p45 were 5'-GGATCCACGACTAATCCCAAACCG-3' and 5'-GAACATGCAATGTTGGAGGTGGAG3', respectively. The sequence-confirmed clones were used to generate a 205-bp fragment (F2 fragment). The primers HS40–26 (5'-CCACAGTCGAGGACTCTCACCTAAG3') and H590–215 (5'-CAGTACCATCCACCCCTTTCTCCCTG-3') were used to amplify the HS-40 enhancer.

Statistical analysis. Quantitative data are presented as means ± SE. Statistically significant differences between groups were analyzed using the Student’s t-test. P < 0.05 was considered significant.

RESULTS

IFN-γ suppressed activation of A-mediated erythroid gene expression. Our previous study showed that activin A induced α-globin and ζ-globin promoter activation in K562 cells (24). We used this system to determine whether IFN-γ suppresses activin A-induced ζ-globin promoter activation. The inflammatory cytokines, interleukin (IL)-10, IL-13, and IL-1 (IL-1α and IL-1β), also play a role in inhibiting erythropoiesis (10, 26, 33, 36, 48). Therefore, we also analyzed the effect of the inflammatory cytokines on activin A. K562 cells were transiently transfected with a reporter construct that expresses the
Luciferase gene controlled by the ζ-globin promoter (containing the HS-40 enhancer) pH540-ζ597Luc. The cells were then stimulated with cytokine or with activin A combined with various concentrations of erythropoiesis-inhibiting cytokines (IFN-γ, IL-1α, IL-10, and IL-13) for 24 h. The luciferase activity of the reporter gene was calculated compared with the activity of untreated cells (normalized as 1). The results in Fig. 1A show that IFN-γ reduced the luciferase activity of ζ-globin promoter. IFN-γ suppressed activin A-induced ζ-globin promoter activation in a dose-dependent manner. The luciferase activity of ζ-globin promoter did not significantly change when IL-1α, IL-10, or IL-13 was cotreated with activin A compared with when only activin A was used (Fig. 1A). Conversely, activin A reversed the suppressive effect of IFN-γ on ζ-globin promoter activity in a dose-dependent manner (Fig. 1B). Similarly, IFN-γ suppressed the luciferase activity of α-globin promoter (containing the HS-40 enhancer) and activin A-induced α-globin promoter (Fig. 2). To determine whether IFN-γ suppressed erythroid gene expression, K562 cells were treated with activin A, IFN-γ, or activin A and IFN-γ for 3 days. RT-PCR and quantitative real-time PCR data showed that activin A increased and IFN-γ reduced the RNA levels of α-globin, ζ-globin, and erythroid-specific transcription factors NF-E2p45 and GATA-1 (Fig. 3, A and B). IFN-γ significantly reduced the activin A-induced expression levels of these genes (Fig. 3, A and B).

IFN-γ increased c-Jun protein levels and the simultaneous binding of NF-κBp65 and c-Jun to two AP-1-binding sites on the c-Jun promoter. NF-κB and c-Jun have been suggested to be suppressors that inhibit erythroid differentiation (32, 37). NF-κBp65 can directly interact with c-Jun to increase transcription of AP-1-responsive genes (40). Therefore, we examined whether IFN-γ suppresses α-globin and ζ-globin gene expression through the NK-β/c-Jun pathway. As shown in Fig. 4A, IFN-γ increased c-Jun protein levels in K562 cells. Cotreatment with IFN-γ and the NF-κB inhibitor Bay117082 reduced the c-Jun level to below the level in cells treated with only IFN-γ (Fig. 4A). NF-κBp65 overexpression increased c-Jun levels in K562 cells (Fig. 4B). In addition, the potential κB binding site was not observed on the c-Jun promoter when using the TFSEARCH computer program (www.cbrc.jp/research/db/TFSEARCH.html). Two AP-1 binding sites on the c-Jun promoter [a proximal (p)AP-1 site and a distal (d)AP-1 site] were, respectively, located at −71 to −64 (4) and −190 to −183 (39)-5′ upstream of the start of transcription on the c-Jun promoter (Fig. 4C). To determine whether NF-κBp65 and c-Jun both bind to the two AP-1 sites on the c-Jun promoter in K562 cells in vivo, we performed chromatin immunoprecipitation (ChIP) assays by using specific antibodies against NF-κBp65 and c-Jun. As shown in Fig. 4D, the pAP-1- and dAP-1-containing regions of the c-Jun promoter were immunoprecipitated with antibodies against NF-κBp65 and c-Jun.

IFN-γ inhibits activin A-induced erythroid gene expression.

**Fig. 1.** Effects between activin A and erythropoiesis-inhibiting cytokines on the luciferase activity of ζ-globin promoter in K562 cells. Cells were cotransfected with pH540-ζ597 Luc and pRL-TK plasmids (as an internal transfection control). A: after 5 h of transfection, the cells were subsequently treated with activin A, erythropoiesis-inhibiting cytokines (IFN-γ, IL-1α, IL-10, or IL-13), or activin A and various concentrations of erythropoiesis-inhibiting cytokines. B: after 5 h of transfection, the cells were subsequently treated with activin A, IFN-γ, or IFN-γ and various concentrations of activin A. The luciferase activity was measured 24 h after adding the cytokine and was normalized to RL expression. Values are expressed relative to the untreated control (normalized as 1). Data from 4 independent experiments are shown as means ± SE. #P < 0.05 vs. untreated cells; *P < 0.05 vs. activin A treatment.

**Fig. 2.** Suppressive effect of IFN-γ on activin A-stimulated α-globin promoter activation in K562 cells. Cells were cotransfected with pH540-α597 Luc and pRL-TK plasmids. After 5 h of transfection, the cells were cultured in the presence or absence of 50 ng/ml of activin A, 50 ng/ml of IFN-γ, or activin A and IFN-γ. Luciferase activity was measured 24 h after addition the cytokine and was normalized to RL expression. Values are expressed relative to the untreated control (normalized as 1). Data from 4 independent experiments are shown as means ± SE. #P < 0.05 vs. untreated cells; **P < 0.05 vs. activin A treatment.
These results suggested that c-Jun expression was modulated by the simultaneous binding of NF-κBp65 and c-Jun to two AP-1 sites on the c-Jun promoter after IFN-γ stimulation.

**c-Jun participated in IFN-γ-suppressed α-globin and β-globin expression.** To demonstrate the effect of c-Jun on IFN-γ-suppressed α-globin and ζ-globin expression, the luciferase activity of α-globin and ζ-globin promoters were analyzed. K562 cells were transiently cotransfected with reporter constructs pHs40-α590Luc or pHs40-ζ597Luc, and the indicated plasmids. The results in Fig. 5, A and B, show that 24 h of exposure to IFN-γ significantly suppressed the luciferase activity of α-globin and ζ-globin promoters in the vector control group. c-Jun overexpression inhibited the luciferase activity of α-globin and ζ-globin promoters, which was further inhibited by IFN-γ (Fig. 5, A and B). A cotransfection analysis also showed that the inhibition of c-Jun transcriptional activity by the c-Jun DN mutant TAM-67 eliminated the IFN-γ-suppressed promoter activity of α-globin and ζ-globin in K562 cells (Fig. 5, A and B). The real-time PCR analysis that used α-globin- and ζ-globin-specific primers indicated that the inhibition of c-Jun by TAM-67 reversed the suppressive effects of IFN-γ on α-globin and ζ-globin expression compared with vector control (Fig. 5C). These results suggested that c-Jun is involved in IFN-γ-suppressed α-globin and ζ-globin expression.

**NF-E2 enhanced activin A-mediated stimulation of α-globin and ζ-globin promoter activity, which was inhibited by IFN-γ/c-Jun.** The NF-E2 erythroid-specific transcription factor is important for globin gene induction. Both bZip proteins, NF-E2p18 and c-Jun, can form heterodimers (27). To examine whether NF-E2 and c-Jun interact to affect globin gene induction, plasmid-expressing HA epitope-tagged NF-E2p18 or NF-E2p45 was generated by inserting full-length cDNAs, encoding the entire NF-E2p18 or NF-E2p45 protein, downstream of the HA tag. An immunoblot analysis showed that the HA-NF-E2p18 and HA-NF-E2p45 constructs were expressed when and c-Jun but not by an IgG control antibody. Furthermore, IFN-γ stimulation of K562 cells resulted in the inducible binding of NF-κBp65 and c-Jun to both the pAP-1 and dAP-1-containing regions of the c-Jun promoter (Fig. 4E).

![Image](http://ajpcell.physiology.org/)
c-Jun is involved in the suppressive effect of IFN-γ on activin A-induced erythroid gene expression. K562 cells were cotransfected with the indicated plasmid and the reporter construct pHS40-α590 Luc (A) or the reporter construct pHS40-ζ597 Luc (B). After 5 h of transfection, the cells were subsequently treated with or without (control) 50 ng/ml of IFN-γ. Luciferase activity was measured 24 h after addition of IFN-γ and was normalized to RL expression. Values are expressed relative to the untreated control (normalized as 1). C: K562 cells were transfected with the pCMV-c-Jun or pCMV-TAM-67 plasmid. After 5 h of transfection, the cells were subsequently treated with or without (control) 50 ng/ml of IFN-γ. After 72 h, RNA was isolated. α-globin and ζ-globin expression was analyzed by using a real-time PCR. β-actin was used as a control. Values are shown as the means ± SE from 3 experiments. *P < 0.05 vs. the vector/untreated control (A-C); **P < 0.005 (A and B); *P < 0.05 vs. the vector/IFN-γ-treated group (C).

The overexpression of NF-E2p18 or NF-E2p45 enhanced activin A-induced ζ-globin promoter activation compared with vector control (Fig. 6B). The enhancing effect of NF-E2p18 or NF-E2p45 on activin A-induced ζ-globin promoter activation decreased when c-Jun was present, and the presence of IFN-γ further enhanced the inhibitory effect of c-Jun (Fig. 6B). These results indicated that c-Jun is involved in the suppressive effect of IFN-γ on activin A/NF-E2-mediated ζ-globin promoter activation.

Activated c-Jun eliminated activin A-induced NF-E2p45 binding to the HS-40 enhancer on the α-globin gene. We performed ChIP assays to verify whether NF-E2 binds the HS-40/AP-1 (NA) sites at the upstream HS-40 element of the α-globin gene after activin A treatment in K562 cells. HS-40 is the major regulatory element of the α-globin gene cluster, which is located 40 kb upstream of the ζ-globin gene (51). HS-40 is a 300 to 400 bp enhancer element that consists of specific sequence motifs, such as two NA sites (41, 51) (Fig. 7A). As shown in Fig. 7B, activin A significantly increased NF-E2p45 binding to the NA sites at the HS-40 element. Studies have shown that c-Jun heterodimerizes with NF-E2p18 (27, 28) and then inhibits NF-E2 transcriptional activity (20). To determine whether c-Jun suppresses NF-E2 binding to the HS-40 element NA sites, we used a ChIP assay to measure the level of NF-E2p45 at the NA site region. As shown in Fig. 7C, c-Jun overexpression significantly reduced activin A-mediated NF-E2p45 binding to the HS-40 element NA sites. These results suggested that c-Jun eliminates activin A-induced bind-

Fig. 5. Effects of wild-type c-Jun and the c-Jun dominant-negative mutant TAM-67 on IFN-γ-suppressed α-globin and ζ-globin expression. K562 cells were cotransfected with the indicated plasmid and the reporter construct pHS40-α590 Luc (A) or the reporter construct pHS40-ζ597 Luc (B). After 5 h of transfection, the cells were subsequently treated with or without (control) 50 ng/ml of IFN-γ. Luciferase activity was measured 24 h after addition of IFN-γ and was normalized to RL expression. Values are expressed relative to the untreated control (normalized as 1). C: K562 cells were transfected with the pCMV-c-Jun or pCMV-TAM-67 plasmid. After 5 h of transfection, the cells were subsequently treated with or without (control) 50 ng/ml of IFN-γ. After 72 h, RNA was isolated. α-globin and ζ-globin expression was analyzed by using a real-time PCR. β-actin was used as a control. Values are shown as the means ± SE from 3 experiments. *P < 0.05 vs. the vector/untreated control (A-C); **P < 0.005 (A and B); *P < 0.05 vs. the vector/IFN-γ-treated group (C).

Fig. 6. NF-E2 and c-Jun are involved in activin A- and IFN-γ-mediated ζ-globin promoter activation, respectively. A: Expression analysis of the HA-NF-E2p18 and HA-NF-E2p45 proteins. K562 cells were transfected with the control plasmid (vector) or plasmid producing HA-tagged NF-E2p18 or NF-E2p45. Cell lysates were detected by performing immunoblotting using anti-HA and anti-α-tubulin antibodies. B: The reporter plasmid pHS40-ζ597 Luc was cotransfected with a plasmid that expresses the indicated protein in K562 cells. The cells were treated with or without (control) 50 ng/ml of activin A or IFN-γ. Relative luciferase activity was determined based on whole cell extracts, and the values are expressed relative to the activity of the untreated control value (normalized as 1). Values are shown as the means ± SE from 4 experiments. *P < 0.05 vs. the vector/activin A-treated group (B); **P < 0.005 (C).
IFN-γ inhibits activin A-induced erythroid gene expression

**DISCUSSION**

In this study, we demonstrated that activin A-induced RNA expression and promoter activation of α-globin and ζ-globin were inhibited by IFN-γ in K562 cells but not by IL-1α, IL-10, or IL-13. Previous studies using this cell line have also demonstrated the TNF-α inhibition of globin expression and hemoglobin synthesis (32, 35). Thus K562 cells provide a useful in vitro system for studying the molecular regulation of the proinflammatory cytokines IFN-γ and TNF-α during erythroid differentiation.

IFN-γ plays a role in proinflammatory and antimicrobial responses. IFN-γ also plays a role in regulating hematopoiesis, including inhibiting the formation of B cells (5) and eosinophilic (12) and neutrophilic granulocytes (13). IFN-γ also exhibits a direct suppressive effect on the colony formation of bone marrow-derived hematopoietic progenitor cells in vitro (34, 46). A recent study demonstrated that IFN-γ reduces the proliferation of hematopoietic stem cells in mice (14). Neutralizing IFN-γ in a mouse model illustrated the importance of this cytokine in inhibiting erythropoiesis in vivo (43). Our study showed that IFN-γ directly inhibited the activin A induction of α-globin and ζ-globin expression. IFN-γ was determined to inhibit the secretion of activin A in bone marrow-derived stromal fibroblasts (1). Thus the IFN-γ inhibition of erythroid differentiation is mediated by a decrease of activin A production in the bone marrow microenvironment. However, when activin A and IFN-γ work together on erythroid progenitor cells, IFN-γ counteracts the actions of activin A. Our study also showed that an increasing concentration of activin A may reverse IFN-γ-inhibited α-globin and ζ-globin expression. These results indicated that the interplay of activin A and IFN-γ modulates the erythroid differentiation of erythroid progenitor cells.

The inhibitory effect of IFN-γ on erythroid differentiation at the molecular level demonstrates that IFN-γ upregulates PU.1 by inducing the transcription factor IRF1 to suppress erythroid differentiation (31). IFN-γ also increases the expression of proapoptotic molecules, such as CD95(L), TRAIL, and TWEAK, which contributes to cell death in differentiating erythroblasts (11, 19). In our study, IFN-γ did not induce apoptosis in K562 cells (data not shown). Therefore, we used this cell line to study the molecular mechanism of the IFN-γ-inhibited activin A induction of globin expression.

The NF-κB transcription factor was highly expressed in early erythroid precursors, but the NF-κB transcription factor levels declined during erythroid differentiation (50). Overexpression of the NF-κBp65 subunit reduced globin gene expression in K562 cells (32). In addition, the transcription factors c-myc and c-myc may be associated with erythroid development (30, 42, 45). Zhang et al. (50) reported that NF-κB binds to κB sites in c-myc and c-myc promoters in burst-forming unit-erythrocyte/derived cells, suggesting that c-myc and c-myc may be involved in NF-κB-inhibited erythropoiesis. However, the NF-κB downstream target genes involved in inhibiting erythroid differentiation have not been identified. NF-κBp65 can physically interact with c-Jun, which can enhance DNA binding through the κB- and AP-1-response elements on HIV-1 LTR (40). This study showed that IFN-γ increased c-Jun levels in K562 cells through the NF-κB pathway. A ChIP assay demonstrated that IFN-γ induced NF-κBp65 and c-Jun to simultaneously bind to the pAP-1 and dAP-1 sites on the c-Jun promoter, which lacked a consensus κB element. This suggested that NF-κB may mediate the effect of IFN-γ in regulating c-Jun expression by interacting with c-Jun on the two AP-1-binding sites of the c-Jun promoter in K562 cells. Elagib et al. (18) also showed that c-Jun inhibits erythroid differentiation in primary human hematopoietic progenitors. The results of this study showed that IFN-γ suppression of ζ-globin promoter activation was enhanced by wild-type c-Jun and was eliminated by DN c-Jun in K562 cells. These results indicated that NF-κB/c-Jun is involved in the suppressive effect of IFN-γ on erythroid gene expression.

The NF-E2 heterodimer binds to two NA sites in the upstream HS-40 enhancer of mammalian α-like globin genes, such as α-globin and ζ-globin, and induces their expression (29, 41, 51). c-Jun can heterodimerize with NF-E2p18 (27, 28) and inhibits NF-E2 transcriptional activity in Friend erythroblast cell lines (20). Confirming previous studies, our reporter assay demonstrated that c-Jun reduced the activity of NF-E2p18 in enhancing activin A-induced ζ-globin promoter activation. c-Jun also reduced the NF-E2p45-increased activin A induction of ζ-globin promoter activation. This study showed that the individual exogenous expression of NF-E2p18 and NF-E2p45 interacted with endogenous NF-E2p45 and NF-E2p18, respectively. Furthermore, activin A may increase NF-E2 heterodimers by inducing NF-E2p45 expression. Therefore, NF-E2 can enhance activin A-induced ζ-globin promoter activation, and c-Jun inhibits this effect. ChIP assays confirmed the direct involvement of NF-E2p45 in activin A-mediated...
α-like globin gene induction. The reduction in NF-E2p45 binding to the upstream HS-40 element of the α-like globin gene in the ChIP assay demonstrated the function of c-Jun. These results suggested that c-Jun interferes with NF-E2 binding to its DNA element and then reduces the activin A induction of β-globin promoter activation. K562 cells treated only with IFN-γ exhibited markedly reduced levels of α-globin and β-globin promoter activity and mRNA expression in the absence of activin A. The K562 cells expressed low levels of NF-E2. Our data also suggested that the increase in c-Jun caused by IFN-γ may inhibit the low level of NF-E2 in K562 cells, leading to a significant decrease in the basal luciferase activity of the α-globin and β-globin promoters as well as the expression of these globin genes. In addition, c-Jun was shown to inhibit erythroid differentiation by downregulating GATA-1, a key erythroid transcription factor (18). NF-E2p45 is a GATA-1 target (21, 44, 47). Our study showed that IFN-γ suppresses activin A-induced NF-E2p45 and GATA-1 expression. These results suggested that the suppression of the activin A induction of globin expression by IFN-γ may occur through two mechanisms: c-Jun inhibits NF-E2 transcriptional activity and c-Jun reduces NF-E2 through GATA-1.

In conclusion, IFN-γ suppresses activin A/NF-E2-induced globin expression through the NF-κB/c-Jun pathway. It is crucial to ascertain the contribution of the NF-κB/c-Jun pathway to the development of anemia in several chronic diseases and whether targeting the NF-κB/c-Jun pathway can eliminate the suppression of erythroid differentiation in ACDs.

ACKNOWLEDGMENTS
We thank Dr. Che-Kun James Shen and Dr. Michael J. Birrer for the generous gift of plasmids.

GRANTS
This study was supported by National Science Council (Taiwan) Grant NSC 99–2320-B-038–005-MY3 and the Taipei Medical University-Shuang Ho Hospital (Taiwan) Grant 100TMYU-SHH-16.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


