0026-895X/08/7303-778–788$20.00
MOLECULAR PHARMACOLOGY
Vol. 73, No. 3
40824/3311583
Mol Pharmacol 73:778–788, 2008
Printed in U.S.A.

Interferon β Augments Tuberous Sclerosis Complex 2 (TSC2)-Dependent Inhibition of TSC2-Null ELT3 and Human Lymphangioleiomyomatosis-Derived Cell Proliferation

Elena A. Goncharova, Dmitry A. Goncharov, Amelia Chisolm, Matthew S. Spaits, Poay N. Lim, Gregory Cesarone, Irene Khavin, Omar Tliba, Yassine Amrani, Reynold A. Panettieri, Jr., and Vera P. Krymskaya

Pulmonary, Allergy & Critical Care Division, Department of Medicine, Abramson Cancer Center, University of Pennsylvania, Philadelphia, Pennsylvania

Received August 17, 2007; accepted December 18, 2007

ABSTRACT

Lymphangioleiomyomatosis (LAM), a rare pulmonary disorder, manifests as an abnormal neoplastic growth of smooth muscle-like cells within the lungs. Mutational inactivation of tumor suppressor tuberous sclerosis complex 2 (TSC2) in LAM constitutively activates the mammalian target of rapamycin (mTOR)/p70 S6 kinase 1 (S6K1) signaling pathway and promotes neoplastic growth of LAM cells. In many cell types, type I interferon β (IFNβ) inhibits proliferation and induces apoptosis through signal transducers and activators of transcription (STAT)-dependent and STAT-independent signaling pathways, one of which is the mTOR/S6K1 signaling pathway. Our study shows that IFNβ is expressed in LAM tissues and LAM-derived cell cultures; however, IFNβ attenuates LAM-derived cell proliferation only at high concentrations, 100 and 1000 U/ml (IC_{50} value for IFNβ is 20 U/ml compared with 1 U/ml for normal human mesenchymal cells, human bronchus fibroblasts and human airway smooth muscle cells). Likewise, IFNβ only attenuates proliferation of smooth muscle TSC2-null ELT3 cells. Analysis of IFNβ signaling in LAM cells showed expression of IFNβ receptor α (IFNβRα) and IFNβRβ, activation and nuclear translocation of STAT1, and phosphorylation of STAT3 and p38 mitogen-activated protein kinase (MAPK), but IFNβ had little effect on S6K1 activity. However, the re-expression of TSC2 or inhibition of mTOR/S6K1 with rapamycin (sirolimus) augmented antiproliferative effects of IFNβ in LAM and TSC2-null ELT3 cells. Our study demonstrates that IFNβ-dependent activation of STATs and p38 MAPK is not sufficient to fully inhibit proliferation of cells with TSC2 dysfunction and that TSC2-dependent inhibition of mTOR/S6K1 cooperates with IFNβ in inhibiting human LAM and TSC2-null ELT3 cell proliferation.

LAM, a rare lung disorder, manifests as abnormal neoplastic proliferation of smooth muscle-like cells within the lung, which leads to progressive loss of pulmonary function and death and affects predominantly women of childbearing age (Johnson, 2006; Taveira-DaSilva et al., 2006; Juvet et al., 2007). LAM occurs from loss of heterozygosity or from somatic inactivating mutations of the tumor suppressor genes *tuberous sclerosis complex 1* (*TSC1*) and *TSC2*. Loss of tumor suppressor function of either TSC1 or TSC2, also known as hamartin and tuberin, respectively, promotes the constitutive activation of the mammalian target of rapamycin (mTOR)/p70S6 kinase 1 (S6K1) signaling pathway, leading to abnormal proliferation of LAM cells (Goncharova et al., 2002b, 2006a; Kwiatkowski et al., 2002; Krymskaya, 2003, 2008; Krymskaya and Shipley, 2003; Inoki et al., 2005; Goncharova and Krymskaya, 2008). Although some understanding of TSC1/TSC2 regulatory signaling mechanisms has been gained (Inoki et al.,

This work was supported by National Institutes of Health grants 2R01-HL071106 (to V.P.K.), HL55301 (to R.A.P.), HL64063 (to R.A.P.), by National Institute of Environmental Health Sciences grant ES0135080 (to R.A.P. and V.P.K.), and by The LAM Foundation (to V.P.K. and E.A.G.).
R.A.P. and V.P.K. contributed equally to this work.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.107.040824.

ABBREVIATIONS: LAM, lymphangioleiomyomatosis; TSC, tuberous sclerosis complex; mTOR, mammalian target of rapamycin; STAT, signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase; HBF, human bronchus fibroblast; BrdU, 5-bromo-2′-deoxyuridine; PDGF, platelet-derived growth factor; SM, smooth muscle; GFP, green fluorescent protein; IFN, interferon; IFNβR, interferon β receptor; FITC, fluorescein isothiocyanate; RT-PCR, reverse-transcriptase polymerase chain reaction; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; HASM, human airway smooth muscle; pEGFP, plasmid encoding for enhanced green fluorescent protein; ANOVA, analysis of variance; S6K1, p70 S6 kinase 1; JAK, Janus tyrosine kinase; RAPA, rapamycin; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole.

2005), precise molecular mechanisms of how dysregulation of TSC1/TSC2 function leads to abnormal neoplastic cell proliferation in LAM remains unknown.

In cancer, type I interferons (IFNs) α and β play important roles by suppressing cell growth and proliferation and promoting apoptosis (Takaoka and Taniguchi, 2003). The biological effects of type I IFNs, however, are cell type-specific; thus, for many cell types, IFNα and IFNβ inhibit proliferation and are proapoptotic but promote survival of memory T cells (Platanias, 2005). Type I IFNβ (Karpusas et al., 1998) signals by binding to cognate receptors, IFNβ receptor α (IFNβRα), and IFNβRβ, leading to the activation of the Janus kinases JAK1 and Tyk2, which promote signal transducer and activator of transcription 1 (STAT1) and STAT2 activation. Subsequent dimerization and translocation into the nucleus of JAK/STAT complex regulate the transcription of target genes, several of which encode proteins that have tumor suppressor activity (Takaoka and Taniguchi, 2003). Antiproliferative and proapoptotic effects of IFNβ can also be JAK/STAT-independent and involve different signaling pathways, including phosphatidylinositol 3 kinase, mTOR/S6K1, and Rac1/p38 MAPK; activation of these signaling cascades is tissue- and cell type-specific (Thyrell et al., 2004; Platanias, 2005; de Weerd et al., 2007). Few investigators have examined the role of type II IFNγ in TSC2-null cell and tumor growth (Hino et al., 2003; El-Hashemite et al., 2004); however, whether TSC2 dysfunction modulates growth-inhibitory and proapoptotic functions of type I IFNs remains controversial.

Here, we report that proliferation of rat TSC2-null ELT3 cells and human LAM cells is attenuated by type I IFNβ through cell cycle arrest in G_0/G_1 phase and induction of apoptosis. Inhibition of mTOR/S6K1 signaling pathway by re-expression of TSC2 or mTOR inhibitor rapamycin (sirolimus) (Hartford and Ratain, 2007) enhances IFNβ-dependent cell cycle G_0/G_1 phase blockade and promotes proapoptotic activity that augments the inhibition of TSC2-null and LAM cell growth. These data show that IFNβ augments TSC2-dependent inhibition of cell proliferation and suggest that combination of IFNβ with rapamycin may offer potential therapeutic advantages for the treatment of LAM disease.

Materials and Methods

Cell Culture. LAM cells were dissociated from LAM nodules from the lung of patients with LAM who have undergone lung transplant according to the protocol approved by the University of Pennsylvania Institutional Review Board and provided by the National Heart, Lung, and Blood Institute LAM Registry (Goncharova et al., 2002b, 2006a; Krymskaya, 2008). In brief, cells were dissociated by enzymatic digestion in M199 medium containing 0.2 mM $CaCl_2$, 2 mg/ml collagenase D (Roche, Indianapolis, IN), 1 mg/ml trypsin inhibitor (Sigma Chemical Co., St. Louis, MO), and 3 mg/ml elastase (Worthington, Lakewood, NJ). The cell suspension was filtered and then washed with equal volumes of cold DF8 medium, consisting of equal amounts of Ham's F-12 and Dulbecco's modified Eagle's medium supplemented with 1.6×10^{-6} M ferrous sulfate, 1.2×10^{-5} U/ml vasopressin, 1.0×10^{-9} M triiodothyronine, 0.025 mg/ml insulin, 1.0×10^{-8} M cholesterol, 2.0×10^{-7} M hydrocortisone, 10 pg/ml transferrin, and 10% fetal bovine serum. The cells were cultured in DF8 medium and were passaged twice per week. LAM cells from each patient were characterized on a basis of smooth muscle (SM) α-actin expression, S6K1 activity, ribosomal protein S6 phosphorylation, and DNA synthesis. All LAM cells used in this study had constitutively activated S6K1, hyperphosphorylated ribosomal protein S6, and a high degree of proliferative activity in the absence of any stimuli, as well as a filamentous expression pattern of smooth muscle α-actin (Goncharova et al., 2006a). As we reported in a study published previously, LAM cells from two LAM patients carry *TSC2* gene mutations and have no immunoreactivity to HMB45 (Goncharova et al., 2002b). LAM cells in subculture during the 3rd through 12th cell passages were used. Human bronchus fibroblasts (HBFs) were dissociated from the bronchus of the same patients with LAM according to the protocol used for LAM cell dissociation (Goncharova et al., 2002b, 2006a). Human airway smooth muscle (HASM) cells were dissociated from human trachea, which was obtained from human lung transplant donors, and have been described previously (Goncharova et al., 2002b). TSC2-null ELT3 cells were derived from the Eker rat uterine leiomyoma and maintained as described previously (Goncharova et al., 2002b). Prior experiments cells were serum-deprived for 48 h. All experiments were performed with a minimum of three different LAM, HBFs, and HASM cell lines.

DNA Synthesis Analysis

[^{3}H]Thymidine Incorporation Assay. DNA synthesis was measured using [^{3}H]thymidine incorporation assay (Goncharova et al., 2006b). In brief, near-confluent cells that were serum-deprived for 48 h were incubated with different concentrations of IFNβ or diluent in the presence or absence of 200 nM rapamycin and/or 10 ng/ml PDGF. After 18 h of incubation, cells were labeled with 3 μCi/ml [methyl-^{3}H]thymidine (60 Ci/mmol; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) for 24 h. The cells were then scraped and lysed, and DNA was precipitated with 10% trichloroacetic acid. The precipitants were aspirated on glass filters and extensively washed and dried, and [^{3}H]thymidine incorporation was counted.

5-Bromo-2′-deoxyuridine Incorporation Assay. Nontransfected cells or cells transfected with plasmids expressing GFP-conjugated TSC2 or control GFP were maintained for 48 h in serum-free medium, and then 5-bromo-2′-deoxyuridine (BrdU) incorporation was assessed (Goncharova et al., 2002a, 2006b). In brief, cells were treated with rapamycin and IFNβ separately or in combination or with diluent in the presence or absence 10 ng/ml PDGF for 18 h, and then 10 μM BrdU was added. Twenty-four hours later, cells were fixed with 3.7% paraformaldehyde (Polysciences, Warrington, PA) and then permeabilized with 0.1% Triton X-100 followed by immunocytochemical analysis with 2 μg/ml murine anti-BrdU antibody (BD Biosciences, San Jose, CA) and then with 10 μg/ml Texas Red-conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) to detect BrdU-positive cells. To identify transfected cells, cells were incubated with anti-GFP rabbit serum and then with Alexa Fluor 594 goat anti-rabbit IgG conjugate (Invitrogen, Carlsbad, CA). Cells were then incubated with 1 μg/ml 4,6-diamidino-2-phenylindole to detect the total number of nuclei. The cells were examined using Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) at 200× magnification with the appropriate fluorescent filters. The mitotic index of nontransfected cells was defined as the percentage of BrdU-positive cells per field/total number of cells per field. The mitotic index of transfected cells was calculated as the percentage of BrdU- and GFP-positive cells per field/GFP-positive cells per field. A total of 200 cells were counted per each condition in each experiment.

Transient Transfection

Plasmids were prepared using EndoFree Plasmid Maxi Kit (QIAGEN Inc., Valencia, CA). Transient transfection was performed using the Effectene transfection reagent (QIAGEN) according to the manufacturer's protocol. In brief, cells were incubated with pEGFP or pEGFP-TSC2 for 6 h and then washed with PBS, incubated in complete medium, and then maintained for 24 h in serum-free media before DNA synthesis assays. Transient transfection of pEGFP-TSC2 plasmid was verified by immunoblot assay using anti-tuberin

(C20) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (Goncharova et al., 2002b, 2004, 2006a).

Immunoblot Analysis

Serum-deprived cells were treated with 100 U/ml IFNβ, 200 nM rapamycin (RAPA) alone or in combination, or diluent in the presence or absence of 10 ng/ml PDGF for 30 min followed by immunoblot analysis with anti-phospho-STAT1 (Tyr701), anti-STAT1, anti-phospho STAT3 (Tyr705), anti-STAT3, anti-phospho S6, anti-total S6, anti-phospho p38, and anti-p38 antibodies (Cell Signaling Technology Inc., Danvers, MA) (Goncharova et al., 2002a).

Immunohistochemical Analysis

LAM and normal lung tissue sections were immunostained with primary anti-IFNβ (Santa Cruz Biotechnology) or anti-smooth muscle α-actin clone 1A4 FITC conjugate (Sigma Chemical) and secondary Alexa Fluor 594 chicken anti-mouse IgG conjugate (Molecular Probes) antibodies (Goncharova et al., 2002b). Negative controls included replacement of the primary antibody with isotype-matched IgG.

Immunocytochemical Analysis

LAM cells and HBFs serum-deprived for 24 h were treated with 100 U/ml IFNβ for 30 min, fixed with 3.7% paraformaldehyde (Polysciences) for 15 min, incubated with 0.1% Triton X-100 (Sigma Chemical) for 30 min at room temperature, and then blocked as described previously (Goncharova et al., 2002b, 2004). Cells were incubated with primary anti-STAT1/anti-phospho-S6 or anti-GFP antibodies (Cell Signaling Technology) and then with secondary Alexa Fluor 594 or Alexa Fluor 488 goat anti-rabbit IgG conjugate (Molecular Probes) antibodies for 1 h at 37°C. Cells were visualized using a Nikon Eclipse E400 microscope under appropriate filters.

Analysis of IFNβ Receptor Expression

Cell surface expression of IFNβ receptor subunits was detected using flow cytometry analysis (Krymskaya et al., 2001). In brief, cells were resuspended in EDTA solution followed by immunocytochemical analysis using primary anti-IFNβRα and anti-IFNβRβ antibodies (Santa Cruz Biotechnology) and FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Fluorescence intensity was analyzed using a BD Biosciences FACScan. Normal IgG was used as isotype controls.

Analysis of Cell Cycle Checkpoint Status

Cell cycle checkpoint status was examined using flow cytometry analysis (Krymskaya et al., 2001). In brief, cells were serum-deprived for 48 h, treated with 200 nM rapamycin, 100 U/ml IFNβ, 200 nM rapamycin plus 100 U/ml IFNβ, or diluent for 18 h, and then 10 μM BrdU was added. After 24 h of incubation, cells were resuspended in 0.05% trypsin/EDTA, labeled with anti-BrdU FITC-conjugated antibody, and propidium iodine followed by flow cytometry analysis using a BD Biosciences FACScan.

RT-PCR Analysis

Total RNA was extracted from serum-deprived LAM cells, HBFs, and HASM cells using an RNeasy mini kit (Qiagen) according to the manufacturer's protocol. RT-PCR reactions were performed using IFNβ and glyceraldehyde-3-phosphate dehydrogenase primers for semiquantitative analysis, as described previously (Amrani et al., 2003). Primers for human IFNβ and GAPDH detection were identical with those reported previously (Talon et al., 2000; Tliba et al., 2003). Polymerase chain reaction products were separated on 1% agarose gels and stained with ethidium bromide.

Apoptosis Analysis

Analysis of apoptosis was performed using In Situ Cell Death Detection Kit based on TUNEL technology (Roche, Nutley, NJ) according to the manufacturer's protocol. In brief, cells serum-deprived for 24 h were incubated with 200 nM rapamycin, 100 U/ml IFNβ, separately or in combination, or diluent in the presence or absence of 10 ng/ml PDGF for 18 h. Cells were then fixed with 3.7% paraformaldehyde (Polysciences) for 15 min and treated with 0.1% Triton X-100 (Sigma Chemical) for 30 min at room temperature followed by 1-h incubation with TUNEL reaction mixture at 37°C. After incubation, cells were mounted in Vectashield mounting medium with 4,6-diamidino-2-phenylindole to detect cell nuclei (Vector Laboratories, Burlingame, CA) and then visualized on the Nikon Eclipse E400 microscope under appropriate filters. A total of 200 cells were counted per each condition in each experiment.

Data Analysis

Data points from individual assays represent the mean values $\pm$ S.E. Statistically significant differences among groups were assessed with the analysis of variance (ANOVA) (Bonferroni-Dunn), with values of $p < 0.05$ sufficient to reject the null hypothesis for all analyses. All experiments were designed with matched control conditions within each experiment to enable statistical comparison as paired samples.

Results

IFNβ Is Expressed in LAM Lung Tissues and LAM Cell Cultures. Because autocrine IFNβ is involved in suppressing tumor development and growth (Takaoka and Taniguchi, 2003), we examined whether IFNβ is expressed in LAM lungs. We performed the immunohistochemical analysis of IFNβ expression in LAM lung specimens from eight

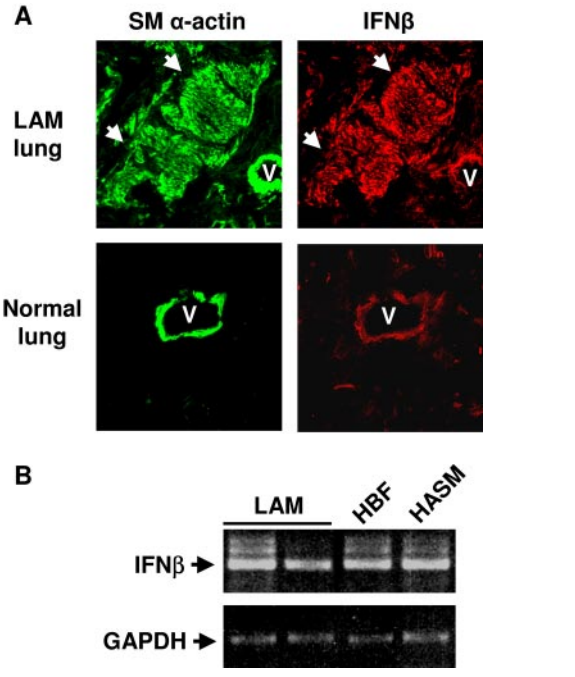


Fig. 1. A, expression of IFNβ in LAM lungs. Tissue specimens from LAM lung (top) and normal lung (bottom), were immunostained with anti-SM α-actin FITC-conjugated antibody (green) and anti-IFNβ antibody (red). Images were taken on a Nikon Eclipse E400 microscope under 200$\times$ magnification. White arrows indicate LAM nodules. V indicates blood vessel. Tissue samples from eight different patients were analyzed. B, IFNβ mRNA expression in LAM cell cultures. Total mRNA (2 μg), extracted from serum-deprived LAM cells from the lung of two different patients, HBFs, and HASM cells, was subjected to RT-PCR analysis with the primers for IFNβ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. Polymerase chain reaction products were separated on a 1% gel and stained with ethidium bromide. Image is representative from two different experiments yielded similar results.

patients with LAM. As seen in Fig. 1A, many of the smooth muscle α-actin-positive LAM cells (green) stained positive for IFNβ (red). However, our data demonstrated that not all SM α-actin-positive cells in LAM lungs were IFNβ-positive. IFNβ expression in LAM tissue sections was detected in four (50%) of the eight analyzed patients with LAM. It is interesting that smooth muscle cells from blood vessel wall expressed IFNβ in both LAM and normal lungs (Fig. 1A). RT-PCR analysis of IFNβ mRNA levels also demonstrated IFNβ mRNA expression in serum-deprived LAM cell cultures on the levels comparable with control HBFs and HASM cells (Fig. 1B). These data show that IFNβ was expressed in both smooth muscle-positive cells in LAM lung tissue sections and LAM cell cultures.

Effect of IFNβ on TSC2-Null and LAM Cell Proliferation. Next, we examined whether IFNβ modulate TSC2-null and LAM cell proliferation. We used three primary LAM cell cultures dissociated from the LAM nodules from the lung of three different patients with LAM and control HBFs from bronchus of the same patients as described previously (Goncharova et al., 2006a). Cells were treated with different concentrations of IFNβ or diluent in the presence or absence of 10 ng/ml PDGF for 18 h and then subjected to DNA synthesis analysis assessed by [^{3}H]thymidine incorporation. As seen in Fig. 2, IFNβ inhibited both basal (Fig. 2A) and PDGF-induced (Fig. 2B) LAM cell proliferation in a concentration-dependent manner. It is noteworthy that basal LAM cell DNA synthesis is considerably greater compared with that of HBFs (Goncharova et al., 2002b, 2006a). These data are consistent with the in vivo aberrant growth of LAM cells (Johnson, 2006; Taveira-DaSilva et al., 2006; Juvet et al., 2007; Goncharova and Krymskaya, 2008). It is interesting that IFNβ induced the greatest inhibition of DNA synthesis in HBF cells compared with that seen in LAM cells: the IC_{50} value for IFNβ was markedly higher for LAM cells compared with the IC_{50} value for HBFs, 20 versus 1 U/ml, respectively. Furthermore, the maximal inhibition IFNβ in LAM was 54.5 ± 9.1 compared with 92.2 ± 7.2 in HBF. Likewise, IFNβ significantly but not completely inhibited both basal and PDGF-induced proliferation of TSC2-null ELT3 cells with IC_{50} value of ~20 U/ml (Fig. 2C). These data demonstrate that proliferation of both LAM and TSC2-null ELT3 cells is inhibited by IFNβ far less than that inhibited in HBF.

IFNβ Receptor Expression and IFNβ-Dependent STAT Activation. To examine whether decreased sensitivity of LAM cells to growth-inhibitory effects of IFNβ were associated with alterations in IFNβ receptor expression, we performed flow cytometry analysis with anti-IFNβ receptor α (IFNβRα) and anti-IFNβRβ antibodies. As seen in Fig. 3A, both α and β subunits of IFNβ receptor are expressed in LAM cells. Quantitative analysis of flow cytometry experiments shows that there is no significant difference between IFNβRα and IFNβRβ expression in LAM, HASM cells, and HBFs (Fig. 3A, bottom), demonstrating that the levels of receptor expression were comparable in LAM cells, HASM cells, and HBFs.

Because binding of IFNβ with its specific receptors activate JAK/STAT signaling pathways (Platanias, 2005), we examined whether IFNβ stimulate STAT activation in LAM cells. We found that IFNβ stimulates STAT1 phosphorylation at Tyr701 (Fig. 3B) and STAT1 nuclear translocation comparable with those in control HBFs (Fig. 3C). To examine the effect of IFNβ on STAT3 phosphorylation, serum-deprived LAM cells were stimulated with IFNβ or diluent for 30, 60, 120, or 180 min followed by immunoblot analysis with phosphospecific antibody recognizing STAT3 phosphorylated at Tyr705. As seen in Fig. 3D, basal levels of Tyr705 STAT3 phosphorylation were detected in serum-deprived LAM cells; PDGF stimulation increased Tyr705 STAT3 phosphorylation at 30 min of incubation. The incubation with 100 U/ml IFNβ for 30 and 60 min increased Tyr705 STAT3 phosphorylation

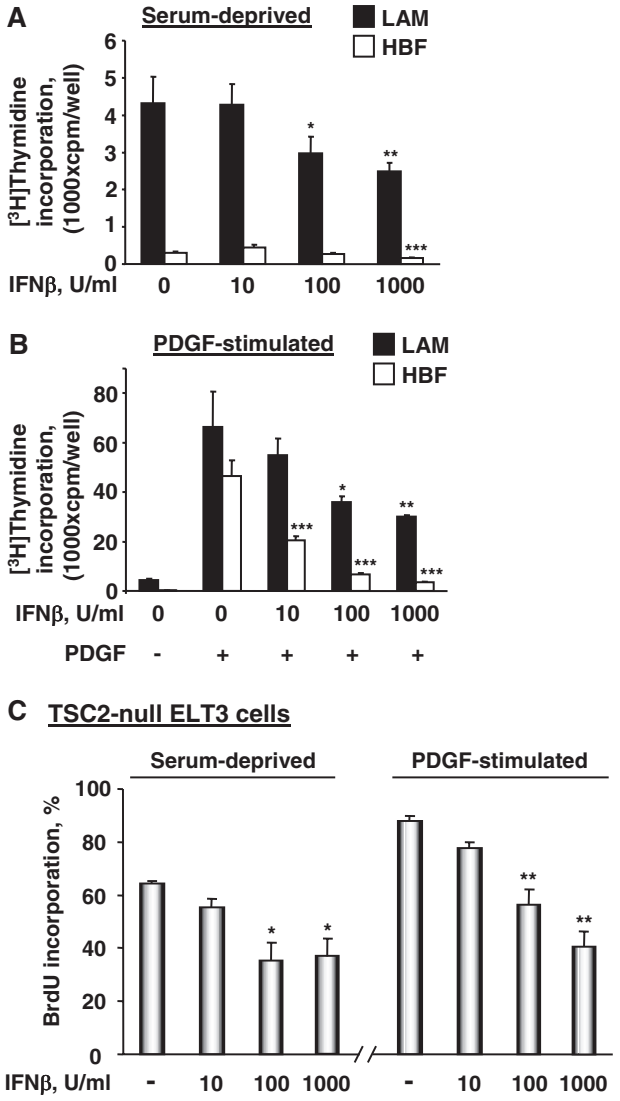


Fig. 2. A and B, effect of IFNβ on LAM cell proliferation. Serum-deprived LAM cells and HBFs were treated with 10, 100, 1000 U/ml human IFNβ or diluent for 18 h in the absence (A) or presence (B) of 10 ng/ml PDGF followed by [^{3}H]thymidine incorporation assay. A, *, $p < 0.05$ for LAM cells treated with 100 U/ml IFNβ versus diluent-treated LAM cells; **, $p < 0.01$ for LAM cells treated with 1000 U/ml IFNβ versus diluent-treated LAM cells; ***, $p < 0.01$ for HBFs treated with 1000 U/ml IFNβ versus diluent-treated HBFs. B, *, $p < 0.01$ for LAM cells treated with 100 U/ml IFNβ + PDGF versus LAM cells treated with PDGF; **, $p < 0.001$ for LAM cells treated with 1000 U/ml IFNβ + PDGF versus PDGF-treated LAM cells; ***, $p < 0.001$ for HBFs treated with IFNβ + PDGF versus PDGF-treated HBFs by ANOVA (Bonferroni-Dunn). C, effect of IFNβ on TSC2-null ELT3 cell proliferation. Serum-deprived rat ELT3 cells were treated with 10, 100, and 1000 U/ml rat IFNβ or diluent for 18 h in the absence or presence of 10 ng/ml PDGF followed by BrdU incorporation assay. Data represent means ± S.E. from three independent experiments. *, $p < 0.01$ for 100 and 1000 U/ml IFNβ versus control; **, $p < 0.01$ for PDGF + 100 U/ml IFNβ and PDGF + 1000 U/ml IFNβ versus PDGF by ANOVA (Bonferroni-Dunn).

in both serum-deprived and PDGF-treated LAM cells (Fig. 3D) and control HBFs (Data not shown). These data demonstrate that IFNβ activates classic receptor-associated JAK/STAT signaling pathways in LAM cells on the levels comparable with control HBFs.

Because recent studies show that p38 MAPK is phosphor-

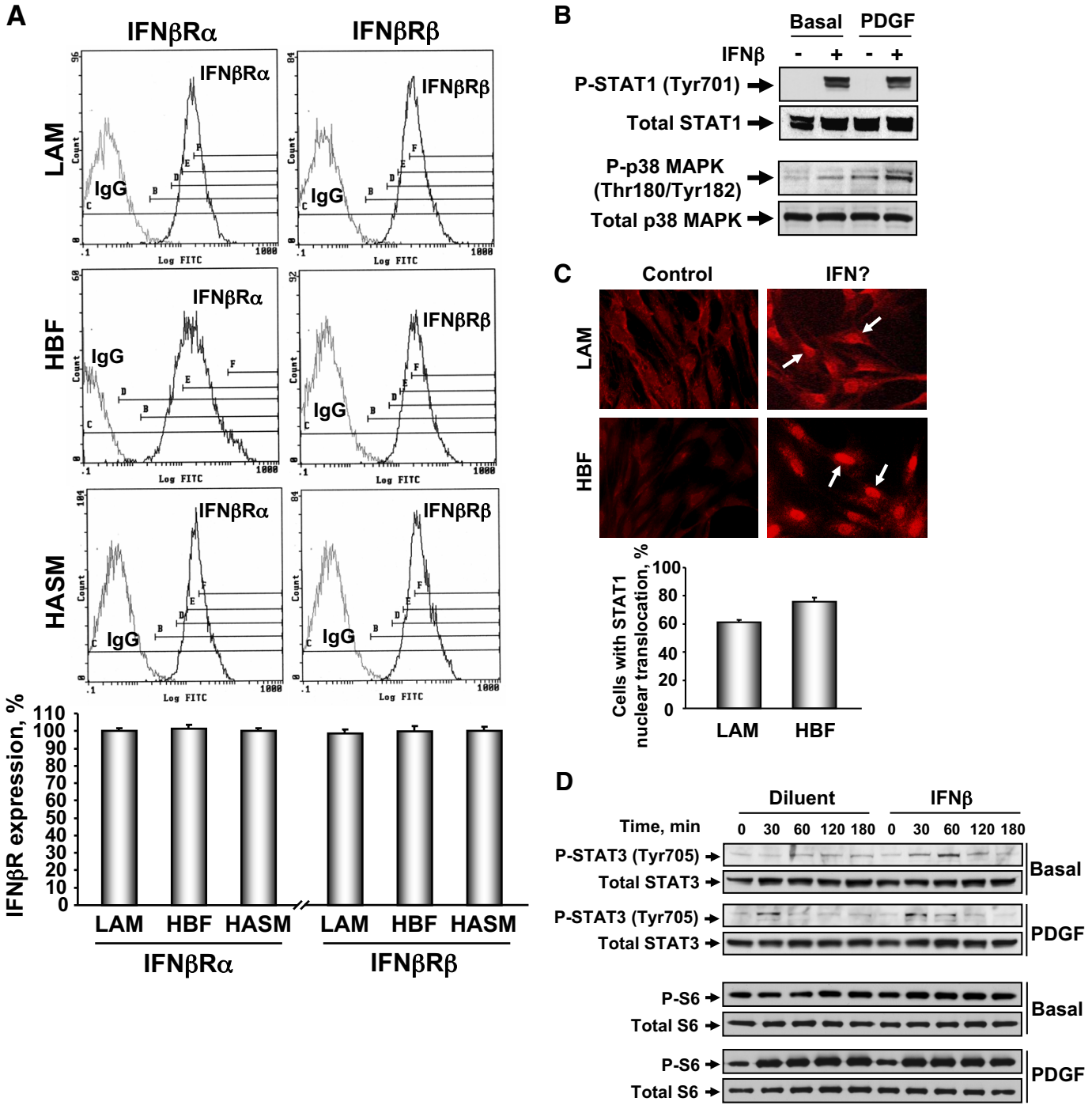
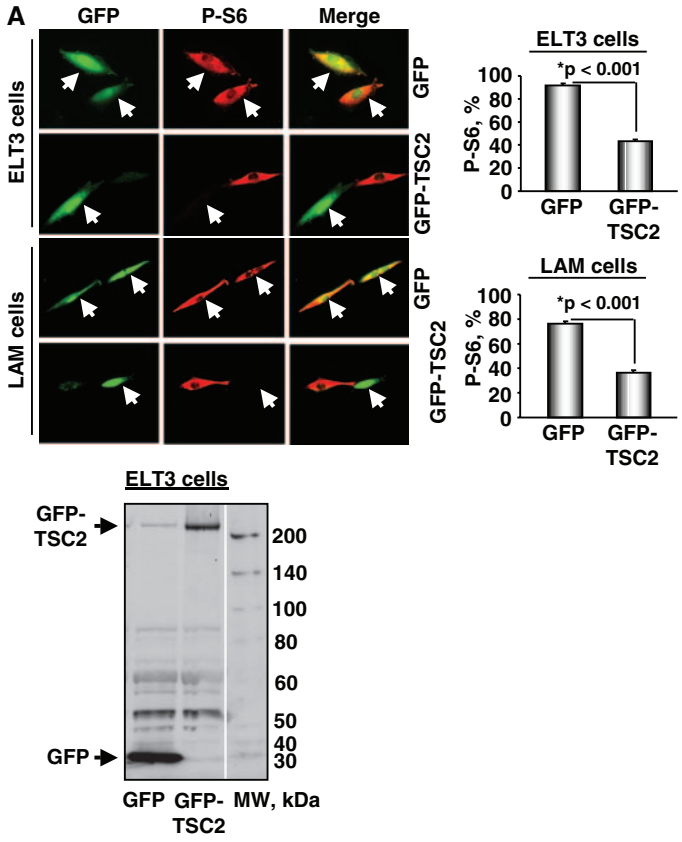
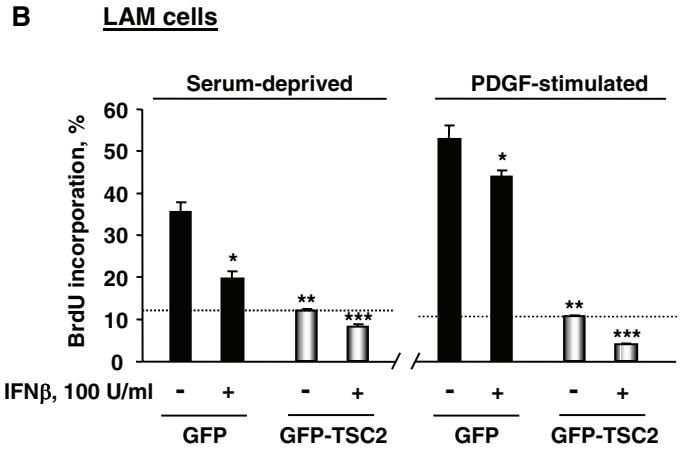


Fig. 3. A, expression of IFNβ receptors in LAM cells. Serum-deprived LAM, HBFs, and HASM cells were subjected to flow cytometry analysis with antibodies specifically recognizing IFNβRα and IFNβRβ. Normal IgG was used as an isotype control. Top, images are representative of three separate experiments. Bottom, quantitative analysis of experiments. Data are means ± S.E. from three independent experiments. IFNβ receptor expression levels in HASM cells were taken as 100%. B, effect of IFNβ on STAT1 and p38 phosphorylation in LAM cells. Serum-deprived LAM cells were treated with 100 U/ml IFNβ or diluent for 30 min in the presence or absence of 10 ng/ml PDGF; cell lysates then were subjected to immunoblot analysis with anti-phospho-STAT1 (Tyr701), anti-Stat1, anti-phospho p38, and anti-p38 antibodies. Images are representative of three independent experiments. C, IFNβ induces STAT1 nuclear translocation in LAM cells. Serum-deprived LAM cells and HBFs were treated with 100 U/ml IFNβ or diluent for 30 min, and then immunostaining with anti-STAT1 antibodies was performed. Images representative of two separate experiments were taken at Nikon Eclipse 2000 microscope under 200× magnification. Data represent a percentage of cells with STAT1 nuclear localization per total number of cells. Data represent means ± S.E. from two independent experiments. D, effect of IFNβ on STAT3 and ribosomal protein S6 phosphorylation. LAM cells were serum-deprived for 24 h and then incubated with 100 U/ml IFNβ or diluent for 30, 60, 120, or 180 min followed by immunoblot analysis with anti-phospho-STAT3 (Tyr705), anti-STAT3, anti-phospho S6, and anti-S6 antibodies. Images are representative of two independent experiments.





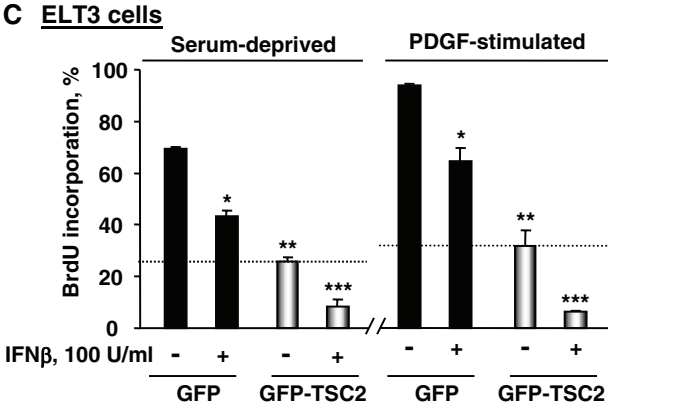


Fig. 4. A, TSC2 inhibits ribosomal protein S6 phosphorylation in LAM and ELT3 cells. Cells were transfected with plasmids expressing GFP-conjugated TSC2 (GFP-TSC2) or control GFP, then cells were serum-deprived for 24 h followed by immunocytochemical analysis with anti-phospho-S6 (P-S6) antibody. Anti-GFP antibody was used to detect transfected cells. Images taken by a Nikon Eclipse 2000 microscope under 300× magnification are representative of three independent experiments. Arrows indicate transfected cells. Slight green signal in nontransfected cells is due to autofluorescence. Data demonstrate the percentage of phospho-S6-positive GFP- or GFP-TSC2-transfected cells per total number of transfected cells. Data represent means ± S.E. from three independent experiments (top). GFP-TSC2 and GFP expression was confirmed by immunoblot analysis with anti-GFP antibody (bottom). B and C, IFNβ augments the inhibitory effect of TSC2 on LAM (B) and TSC2-null ELT3 (C) cell proliferation. LAM (B) and ELT3 (C) cells, transfected with plasmids expressing GFP-conjugated TSC2 (GFP-TSC2) or control GFP, were treated with 100 U/ml IFNβ, human (B) or rat (C), respectively, or diluent in the presence or absence of 10 ng/ml PDGF for 18 h, and then BrdU incorporation assay was performed. Data represent a percentage of GFP- and BrdU-positive cells per total number of GFP-positive cells. Data represent means ± S.E. from three independent experiments. B, *, $p < 0.01$ for IFNβ versus diluent or for IFNβ + PDGF versus PDGF; **, $p < 0.001$ for GFP-TSC2-transfected cells versus GFP-transfected cells; ***, $p < 0.01$ for GFP-TSC2 + IFNβ versus GFP-TSC2 and GFP + IFNβ. C, *, $p < 0.001$ for IFNβ versus diluent or for IFNβ + PDGF versus PDGF; **, $p < 0.001$ for GFP-TSC2 transfected cells versus GFP-transfected cells; ***, $p < 0.01$ for GFP-TSC2 + IFNβ versus GFP-TSC2 and GFP + IFNβ by ANOVA (Bonferroni-Dunn).

ylated in some cell types in response to treatment with IFNβ and may be involved in IFN-induced biological responses (Platanias, 2003), we examined whether p38 MAPK activity is involved in IFNβ-dependent LAM cell growth. We found that IFNβ induces p38 phosphorylation in both serum-deprived and PDGF-stimulated cells (Fig. 3B). Next we examined whether IFNβ-dependent p38 phosphorylation is involved in modulating growth-inhibitory effects of IFNβ by using p38 inhibitor SB203580. Cells were treated with either 10 μM SB203580 or 100 U/ml IFNβ alone or in combination in the presence or absence of 10 ng/ml PDGF, and then DNA synthesis analysis was performed as described under *Materials and Methods*. We found that SB203580 had little effect on DNA synthesis of either diluent- or IFNβ-treated LAM cells (Data not shown), suggesting that it is very unlikely that p38 MAPK may be involved in modulating LAM cell proliferation.

It is interesting that we did not detect a significant effect of IFNβ on ribosomal protein S6 phosphorylation in both serum-deprived and PDGF-stimulated LAM cells at 30, 60, 120, and 180 min of treatment (Fig. 3D). However, because S6 in LAM cells is hyperphosphorylated without any stimuli, more studies are needed to establish whether there is a cross-talk between IFNβ-dependent signaling and mTOR/S6K1 signaling pathways or whether these pathways act in parallel.

Inhibition of mTOR/S6K1 Signaling Pathway by Re-Expression of TSC2 or Rapamycin Enhances IFNβ-Dependent Inhibition of TSC2-Null and LAM Cell Proliferation. We demonstrated that TSC2 functions as a negative regulator of mTOR/S6K1 signaling pathway (Goncharova et al., 2002b, 2006a). As seen in Fig. 4A, TSC2-dependent inhibition of ribosomal protein S6 hyperphosphorylation was confirmed by immunocytochemical analysis of GFP-TSC2- transfected cells with anti-phospho-S6 and anti-GFP antibodies. Quantitative analysis shows the percentage of phospho-S6-positive cells transfected with plasmids expressing GFP-TSC2 or control GFP per total number of transfected cells. Transfected cells were detected using anti-GFP antibody (Fig. 4A, top). Our data indicate that TSC2

markedly reduced the percentage of phospho-S6-positive cells compared with cells transfected with control GFP in both LAM and ELT3 cells (Fig. 4A, top). Because abnormal proliferation of LAM and TSC2-null ELT3 cells is promoted by constitutive activation of mTOR/S6K1 signaling pathway caused by loss of TSC2 function and inhibited by TSC2 re-expression (Goncharova et al., 2002b, 2006a), we next examined whether TSC2 modulates IFNβ-dependent LAM cell proliferation. LAM and ELT3 cells were transfected with plasmids expressing GFP-conjugated TSC2 (GFP-TSC2) or control GFP, treated with 100 U/ml IFNβ or diluent in the presence or absence of 10 ng/ml PDGF for 18 h, and DNA synthesis was measured using BrdU incorporation assay (Goncharova et al., 2002b, 2006a). GFP-TSC2 and GFP expression was confirmed by immunoblot analysis with anti-GFP antibody (Fig. 4A, bottom). DNA synthesis analysis in LAM cells demonstrated that IFNβ only partially reduced the proliferation of GFP-transfected cells. It is noteworthy that IFNβ further inhibited basal and PDGF-induced BrdU incorporation in cells transfected with TSC2 compared with diluent-treated TSC2-transfected cells (Fig. 4B). To confirm our finding, we examined whether re-expression of TSC2 modulates the effects of IFNβ on the TSC2-null ELT3 cell proliferation. Likewise, IFNβ attenuated the proliferation of GFP-transfected ELT3 cells, and proliferation of ELT3 cells transfected with GFP-TSC2 and treated with IFNβ of cells was inhibited significantly (Fig. 4C). These data demonstrate that IFNβ augments TSC2-dependent inhibition of human LAM and rat TSC2-null ELT3 cell proliferation.

Because TSC2 modulates cell proliferation through inhibition of mTOR/S6K1 signaling pathway, we examined whether mTOR inhibitor rapamycin will affect antiproliferative activity of IFNβ. Examination of concentration-dependent effects of rapamycin demonstrated (Goncharova et al., 2002b) that the constitutive activity of S6K1 was abrogated and LAM cell proliferation was maximally inhibited by rapamycin at a concentration of 200 nm (Fig. 5A). In the current study, cells were treated with different concentrations of IFNβ with or without 200 nM rapamycin in the presence or absence of PDGF for 18 h followed by [^{3}H]thymidine-incorporation assay. As seen in Fig. 5, A and B, rapamycin, as demonstrated previously (Goncharova et al., 2002b, 2006a), inhibited LAM cell proliferation; it is important to note that rapamycin markedly enhanced the inhibitory effect of IFNβ on basal (Fig. 5B, top) and PDGF-induced (Fig. 5B, bottom) LAM cell proliferation in a concentration-dependent manner. Similar results were obtained using TSC2-null ELT3 cells: rapamycin alone inhibited cell proliferation in the concentration-dependent manner (Fig. 5C), and the combination of IFNβ with rapamycin further suppressed ELT3 cell proliferation compared with the effects of IFNβ and rapamycin alone (Fig. 5D). However, rapamycin, which inhibits S6 phosphorylation (Goncharova et al., 2002b, 2006a), has little effect on IFNβ-induced STAT1 phosphorylation (data not shown). Together, these data demonstrate that combination of rapamycin and IFNβ provide greater inhibition of LAM cell proliferation, potentially in the additive manner, than each agent alone.

IFNβ and Rapamycin Induce Cell Cycle Arrest in G_0/G_1 Phase. Because rapamycin augments the growth-inhibitory effect of IFNβ on LAM cell proliferation, we determined whether these agents modulate LAM cell cycle progression. We found that abnormal LAM cell proliferation is associated with alterations in cell cycle progression. The percentage of LAM cells was markedly increased in S-phase and reduced in G_0/G_1 phase compared with control cells: 21.96 $\pm$ 2.04% of serum-deprived LAM cells were accumulated in the S-phase compared with 1.75 $\pm$ 0.19% of HASM cells and 5.3 $\pm$ 0.78% of HBFs; in addition, the percentage of LAM cells in G_0/G_1 phase was 78 $\pm$ 0.64%, which is significantly lower then 94.12 $\pm$ 0.66% for HASM cells and 83.69 $\pm$ 0.31% for HBFs (Fig. 6, A and B). Both IFNβ and rapamycin significantly decreased the quantity of LAM cells in S-phase (Fig. 6C, top). The combination of IFNβ and rapamycin further reduced the percentage of LAM cells in S-phase compared with each agent alone and enriched G_0/G_1 phase (Fig. 6C, bottom). Similar results were obtained for PDGF-stimulated LAM cells (Fig. 6D). These data suggest that the combination of IFNβ and rapamycin may inhibit the proliferation of serum-deprived and PDGF-stimulated LAM cells as a result of cell cycle arrest in G_0/G_1 phase.

Rapamycin Augments IFN-Dependent Apoptosis in LAM Cells. Because it is shown that IFNβ promotes apoptosis in some cell types (Stark et al., 1998), we examined whether IFNβ alone or in combination with rapamycin promotes LAM cell apoptosis. As shown in Fig. 7, IFNβ and rapamycin alone slightly induced apoptosis in serum-deprived LAM cells. The combination of rapamycin and IFNβ markedly increased apoptosis compared with each agent added separately (Fig. 7). These data suggest that the combination of IFNβ and rapamycin may inhibit LAM cell growth through the induction of apoptosis.

Discussion

The elucidation of cellular and molecular mechanisms of cell growth modulated by loss of function of tumor suppressor TSC2 is critically important not only for better understanding of the pathobiology of a disease such LAM but also for identifying new therapeutic targets to treat LAM. In this study, we have demonstrated that TSC2-null ELT3 cells and primary cultures of human LAM cells, in contrast with other mesenchymal-derived cells such as HASM cells and HBFs, have reduced sensitivity to growth-inhibitory effects of IFNβ. Inhibition of mTOR/S6K1 signaling pathway by re-expression of TSC2 or by treatment with rapamycin augments IFNβ-induced inhibition of cell proliferation. Cotreatment of cells with IFNβ and rapamycin promotes cell cycle arrest in G_0/G_1 phase, induces apoptosis, and inhibits cell proliferation with greater efficiency than each agent alone. These data demonstrate that TSC2 dysfunction and the constitutive activation of the mTOR/S6K1 in LAM cells attenuates the growth-inhibitory effects of IFNβ in LAM and TSC2-null ELT3 cells.

IFNs play a critical role in immune surveillance against some types of cancer because of their proinflammatory, antiproliferative, and proapoptotic activities (Dunn et al., 2002; Smyth et al., 2002; Verma et al., 2003; Wilderman et al., 2005). Recent data demonstrate that type II IFNγ inhibits animal tumor and cell growth that is associated with loss of TSC2 (Hino et al., 2003; El-Hashemite et al., 2004; Lee et al., 2006). Alterations in the IFNγ-JAK-STAT pathway were reported in TSC-related and sporadic LAM (El-Hashemite and Kwiatkowski, 2005), and the correlation was demonstrated between high-expressing IFNγ allele and lower frequency of

kidney angiomyolipomas in patients with TSC (Dabora et al., 2002). Together, these findings suggest that IFNγ may suppress tumor growth associated with the loss of TSC2 function. However, the effects of IFNβ on abnormal proliferation of LAM and TSC2-deficient cells remain unclear. We found that IFNβ is expressed in LAM cells at levels similar to those seen in HASM cells and HBFs. Autocrine secretion of IFNs inhibits HASM cell proliferation (Tliba et al., 2003), and our new data show that IFNβ also inhibits proliferation of HBFs with an IC_{50} value of ~1 U/ml. In contrast, IFNβ, attenuated

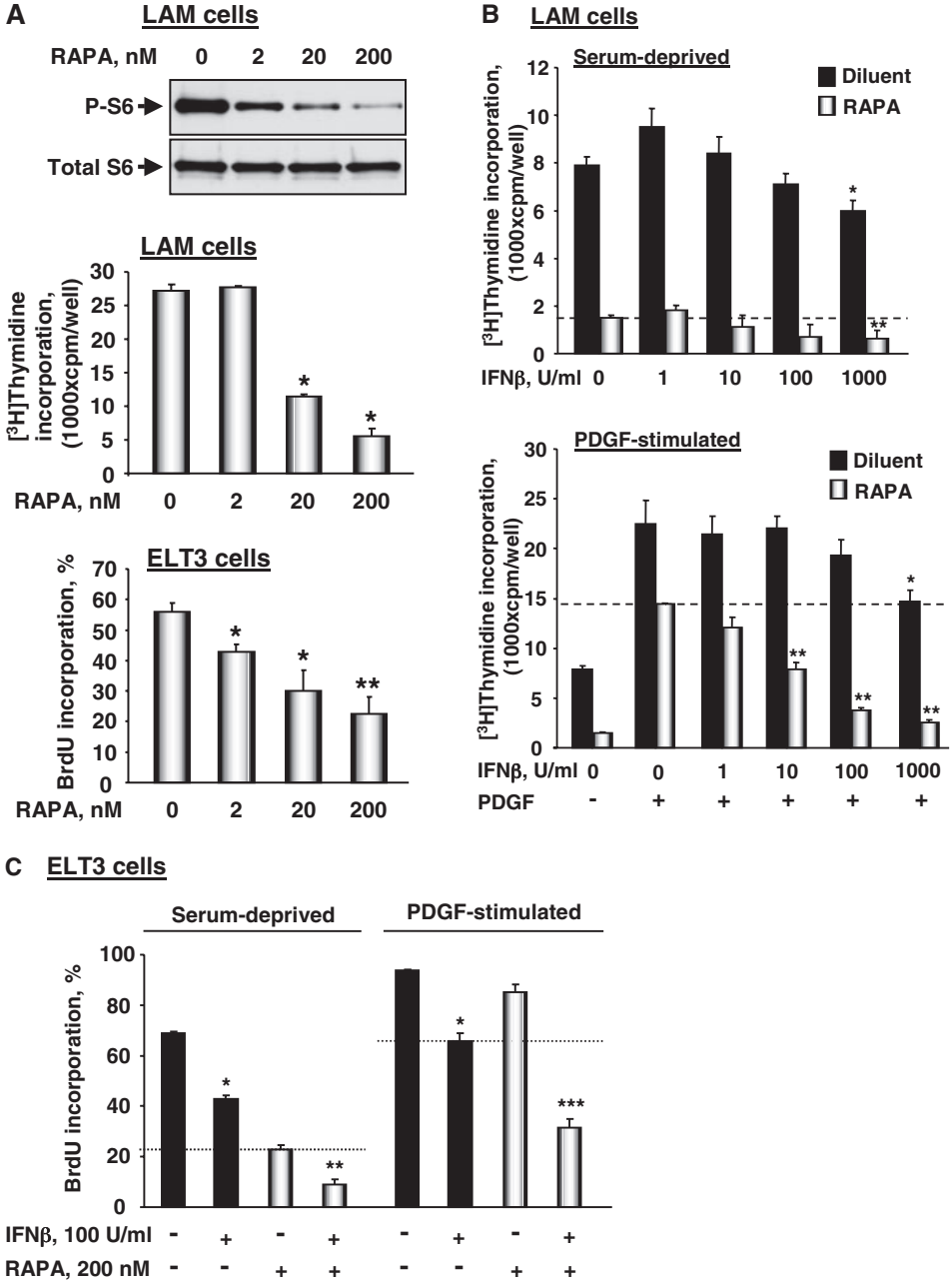


Fig. 5. A, rapamycin inhibits S6 phosphorylation and proliferation of LAM and ELT3 cells in a concentration-dependent manner. Top, serum-deprived for 24 h LAM cells were treated with 2, 20, or 200 nM RAPA or diluent for 30 min followed by immunoblot analysis with anti-phospho-S6 and anti-S6 antibodies. Images are representative of two independent experiments. Middle and bottom, DNA synthesis analysis in LAM (middle) and ELT3 (bottom) cells treated with 2, 20, and 200 nM RAPA or diluent for 18 h. Data are means ± S.E. from three independent experiments. Middle, *, $p < 0.001$ for RAPA versus diluent; bottom, *, $p < 0.05$ for 2 or 20 nM RAPA versus diluent; **, $p < 0.001$ for 200 nM RAPA versus diluent by ANOVA (Bonferroni-Dunn). B, rapamycin augments inhibitory effect of IFNβ on LAM cell proliferation. Serum-deprived cells (top) or cells treated with 10 ng/ml PDGF (bottom) were preincubated with 1, 10, 100, and 1000 U/ml human IFNβ or diluent in the presence or absence of 200 nM RAPA for 18 h, and then the [^{3}H]thymidine incorporation assay was performed. Data represent means ± S.E. from three independent experiments. Top, *, $p < 0.001$ for IFNβ versus diluent; **, $p < 0.01$ for 1000 U/ml IFNβ + RAPA versus RAPA. Bottom, *, $p < 0.001$ for IFNβ + PDGF versus PDGF; **, $p < 0.001$ for PDGF + IFNβ versus PDGF + IFNβ + RAPA by ANOVA (Bonferroni-Dunn). C, IFNβ augments rapamycin-dependent inhibition of TSC2-null ELT3 cell proliferation. Serum-deprived ELT3 cells were preincubated with 200 nM RAPA and 100 U/ml rat IFNβ separately or in combination or diluent in the presence or absence of 10 ng/ml PDGF for 18 h followed by BrdU incorporation analysis to detect DNA synthesis. Data represent means ± S.E. from three independent experiments. *, $p < 0.001$ for IFNβ versus diluent or for IFNβ + PDGF versus PDGF; **, $p < 0.01$ for IFNβ + RAPA versus IFNβ or for IFNβ + RAPA versus RAPA; ***, $p < 0.001$ for PDGF + IFNβ + RAPA versus PDGF + IFNβ and PDGF + IFNβ + RAPA versus PDGF + RAPA by ANOVA (Bonferroni-Dunn).

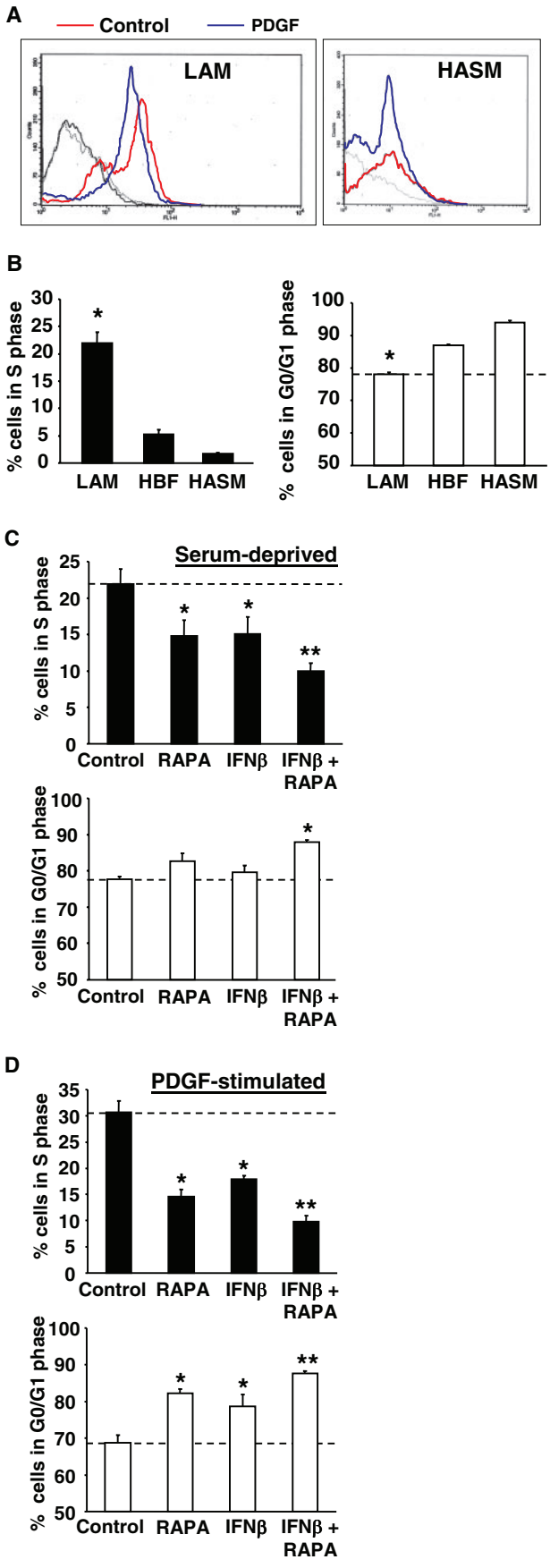


Fig. 6. A, cell cycle checkpoint status of LAM cells. Serum-deprived for 48 h LAM cells, HBFs, and HASM cells were labeled with anti-BrdU FITC-conjugated antibody and propidium iodine, and flow cytometry analysis then was performed. A, representative DNA histograms from flow cytometry analysis of LAM and HASM cells in the presence or absence of PDGF from two separate experiments. B, statistical analysis of cell cycle checkpoint. Data represent means ± S.E. from two independent experiments. *, $p < 0.001$ for LAM cells versus HASM cells and HBFs; **, $p < 0.05$ for LAM cells versus HASM cells and HBFs by ANOVA (Bonferroni-Dunn). C and D, IFNβ and rapamycin modulate LAM cell cycle progression. Serum-deprived cells were preincubated with 100 U/ml human IFNβ, 200 nM RAPA, 100 U/ml IFNβ + 200 nM RAPA, or diluent in the absence (C) or presence (D) 10 ng/ml PDGF for 40 h; then cells were labeled with anti-BrdU FITC-conjugated antibody and propidium iodine followed by flow cytometry analysis. Data represent means ± S.E. from three independent experiments. C, *, $p < 0.01$ for IFNβ or RAPA versus control; **, $p < 0.05$ for IFNβ + RAPA versus IFNβ or RAPA; ***, $p < 0.05$ for IFNβ + RAPA versus control; D, *, $p < 0.01$ for PDGF + IFNβ or PDGF + RAPA versus PDGF; **, $p < 0.05$ for PDGF + IFNβ + RAPA versus PDGF + IFNβ or PDGF + RAPA by ANOVA (Bonferroni-Dunn).

proliferation of LAM cells with an IC_{50} value of ~20 U/ml, which is 20 times higher compared with normal HBFs. Furthermore, despite the concentration of IFNβ, IFNβ inhibits LAM and ELT3 cell proliferation to approximately 30%. Our data, however, indicated no alterations in IFNβ receptor expression and IFNβ-dependent stimulation of classic receptor-dependent JAK/STAT1–2 signaling pathway in LAM cells, which, according to studies, leads to the inhibition of proliferation in different types of cells (Platanias, 2005). IFNβ may be involved in the activation of STAT3 protein, which may promote tumor progression as a result of activation of proangiogenic factors (Platanias, 2003). Abnormal Tyr705 STAT3 phosphorylation had been detected in LAM and TSC tissues (El-Hashemite and Kwiatkowski, 2005), TSC2-null mouse tumors, and mouse embryonic fibroblasts (El-Hashemite et al., 2004). We also detected increased Tyr705 STAT3 phosphorylation in serum-deprived LAM cells compared with HBFs (data not shown). However, IFNβ-dependent Tyr705 STAT3 phosphorylation was comparable in both LAM cells and HBFs, suggesting that STAT3 phosphorylation apparently is not involved in the IFNβ-dependent modulating LAM cell growth.

Evidence suggests that p38 MAPK signaling pathway may play an important role in type I IFN-dependent responses (Platanias, 2003). We found that p38 MAPK is activated by IFNβ in LAM cells. However, we found that pharmacological inhibition of p38 MAPK had little effect on LAM cell proliferation; this does not exclude that IFNβ-dependent p38 MAPK activation may be involved in other IFNβ functions.

Our previous studies demonstrated that TSC2 dysfunction leads to constitutive activation of S6K1, hyperphosphorylation of ribosomal protein S6, and increased proliferation of LAM and TSC2-null ELT3 cells. Re-expression of TSC2 or inhibiting mTOR/S6K1 activity by rapamycin markedly inhibited LAM and ELT3 cell proliferation (Goncharova et al., 2002b, 2006a). Our finding shows that expression of TSC2 or treatment with rapamycin augmented inhibitory effects of IFNβ on LAM and ELT3 cell proliferation. Because rapamycin had little effect on IFNβ-dependent STAT1 activation (data not shown), and IFNβ had little effect on mTOR/S6K1 in TSC2-null and LAM cells, it is likely that IFNβ-dependent JAK/STAT1–2 signaling and mTOR/S6K1 signaling pathways act in parallel and abrogation of mTOR/S6K1 activation by TSC2 or rapamycin enhances IFNβ-dependent inhibition of cell proliferation in an additive manner. However, further studies are needed to elucidate whether a potential cross-talk between these two signaling pathways occurs.

Abnormal TSC2-deficient tumor growth may be associated with dysregulation of G_0/G_1 to S phase transition (Tapon et al., 2001). Loss of TSC1 or TSC2 function and constitutive activation of mTOR/S6K1 signaling pathway, which associated with LAM pathogenesis (Goncharova et al., 2002b, 2006a; Krymskaya and Shipley, 2003), may result in an aberrations in G_1 to S phase traverse (Soucek et al., 1997; Miloloza et al., 2000; Tapon et al., 2001). Here, we demonstrate that the percentage of serum-deprived LAM cells is markedly increased in S-phase and reduced in G_0/G_1 phase compared with control HASM cells and HBFs. Although IFNβ inhibits cell proliferation as a result of cell cycle arrest at the G_1/S checkpoint, inhibition of mTOR signaling can also abrogate traverse of the cell cycle from the G_1 to S phase. Our findings show that combination of IFNβ and rapamycin markedly reduces the numbers of serum-deprived and PDGF-stimulated cells in S-phase and provides cell cycle arrest of LAM cells in the G_0/G_1 phase, which has led to the inhibition of LAM cell proliferation.

Because rapamycin enhances proapoptotic effects of a number of agents, the combination of IFNγ and rapamycin was markedly synergistic in the induction of apoptosis in TSC2-null mouse embryonic fibroblasts (El-Hashemite et al., 2004), and re-expression of TSC2 in TSC2-null renal tumor cells ERC18 increased its susceptibility to okadaic acid-induced apoptosis (Kolb et al., 2005). Inhibition of mTOR/S6K1 by rapamycin inhibits cap-dependent translation of several antiapoptotic proteins, thus sensitizing some cell type to apoptosis (Yan et al., 2006). We found that rapamycin augments IFNβ-induced apoptosis in both serum-deprived and PDGF-stimulated LAM cells, suggesting that IFNβ may inhibit LAM cell growth as a result of two different mechanisms: cell cycle arrest in the G_0/G_1 phase, and induction of apoptosis.

In summary, our current findings provide evidence that LAM cells express IFNβ on the levels comparable with HASM cells and HBFs but have reduced sensitivity to the growth-inhibitory effects of IFNβ compared with normal cells. IFNβ-dependent activation of classic receptor-dependent JAK/STAT signaling pathway is not abrogated in LAM cells. TSC2-dependent inhibition of constitutively activated mTOR/S6K1 signaling augments antiproliferative and proapoptotic effects of IFNβ. Treatment of LAM cells with rapamycin enhances IFNβ-dependent inhibition of LAM cell proliferation, potentially in an additive manner, because of inhibition of G_1-to-S phase transition and induction of apoptosis. These data demonstrate that TSC2 dysfunction and the constitutive activation of the mTOR/S6K1 in LAM cells attenuates growth-inhibitory effects of IFNβ and suggest that combined inhibition of mTOR/S6K1 with rapamycin and IFNβ treatment may abrogate abnormal cell growth in LAM.

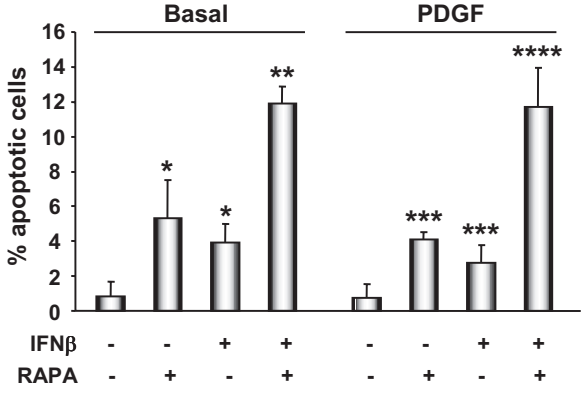


Fig. 7. Rapamycin augments IFNβ-dependent apoptosis in LAM cells. Serum-deprived LAM cells were preincubated with 200 nM RAPA and 100 U/ml IFNβ, separately or in combination, in the presence or absence of 10 mg/ml PDGF followed by apoptosis analysis using In Situ Cell Death Detection Kit based on TUNEL technology. Data represent means ± S.E. from two independent experiments, with three observations in each experiment. *, $p < 0.05$ for control versus RAPA or for control versus IFNβ; **, $p < 0.01$ for IFNβ + RAPA versus IFNβ or for IFNβ + RAPA versus RAPA; ***, $p < 0.05$ for PDGF versus PDGF + RAPA or for PDGF versus PDGF + IFNβ; ****, $p < 0.01$ for PDGF + IFNβ + RAPA versus PDGF + IFNβ or for PDGF + IFNβ + RAPA versus PDGF + RAPA by ANOVA (Bonferroni-Dunn).

Acknowledgments

We thank the patients with LAM and the LAM Registry at National Heart, Lung, and Blood Institute for providing LAM tissue.

References

Amrani Y, Tliba O, Choubey D, Huang CD, Krymskaya VP, Eszterhas A, Lazaar AL and Panettieri RA Jr (2003) IFN-gamma inhibits human airway smooth muscle cell proliferation by modulating the E2F-1/Rb pathway. *Am J Physiol Lung Cell Mol Physiol* **284:**L1063–L1071.

Dabora SL, Roberts P, Nieto A, Perez R, Jozwiak S, Franz D, Bissler J, Thiele EA, Sims K, and Kwiatkowski DJ (2002) Association between a high-expressing interferon-gamma allele and a lower frequency of kidney angiomyolipomas in TSC2 patients. *Am J Hum Genet* **71:**750–758.

de Weerd NA, Samarajiwa SA, and Hertzog PJ (2007) Type I interferon receptors: biochemistry and biological functions. *J Biol Chem* **282:**20053–20057.

Dunn GP, Bruce AT, Ikeda H, Old LJ, and Schreiber RD (2002) Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* **3:**991–998.

El-Hashemite N and Kwiatkowski DJ (2005) Interferon-{gamma}-Jak-Stat signaling in pulmonary lymphangioleiomyomatosis and renal angiomyolipoma: a potential therapeutic target. *Am J Respir Cell Mol Biol* **33:**227–230.

El-Hashemite N, Zhang H, Walker V, Hoffmeister KM, and Kwiatkowski DJ (2004) Perturbed IFN-gamma-Jak-signal transducers and activators of transcription signaling in tuberous sclerosis mouse models: synergistic effects of rapamycin-IFN-gamma treatment. *Cancer Res* **64:**3436–3443.

Goncharova EA, Ammit AJ, Irani C, Carroll RG, Eszterhas AJ, Panettieri RA, and Krymskaya VP (2002a) Phosphatidylinositol 3-kinase is required for proliferation and migration of human pulmonary vascular smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* **283:**L354–L363.

Goncharova EA, Goncharov DA, Eszterhas AJ, Hunter DS, Glassberg MK, Yeung RS, Walker CL, Noonan D, Kwiatkowski DJ, Chou MM, et al. (2002b) Tuberin regulates p70 S6 kinase activation and ribosomal protein S6 phosphorylation: a role for the TSC2 tumor suppressor gene in pulmonary lymphangioleiomyomatosis (LAM). *J Biol Chem* **277:**30958–30967.

Goncharova EA, Goncharov DA, Noonan D, and Krymskaya VP (2004) TSC2 modulates actin cytoskeleton and focal adhesion through TSC1-binding domain and the Rac1 GTPase. *J Cell Biol* **167:**1171–1182.

Goncharova EA, Goncharov DA, Spaits M, Noonan D, Talovskaya E, Eszterhas A, and Krymskaya VP (2006a) Abnormal smooth muscle cell growth in lymphangioleiomyomatosis (LAM): role for tumor suppressor TSC2. *Am J Respir Cell Mol Biol* **34:**561–572.

Goncharova EA and Krymskaya VP (2008) Pulmonary lymphangioleiomyomatosis (LAM): progress and current challenges. *J Cell Biochem,* in press.

Goncharova EA, Lim P, Goncharov DA, Eszterhas A, Panettieri RA Jr, and Krymskaya VP (2006b) Assays for in vitro monitoring proliferation of human airway smooth muscle (ASM) and human pulmonary arterial vascular smooth muscle (VSM) cells. *Nat Protoc* **1:**2905–2908.

Hartford CM and Ratain MJ (2007) Rapamycin: something old, something new, sometimes borrowed and now renewed. *Clin Pharmacol Ther* **82:**381–388.

Hino O, Kobayashi T, Momose S, Kikuchi Y, Adachi H, and Okimoto K (2003) Renal carcinogenesis: genotype, phenotype and dramatype. *Cancer Sci* **94:**142–147.

Inoki K, Corradetti MN, and Guan K-L (2005) Dysregulation of the TSC-mTOR pathway in human disease. *Nat Genet* **37:**19–24.

Johnson SR (2006) Lymphangioleiomyomatosis. *Eur Respir J* **27:**1056–1065.

Juvet SC, McCormack FX, Kwiatkowski DJ, and Downey GP (2007) Molecular pathogenesis of lymphangioleiomyomatosis: lessons learned from orphans. *Am J Respir Cell Mol Biol* **36:**398–408.

Karpusas M, Whitty A, Runkel L, and Hochman P (1998) The structure of human interferon-beta: implications for activity. *Cell Mol Life Sci* **54:**1203–1216.

Kolb TM, Duan L, and Davis MA (2005) Tsc2 expression increases the susceptibility of renal tumor cells to apoptosis. *Toxicol Sci* **88:**331–339.

Krymskaya VP (2003) Tumour suppressors hamartin and tuberin: intracellular signalling. *Cell Signal* **15:**729–739.

Krymskaya VP (2008) Smooth muscle-like cells in lymphangioleiomyomatosis. *Proc Am Thorac Soc* **5:**119–126.

Krymskaya VP, Ammit AJ, Hoffman RK, Eszterhas AJ, and Panettieri RA Jr (2001) Activation of class IA PI3K stimulates DNA synthesis in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* **280:**L1009–L1018.

Krymskaya VP and Shipley JM (2003) Lymphangioleiomyomatosis: a complex tale of

serum response factor-mediated tissue inhibitor of metalloproteinase-3 regulation. *Am J Respir Cell Mol Biol* **28:**546–550.
Kwiatkowski DJ, Zhang H, Bandura JL, Heiberger KM, Glogauer M, el-Hashemite N, and Onda H (2002) A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells. *Hum Mol Genet* **11:**525–534.
Lee L, Sudentas P, and Dabora SL (2006) Combination of a rapamycin analog (CCI-779) and interferon-gamma is more effective than single agents in treating a mouse model of tuberous sclerosis complex. *Genes Chromosomes Cancer* **45:**933–944.
Miloloza A, Rosner M, Nellist M, Halley D, Bernaschek G, and Hengstschlager M (2000) The TSC1 gene product, hamartin, negatively regulates cell proliferation. *Human Molecular Genetics* **9:**1721–1727.
Platanias LC (2003) The p38 mitogen-activated protein kinase pathway and its role in interferon signaling. *Pharmacol Ther* **98:**129–142.
Platanias LC (2005) Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* **5:**375–386.
Smyth MJ, Hayakawa Y, Takeda K, and Yagita H (2002) New aspects of natural-killer-cell surveillance and therapy of cancer. *Nat Rev Cancer* **2:**850–861.
Soucek T, Pusch O, Wienecke R, DeClue JE, and Hengstschlager M (1997) Role of the tuberous sclerosis gene-2 product in cell cycle control. *J Biol Chem* **272:**29301–29308.
Stark GR, Kerr IM, Williams BRG, Silverman RH, and Schreiber RD (1998) How cells respond to interferons. *Annu Rev Biochem* **67:**227–264.
Takaoka A and Taniguchi T (2003) New aspects of IFN-alpha/beta signalling in immunity, oncogenesis and bone metabolism. *Cancer Sci* **94:**405–411.
Talon J, Horvath CM, Polley R, Basler CF, Muster T, Palese P, and Garcia-Sastre A (2000) Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. *J Virol* **74:**7989–7996.
Tapon N, Ito N, Dickson BJ, Treisman JE, and Hariharan IK (2001) The *Drosophila* tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. *Cell* **105:**345–355.
Taveira-DaSilva AM, Steagall WK, and Moss J (2006) Lymphangioleiomyomatosis. *Cancer Control* **13:**276–285.
Thyrell L, Hjortsberg L, Arulampalam V, Panaretakis T, Uhles S, Dagnell M, Zhivotovsky B, Leibiger I, Grander D, and Pokrovskaja K (2004) Interferon α-induced apoptosis in tumor cells is mediated through the phosphoinositide 3-kinase/mammalian target of rapamycin signaling pathway. *J Biol Chem* **279:**24152–24162.
Tliba O, Tliba S, Da Huang C, Hoffman RK, DeLong P, Panettieri RA Jr, and Amrani Y (2003) Tumor necrosis factor α modulates airway smooth muscle function via the autocrine action of interferon β. *J Biol Chem* **278:**50615–50623.
Verma A, Kambhampati S, Parmar S, and Platanias LC (2003) Jak family of kinases in cancer. *Cancer Metastasis Rev* **22:**423–434.
Wilderman MJ, Sun J, Jassar AS, Kapoor V, Khan M, Vachani A, Suzuki E, Kinniry PA, Sterman DH, Kaiser LR, et al. (2005) Intrapulmonary IFN-{beta} gene therapy using an adenoviral vector is highly effective in a murine orthotopic model of bronchogenic adenocarcinoma of the lung. *Cancer Res* **65:**8379–8387.
Yan H, Frost P, Shi Y, Hoang B, Sharma S, Fisher M, Gera JF, and Lichtenstein AK (2006) Mechanism by which mammalian target of rapamycin inhibitors sensitize multiple myeloma cells to dexamethasone-induced apoptosis. *Cancer Res* **66:**2305–2313.

Address correspondence to: Dr. Elena A. Goncharova, Department of Medicine, University of Pennsylvania, TRL Suite 1200, 125 South 31st Street, Philadelphia, PA 19104. E-mail: goncharo@mail.med.upenn.edu