

Peroxisome Proliferator-Activated Receptor- γ and Retinoic Acid X Receptor α Represses the *TGF β 1* Gene via PTEN-Mediated p70 Ribosomal S6 Kinase-1 Inhibition: Role for Zf9 Dephosphorylation^[S]

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ABSTRACT

Peroxisome proliferator-activated receptor (PPAR)- γ and retinoic acid X receptor (RXR) heterodimer regulates cell growth and differentiation. Zinc finger transcription factor-9 (Zf9), whose phosphorylation promotes target genes, is a transcription factor essential for transactivation of the *transforming growth factor (TGF)- β 1* gene. This study investigated whether activation of PPAR γ -RXR heterodimer inhibits *TGF β 1* gene transcription and Zf9 phosphorylation and, if so, what signaling pathway regulates it. Either 15-deoxy- δ (12,14)-prostaglandin J₂ (PGJ₂) or 9-*cis*-retinoic acid (RA) treatment decreased the *TGF β 1* mRNA level in L929 fibroblasts. PGJ₂ + RA, compared with individual treatment alone, synergistically inhibited the *TGF β 1* gene expression, which was abrogated by PPAR γ antagonists. Likewise, PGJ₂ + RA decreased luciferase expression from the *TGF β 1* gene promoter. Promoter deletion analysis of the *TGF β 1* gene revealed that pGL3-323 making up to -323-base pair region, but lacking PPAR-responsive elements, responded to PGJ₂ + RA. PGJ₂ + RA treatment inhibited the activity of p70 ribosomal S6 kinase-1 (S6K1), abolishing Zf9 phos-

phorylation at serine as did rapamycin [a mammalian target of rapamycin (mTOR) inhibitor]. Zf9 dephosphorylation by PGJ₂ + RA was reversed by transfection of cells with the plasmid encoding constitutively active S6K1 (CA-S6K1). Transfection with dominant negative S6K1 inhibited the *TGF β 1* gene. *TGF β 1* gene repression by PGJ₂ + RA was consistently antagonized by CA-S6K1. Ectopic expression of PPAR γ 1 and RXR α repressed pGL3-323 transactivation with S6K1 inhibition, which was abrogated by CA-S6K1 transfection. PGJ₂ + RA induced phosphatase and tensin homolog deleted on chromosome 10 (PTEN), whose overexpression repressed the *TGF β 1* gene through S6K1 inhibition, decreasing extracellular signal-regulated kinase 1/2-90-kDa ribosomal S6 kinase 1 and Akt-mTOR phosphorylations. Data indicate that activation of PPAR γ -RXR heterodimer represses the *TGF β 1* gene and induces Zf9 dephosphorylation via PTEN-mediated S6K1 inhibition, providing insight into pharmacological manipulation of the *TGF β 1* gene regulation.

The human transforming growth factor- β isoforms constitute extracellular signaling molecules that have antiproliferative effects on mammalian cells, promoting the expression of cell adhesion molecules and extracellular matrix proteins.

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In particular, transforming growth factor (TGF)- β 1 serves as a key fibrogenic mediator that can enhance extracellular matrix deposition and inhibit collagenase activity during fibrogenesis (Friedman, 1993). The regulation of TGF β 1 expression is complex and occurs at multiple levels, orchestrated transcriptionally by the multiple transcription factors and post-translationally by maturation of the precursors bound with TGF β 1 binding proteins (Kim et al., 1989a; Oklu and Hesketh, 2000).

The peroxisome proliferator-activated receptors (PPARs) are transcription factors that are members of the nuclear

ABBREVIATIONS: TGF β 1, transforming growth factor; PPAR, peroxisome proliferator-activated receptor; RXR, retinoic acid X receptor; PPRE, peroxisome proliferator-activated receptor response element; RA, 9-*cis*-retinoic acid; PGJ₂, 15-deoxy- δ (12,14)-prostaglandin J₂; AP-1, activator protein-1; S6K1, p70 ribosomal S6 kinase-1; PI3, phosphatidylinositol 3; mTOR, mammalian target of rapamycin; Zf9, zinc finger transcription factor-9; PTEN, phosphatase and tensin homolog deleted on chromosome 10; DN, dominant negative; CA, constitutively active; FBS, fetal bovine serum; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; BADGE, bisphenol A diglycidyl ether; ERK, extracellular signal-regulated kinase; RSK1, p90 ribosomal S6 kinase-1; GW9662, 2-chloro-5-nitrobenzanilide.

receptor superfamily (Dubuquoy et al., 2002). Among the PPAR subtypes, PPAR γ is expressed in the major organs (Chawla et al., 1994). Treatment of animals with thiazolidinediones, synthetic PPAR γ ligands, prevented early phase hepatic fibrogenesis caused by toxicants (Kon et al., 2002) and inhibited bile duct proliferation and fibrosis in animals with cholestasis (Marra et al., 2005). This paralleled the observation that thiazolidinediones inhibited hepatic stellate cell activation (Marra et al., 2000; Hazra et al., 2004). Thus, PPAR γ is considered to be an important target for the prevention or treatment of fibrosis (Marra et al., 2000). The activated PPAR γ by binding of ligand forms a heterodimer with RXR α and binds to specific PPAR response elements (PPREs) in the promoter region of its target genes (Kliwer et al., 1992), contributing to cell survival and differentiation (Ijpenberg et al., 1997). 9-*cis*-Retinoic acid (RA) was identified as an activating ligand that is relatively selective for RXR α , which must heterodimerize with a permissive partner (Mukherjee et al., 1997). A previous study from this laboratory has shown that thiazolidinediones or 15-deoxy- δ (12,14)-prostaglandin J₂ (PGJ₂), when combined with RA at nanomolar levels, promotes PPRE-mediated gene transcription via activation of the PPAR γ -RXR heterodimer (Park et al., 2004). RXR activation inhibited the *TGF β 1* gene by antagonizing activating protein-1 (AP-1) activity (Salbert et al., 1993). Nevertheless, the role of PPAR γ -RXR heterodimer for *TGF β 1* gene regulation has never been studied.

p70 ribosomal S6 kinase-1 (S6K1), which is regulated by the phosphatidylinositol 3 (PI3)-kinase-mTOR pathway, plays as a multifunctional kinase for the phosphorylation of ribosomal S6 protein (Jeno et al., 1988), cAMP response element modulator (de Groot et al., 1994), BAD (Harada et al., 2001), and the eukaryotic elongation factor 2 kinase (Wang et al., 2001). Studies have shown that rapamycin inhibited liver fibrosis and *TGF β 1* expression in rats bile duct-ligated or challenged with toxicants (Zhu et al., 1999; Biecker et al., 2005), accompanying decrease in S6K1 activity. Although S6K1 inhibition by an mTOR inhibitor has been shown to be implicated with antifibrosis, the role of S6K1 in *TGF β 1* gene regulation and the molecular mechanistic basis have not been elucidated.

Activation of zinc finger transcription factor-9 (Zf9), also named as KLF6, is critical in the induction of *TGF β 1* during the activation of hepatic stellate cells (Ratziu et al., 1998). The *TGF β 1* gene contains the DNA response element interacting with Zf9 (Kim et al., 1989a). Zf9 also regulates *TGF β* receptors and collagen α 1(I), thereby promoting accumulation of extracellular matrix (Kim et al., 1998). Thus, Zf9 regulates multiple genes involved in tissue differentiation. In addition, Zf9 as an immediate early gene reduces cell proliferation with the induction of p21^{cip1} and the enhancement of c-Jun degradation (Narla et al., 2001; Slavin et al., 2004), thus functioning as a potential tumor suppressor gene. Activation of Zf9 includes its phosphorylation at serine (or tyrosine) residues (Warke et al., 2003). Thus, phosphorylation of Zf9 leads to transcription of its target genes (Warke et al., 2003; Slavin et al., 2004). However, the kinase catalyzing Zf9 phosphorylation has not been studied yet.

In the present study, we attempted to determine whether PPAR γ -RXR heterodimer represses the *TGF β 1* gene, and if so, what signaling pathway regulates the gene repression and phosphorylation of Zf9. In addition, we tried to deter-

mine whether the nuclear receptor heterodimer activates the putative PPREs located in the promoter region of the *TGF β 1* gene. We found that activation of PPAR γ and RXR heterodimer results in the inhibition of S6K1 activity, which contributes to *TGF β 1* gene repression. Because phosphatase and tensin homolog deleted on chromosome 10 (PTEN) antagonizes the PI3-kinase-mTOR-S6K1-mediated signaling cascade (Liu et al., 2005), we also explored the effect of PGJ₂ + RA on the expression of PTEN and the role of PTEN up-regulation in the S6K1 inhibition for *TGF β 1* gene repression by PGJ₂ + RA.

Materials and Methods

Materials. PGJ₂ were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Pioglitazone and rosiglitazone were supplied from Dong-A Pharmaceutical Co. (Shingal, Korea). RA, rapamycin, and anti-phosphoserine antibody were purchased from Sigma-Aldrich (St. Louis, MO). Anti-NF1 antibody, anti-SP1 antibody, and anti-Zf9 antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibodies directed against S6 protein, phosphorylated S6 protein, and PTEN were supplied from Cell Signaling Technology Inc. (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit and rabbit anti-goat IgGs were purchased from Zymed Laboratories (South San Francisco, CA). A series of deletion constructs of pGL3-TGF β 1 containing the human *TGF β 1* promoter region were kindly provided from Dr. S. J. Kim (National Cancer Institute, Bethesda, MD). The expression construct encoding mouse PPAR γ 1 (pCMX-mPPAR γ 1) was supplied from Dr. C. K. Glass (University of California, San Diego, CA). The human RXR α expression plasmid (PECE-RXR α) was a gift from Dr. M. O. Lee (Seoul National University, Seoul, Korea). The S6K1 expression constructs PRK5 myc-tagged 2BQ (dominant negative, DN-S6K1) and D3E (constitutively active, CA-S6K1) were supplied from Dr. J. H. Han (Sungkyunkwan University, Suwon, Korea), originally provided by Dr. G. Thomas (Friedrich Miescher Institut, Basel, Switzerland) (Hannan et al., 2003; Pesce et al., 2003). The PTEN expression plasmid was donated by Dr. S. G. Rhee (National Institutes of Health, Bethesda, MD).

Cell Culture. L929, a mouse fibroblast cell line was obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μ g/ml streptomycin at 37°C in a humidified atmosphere with 5% CO₂. L929 cells that had been cultured in the medium containing 10% FBS were incubated without serum for 12 h and then exposed to PGJ₂, RA, PGJ₂ + RA, pioglitazone, or rosiglitazone, dissolved in dimethyl sulfoxide, for the indicated time period at 37°C.

Reverse Transcription-Polymerase Chain Reaction and Real-Time Reverse Transcription-Polymerase Chain Reaction. Total RNA was isolated from L929 cells using the improved single-step method of thiocyanate-phenol-chloroform RNA extraction, and RT-PCR analysis was carried out according to the procedures described previously (Kang et al., 2002). In this study, we used semiquantitative RT-PCR analysis, which was proven to be adequate for quantification of *TGF β 1* mRNA levels (Kruse et al., 1999). RT-PCR was performed using the selective primers for *TGF β 1* (sense primer, 5'-CTTCAGCTCCACAG AGAAGAAGCTGC-3' and antisense primer, 5'-CACGATCATGTTGGACAAGCTGCTCC-3'; 298 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes (sense, 5'-TCGTGGAGTCTACTGGCG T-3' and antisense, 5'-GCCTGCTTCACCACCTTCT-3'; 510 bp). PCRs were carried out for 26 to 29 cycles using the following conditions: denaturation at 94°C for 0.5 min, annealing at 54°C for 0.5 min, and elongation at 72°C for 1 min, and the optimal cycle was selected for quantification. Band intensities of the amplified DNAs were compared after visualization on an UV transilluminator (Alpha-Innotech, San Leandro, CA). In some

experiments, real-time PCRs were performed in a Light Cycler 1.5 (Roche Diagnostics, Mannheim, Germany) using Light Cycler DNA Master SYBR Green I kit according to the manufacturer's instruction. A thermal profile for SYBR Green RT-PCR was 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, at 51°C for 5 s, and at 72°C for 15 s. A melting curve analysis was done after amplification to verify the accuracy of the amplicon.

Luciferase Reporter Gene Analysis. To determine TGFβ1 activity, we used the dual-luciferase reporter assay system (Promega, Madison, WI). In brief, L929 cells (7×10^5 cells/well) were replated in six-well plates overnight, serum-starved for 12 h, and transiently transfected with pGL3-TGFβ1 promoter-luciferase construct and 0.3 μg of pCMV-β-galactosidase plasmid (Invitrogen, Carlsbad, CA) in the presence of LipofectAMINE reagent (Invitrogen) for 3 h. The pCMV-β-galactosidase plasmid was used to evaluate the transfection efficiency. Transfected cells were incubated in the medium containing 1% FBS (Invitrogen) for 3 h and exposed to PGJ₂ + RA (30 nM each) in the medium containing 1% FBS for 12 h at 37°C. For β-galactosidase activity, 10 μg of cell lysates was added to the solution containing 0.88 mg/ml *o*-nitrophenyl-β-D-galactopyranoside, 100 μM MgCl₂, and 47 mM β-mercaptoethanol in 100 mM sodium phosphate buffer. The reaction mixture was incubated for 2 h at 37°C, and the absorbance was determined at 420 nm. The relative luciferase activity was calculated by normalizing firefly luciferase activity to that of β-galactosidase. In case of PPARγ and/or RXRα overexpression, cells were cotransfected with pCMX-mPPARγ1 and/or PECE-RXRα in combination with pGL3-323 and incubated in the medium containing 1% FBS for 12 h. In some experiments, cells were transfected with the plasmid encoding CA-S6K1 or DN-S6K1 in combination with pGL3-323 and incubated in the medium containing PGJ₂ + RA for 12 h.

Preparation of Cell Lysates and Nuclear Extracts. Lysates and nuclear extracts were prepared according to previously published methods (Park et al., 2004). In brief, cells were centrifuged at 2300g for 3 min and allowed to swell after the addition of the lysis buffer. The samples were centrifuged at 10,000g for 10 min to obtain cell lysates. To prepare nuclear extracts, cells were allowed to swell after the addition of 100 μl of hypotonic buffer. The lysates were incubated for 10 min on ice and then centrifuged at 7200g for 5 min at 4°C. Pellets containing crude nuclei were resuspended in 50 μl of extraction buffer. Nuclear extracts were prepared from the samples by centrifugation at 15,000g for 10 min and stored -70°C until use. Protein content was determined by the Bradford assay (Bio-Rad protein assay kit; Bio-Rad, Hercules, CA).

S6K1-Immune Complex Kinase Assay. To determine the S6K1 activity, S6K1 in cell lysates (200 μg) was immunoprecipitated, and the samples were washed three times in lysis buffer and once in the kinase buffer containing 25 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 25 mM β-glycerophosphate, 1 mM Na₃VO₄, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 200 μM ATP. Kinase reaction was initiated by adding S6 substrate peptide (5 μg per assay) and 2 μCi of [γ -³²P]ATP to a 20-μl reaction mixture and continued for 30 min at 30°C. After brief centrifugation, the supernatant of reaction mixture was spotted onto p81 phosphocellulose paper (Upstate, Lake Placid, NY). The paper was washed with 0.8% phosphoric acid for 5 min three times and subsequently with 90% ethanol for 5 min. The membrane was dried and transferred to 5 ml of scintillation cocktail, and the radioactivity of phosphorylated substrate was measured using a beta-counter (PerkinElmer Wallac, Gaithersburg, MD).

Immunoprecipitation. To determine serine phosphorylations of Zf9, NF1, or SP1, fractions of lysates or nuclear extracts were incubated with the respective antibodies overnight at 4°C for immunoprecipitation. Immune complex precipitated with protein G-agarose was solubilized in 2× Laemmli buffer and boiled. Samples were resolved in 7.5% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The samples were immunoblotted with anti-phosphoserine antibody. The bands were devel-

oped using an ECL chemiluminescence detection kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Transient Transfection. Cells (5×10^5 cells/well) were replated in six-well plates overnight, serum-starved for 6 h, and transiently transfected with pCMX-mPPARγ1 and/or PECE-RXRα (0.5 μg each) in the presence of LipofectAMINE reagent. The transfected cells were incubated in the medium containing 1% FBS for 3 h and subjected to immunoblot analysis. Cells were also transfected with the plasmid encoding PTEN (0.3 or 1 μg) with or without empty plasmid to adjust the total amount of plasmids transfected to 1 μg.

Immunoblot Analysis. SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to previously published procedures (Park et al., 2004) with antibodies specifically directed against Zf9, NF1, SP1, S6 protein, actin, or PTEN.

Statistical Analysis. Scanning densitometry was performed with Image Scan and Analysis System (Alpha-Innotech). One-way analysis of variance procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, the Newman-Keuls test was used for comparisons of multiple group means. The criterion for statistical significance was set at $p < 0.05$ or $p < 0.01$. All statistical tests were two-sided.

Results

Repression of the TGFβ1 Gene by PGJ₂ + RA. To examine the role of PPARγ activation in TGFβ1 expression, we first assessed the dose-dependent effects of PGJ₂, an endogenous PPARγ agonist, or RA, a RXR agonist, on TGFβ1 expression in L929 cells (Fig. 1A). Semiquantitative RT-PCR analysis showed that PGJ₂, at the concentration of 100 or 1000 nM inhibited TGFβ1 expression 20 to 40% 12 h after treatment, indicating that PGJ₂ at the relatively high concentrations weakly inhibited the gene expression. Treatment of the cells with 30 to 100 nM RA for 12 h also repressed the level of TGFβ1 mRNA by 30 to 40% (Fig. 1A). RA at 1000 nM blocked TGFβ1 expression by >50%. Data showed that either PGJ₂ or RA alone moderately decreased the expression of TGFβ1 gene in L929 fibroblasts.

PPARγ heterodimerizes with RXR for activation, and the PPARγ-RXR heterodimers are widely expressed in major organs (Dubuquoy et al., 2002). Next, we determined whether PGJ₂ + RA inhibited TGFβ1 expression. In our previous study, PGJ₂ + RA enhanced class α glutathione S-transferase gene expression to the greatest extent at the 1:1 molar ratio (Park et al., 2004). Therefore, in the subsequent experiments, the cells were similarly treated with PGJ₂ + RA. Compared with the individual treatment, combination treatment of PGJ₂ and RA at equal molar concentrations synergistically down-regulated TGFβ1. TGFβ1 expression was inhibited 40% by treatment of the cells with PGJ₂ + RA as low as at 10 nM each (Fig. 1B). PGJ₂ + RA at the concentrations of 30 to 100 nM inhibited TGFβ1 expression 70 to 80%. A time-course study using 30 nM each of PGJ₂ and RA showed that the TGFβ1 mRNA levels were markedly decreased 12 to 24 h after treatment (Fig. 1C). Real-time RT-PCR analysis using SYBR Green I confirmed the synergistic repression of TGFβ1 gene expression by PGJ₂ + RA treatment compared with PGJ₂ or RA treatment alone (Fig. 1D), implying that PPARγ activation in combination with that of RXR efficaciously down-regulates the TGFβ1 gene.

PPARγ-Dependent Repression of TGFβ1 Gene by PGJ₂ + RA. The role of PPARγ activation in TGFβ1 repression was examined by the experiments using thiazolidinedi-

one PPAR γ agonists. Treatment of L929 cells with either 10 μ M rosiglitazone or 10 μ M pioglitazone for 12 h significantly decreased the expression of TGF β 1 mRNA (Fig. 2A). The suppressed TGF β 1 transcript by rosiglitazone or pioglitazone confirms the role of ligand activation of PPAR γ in TGF β 1 repression.

To further verify the role of PPAR γ in the regulation of TGF β 1 gene, we examined the effects of PPAR γ antagonists on the TGF β 1 expression and on the repression of TGF β 1 by PGJ $_2$ + RA. Treatment of L929 cells with a PPAR γ antagonist BADGE (10 μ M; 13 h) alone significantly increased the gene expression and abrogated TGF β 1 repression elicited by PGJ $_2$ + RA (30 nM each) (Fig. 2B, left). Increase in TGF β 1 expression by BADGE treatment suggests that activated PPAR γ serves as an endogenous negative regulator for TGF β 1 expression. Another PPAR γ antagonist, GW9662 (1 μ M), also completely blocked TGF β 1 repression by PGJ $_2$ + RA (Fig. 2B, right). These data showed that activation of PPAR γ , which in combination with RXR activation enhances TGF β 1 repression, plays a critical role in the regulation of the TGF β 1 gene.

TGF β 1 Reporter Gene Analysis with Promoter Deletions. The effects of PPAR γ and RXR activation on the TGF β 1 gene transactivation that is regulated by the proximal DNA response elements were examined as an effort to identify the molecular basis of TGF β 1 repression by PGJ $_2$ + RA. The potential regulatory sites responsible for the TGF β 1 gene expression were first explored by using the luciferase reporter gene assays. To precisely define the role of DNA elements interacted with transcription factors in the gene repression, this study used a series of promoter deletion mutants: the deletion mutants of the structural TGF β 1 gene downstream of the -1.36-kilobase promoter region included

pGL3-1362, pGL3-1132, pGL3-731, pGL3-453, pGL3-323, and pGL3-175 (Fig. 3A). The putative PPRES were located at the multiple sites upstream from -453 bp of the promoter region.

Exposure of pGL3-1132-transfected cells to PGJ $_2$ + RA (30 nM each) resulted in a 35% decrease in the luciferase activity (Fig. 3B). PGJ $_2$ + RA decreased the relative luciferase-inducible activity by 50% in cells transfected with pGL3-731, compared with vehicle-treated control. TGF β 1 repression by PGJ $_2$ + RA was unaffected by deletion of the promoter containing the region down to -453 bp. Luciferase activity was down-regulated by PGJ $_2$ + RA treatment in cells transfected with pGL3-453 that contains no putative PPRES, indicating that the putative PPRES sites located at the upstream region were not functional for the gene repression. We further examined whether PGJ $_2$ + RA had the ability to repress reporter gene expression from pGL3-323 that misses the AP-1 binding sites present within the region between -453 and -323 bp. Although the constitutive luciferase expression was largely decreased by deletion of the AP-1-containing region, PGJ $_2$ + RA still inhibited luciferase expression from pGL3-323. Such promoter deletion analyses indicate that PGJ $_2$ + RA significantly inhibits luciferase reporter activity in cells transfected with the TGF β 1 promoter, suggesting that neither the putative PPRES nor the AP-1 binding sites are directly regulated by PGJ $_2$ + RA for the gene repression.

Inhibition of S6K1 by PGJ $_2$ + RA. Because S6K1 has been implicated in the regulation of fibrogenesis (Zhu et al., 1999), we sought to determine the effects of PGJ $_2$ + RA on the activity of S6K1 in association with TGF β 1 gene repression. The S6K1 is a physiological kinase that phosphorylates 40S ribosomal S6 protein in cells (Chung et al., 1992). PGJ $_2$ + RA decreased phosphorylation of S6 protein 3 to 12 h after

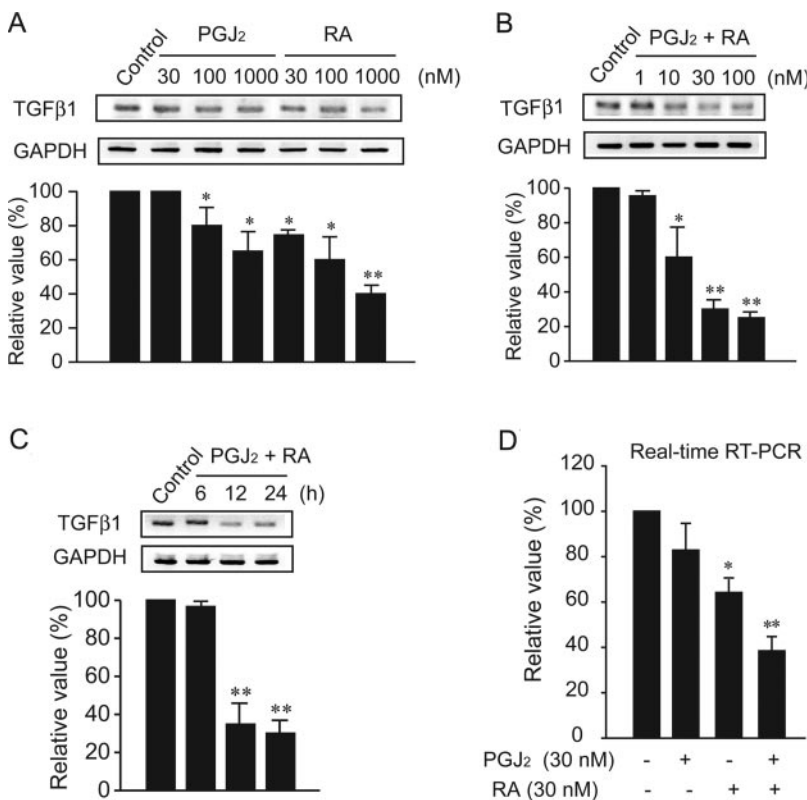
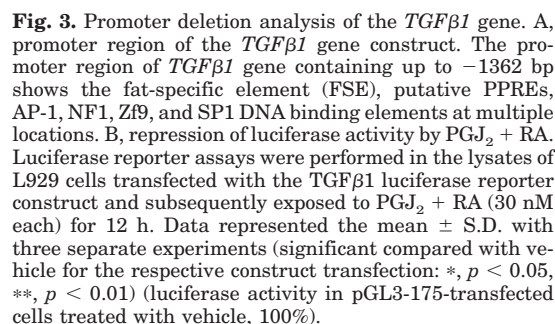
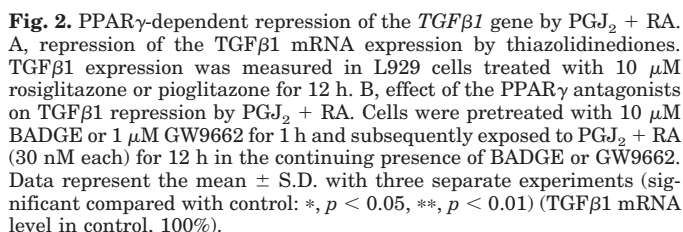


Fig. 1. Effects of PGJ $_2$ and/or RA on TGF β 1 gene expression. A, RT-PCR analysis of the TGF β 1 mRNA levels. Semiquantitative RT-PCR analyses were performed in the total RNA prepared from L929 cells treated with 30 to 1000 nM PGJ $_2$ or RA for 12 h. The GAPDH mRNA levels were monitored as controls. The change in TGF β 1 mRNA relative to that of GAPDH was assessed by scanning densitometry of the band intensities. B, effect of PGJ $_2$ + RA on TGF β 1 mRNA expression. Representative RT-PCR analysis shows the levels of TGF β 1 mRNA in cells treated with PGJ $_2$ + RA at the concentrations of 1 to 100 nM each for 12 h. C, the relative TGF β 1 mRNA levels in cells treated with PGJ $_2$ + RA (30 nM each) for 6 to 24 h. D, real-time RT-PCR analysis. Real-time RT-PCR analysis was performed in the total RNA prepared from cells treated with PGJ $_2$ or RA alone or in combination for 12 h. Data represent the mean \pm S.D. with three separate experiments (significant compared with control: *, $p < 0.05$; **, $p < 0.01$) (TGF β 1 mRNA level in control, 100%).

we measured the kinase activity of S6K1 immunoprecipitated in the lysates of cells treated PGJ₂ + RA. PGJ₂ + RA treatment consistently decreased the immune complex kinase activity in a time- and concentration-dependent manner (Fig. 4B).

phorylation. The transcription factors that interact with the known DNA binding sites on the region downstream within the -323 bp of the *TGF β 1* gene include Zf9, NF1, and SP1 (Fig. 3A). In view of the previous observations that Zf9 is crucial as a transcription factor for TGF β 1 induction in hepatic stellate cells (Kim et al., 1998) and that phosphorylated form of Zf9 plays a role in the transactivation of the target gene promoter (Warke et al., 2003), we next investigated the potential ability of PGJ₂ + RA to inhibit serine phosphorylation of the transcription factor. Immunoblotting for phosphorylated serine in Zf9 immunoprecipitates from lysates revealed that serine phosphorylation of Zf9 was markedly inhibited by PGJ₂ + RA treatment (6 h) (Fig. 5A). In contrast, NF1 and SP1 phosphorylations were unaffected. Therefore, it was presumed that *TGF β 1* gene repression by PGJ₂ + RA might have resulted from dephosphorylation of Zf9.

Given the inhibition of S6K1 activity by $\text{PGJ}_2 + \text{RA}$, we next determined the effect of S6K1 inhibition on Zf9 dephosphorylation. The inhibition of Zf9 phosphorylation by rapamycin that inhibits S6K1 activity via mTOR inhibition supported the role of S6K1 in Zf9 phosphorylation (Fig. 5B). As expected, serine-phosphorylated Zf9 level was also decreased by $\text{PGJ}_2 + \text{RA}$ treatment (6 h). Inhibition of S6 protein phosphorylation by the agents was confirmed (Fig. 5B). To verify the role of S6K1 activity in Zf9 phosphorylation, we tested whether $\text{PGJ}_2 + \text{RA}$ inhibition of Zf9 phosphorylation was reversed by the constitutive activation of S6K1. Multiple analyses showed that Zf9 phosphorylation in untreated cells that express CA-S6K1 was comparable with that in mock-transfected cells, which may have resulted from saturation of Zf9 phosphorylation in L929 cells because of its high constitutive phosphorylation and/or the limit of detection method.



(i.e., Zf9 immunoprecipitation and pan-phosphoserine antibody immunoblot). More importantly, transfection of the cells with CA-S6K1 abrogated dephosphorylation of Zf9 elicited by PGJ₂ + RA (Fig. 5C). We verified good transfection efficiency of CA-S6K1 in the cells by immunocomplex kinase assay of S6K1 (2.3-fold increase relative to mock transfection). Our finding that Zf9 dephosphorylation was antagonized by CA-S6K1 supports the possibility that PGJ₂ + RA inhibits *TGFβ1* gene transcription via Zf9 dephosphorylation because of S6K1 inhibition.

Role of S6K1 in *TGFβ1* Gene Expression. Next, to assess the role of S6K1 for the *TGFβ1* gene expression, pGL3-323 luciferase assay was performed in cells treated with PGJ₂ + RA after transfection with the plasmid encoding CA-S6K1. CA-S6K1 transfection abrogated the ability of PGJ₂ + RA to repress luciferase expression from pGL3-323 (Fig. 6, left). It seems that the basal *TGFβ1* reporter gene activity was rather increased by CA-S6K1 transfection alone. As expected, DN-S6K1 transfection inhibited luciferase ex-

pression from pGL3-323 (Fig. 6, right). Data presented here identifies the role of S6K1 inhibition by PGJ₂ + RA for *TGFβ1* gene repression.

***TGFβ1* Repression by PPARγ1-RXRα-Mediated S6K1 Inhibition.** To further verify the functional role of the PPARγ-RXRα heterodimer in the inhibition of the *TGFβ1* gene, we monitored luciferase expression from pGL3-323 in cells transfected with PPARγ1 or/and RXRα. Either PPARγ1 or RXRα alone significantly inhibited pGL3-323 gene expression (Fig. 7A). Furthermore, transfection of cells with both PPARγ1 and RXRα almost abolished luciferase expression from pGL3-323, the extent of which was comparable with that elicited by PGJ₂ + RA. Results from this receptor overexpression experiment demonstrate that activation of PPARγ-RXR heterodimer indeed contributes to the *TGFβ1* gene repression. Furthermore, we determined whether PPARγ-RXRα overexpression led to inhibition of S6 protein phosphorylation. Either PPARγ or RXRα alone, or in combination, notably inhibited the phosphorylation of S6 protein

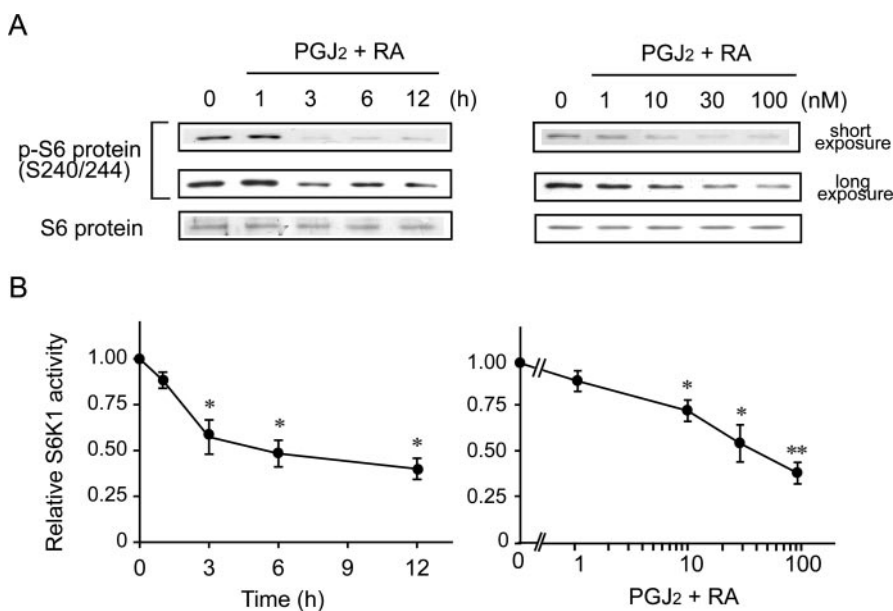


Fig. 4. S6K1 inhibition by PGJ₂ + RA. A, effect of PGJ₂ + RA on the phosphorylation of S6 protein. S6 protein phosphorylation was determined in the lysates prepared from cells treated with PGJ₂ + RA (30 nM each) for 1 to 12 h or those treated with 1 to 100 nM PGJ₂ + RA for 6 h. B, effect of PGJ₂ + RA on S6K1 immune complex kinase activity. The kinase activity of S6K1 toward S6 substrate peptide was determined by monitoring ³²P radioactivity in the S6K1 immune complex precipitated from lysates. Data represent the mean ± S.D. with three separate experiments (significant compared with control: *, *p* < 0.05, **, *p* < 0.01) (S6K1 activity in control, 1).

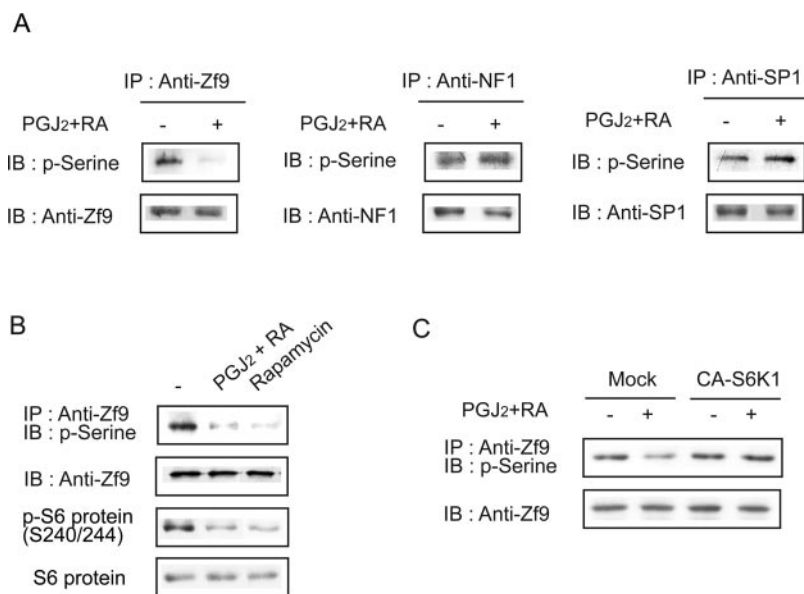


Fig. 5. Role of S6K1 inhibition by PGJ₂ + RA in Zf9 dephosphorylation. A, inhibition of Zf9 dephosphorylation by PGJ₂ + RA. Immunoblot analyses were performed with anti-phosphoserine antibody in Zf9, NF1, or SP1 immunoprecipitates obtained from the lysates (200 μg each) of cells treated with vehicle or PGJ₂ + RA for 6 h. B, Zf9 dephosphorylation by PGJ₂ + RA or rapamycin. Immunoblot analysis for serine-phosphorylated Zf9 was carried out in the nuclear fractions of cells treated with PGJ₂ + RA (30 nM each) or rapamycin (30 nM) for 6 h. C, CA-S6K1 reversal of Zf9 dephosphorylation by PGJ₂ + RA. Cells were transfected with the empty plasmid (mock) or the plasmid encoding CA-S6K1 (0.5 μg each), incubated in the medium containing 1% FBS for 12 h, and then treated with PGJ₂ + RA for 6 h. Immunoblottings for phosphorylated serine were carried out in Zf9 immunoprecipitates from the nuclear extracts. S6K1 immune complex kinase activity in cells transfected with CA-S6K1 construct was 2.3-fold increased compared with that in mock-transfected cells. Results were confirmed by three independent experiments.

(Fig. 7B). As anticipated, pGL3-323 *TGF β 1* gene repression by PPAR γ and RXR α was reversed by transfection with CA-S6K1 (Fig. 7C). Again, CA-S6K1 transfection alone increased the basal gene expression. These results provide evidence that the activation of PPAR γ -RXR α results in the inhibition of S6K1 and that the S6K1 inhibition was responsible for the *TGF β 1* gene repression.

Role of PGJ₂ + RA-Mediated PTEN Induction for S6K1 Inhibition. Functional PPRES are located in the PTEN promoter (Patel et al., 2001). It has been shown that PPAR γ activation induces PTEN, which antagonizes PI3-kinase-mediated cell signaling (Lee et al., 2005). To study more in depth the mechanistic basis of the inhibition of *TGF β 1* gene by PGJ₂ + RA, we determined whether ligand activation of PPAR γ -RXR α was capable of inducing PTEN. A time-course study revealed that PGJ₂ + RA treatment induced PTEN in L929 fibroblast cells, beginning from 3 h at least up to 12 h after treatment (Fig. 8A). We further examined the effect of ectopic PTEN expression on the phosphorylation of S6 protein and *TGF β 1* gene expression. S6 protein phosphorylation notably decreased after PTEN induction presumably through decrease in the formation of phosphatidylinositol-(3,4,5)-trisphosphate, whose production is catalyzed by PI3-kinase (Fig. 8B). *TGF β 1* gene was also repressed by PTEN expression (Fig. 8C). To verify the antagonism of PI3-kinase activity against *TGF β 1* repression by PGJ₂ + RA, PGJ₂ + RA-dependent luciferase gene expression was measured in cells transfected with the plasmid encoding p110, the catalytic subunit of PI3-kinase. The basal *TGF β 1* reporter gene activity from pGL3-323 was increased by p110 transfection (Fig. 8D). More importantly, p110 overexpression inhibited the ability of PGJ₂ + RA to repress luciferase expression from pGL3-323. Together, these data indicate that the induction of PTEN by PGJ₂ + RA may

result in *TGF β 1* gene repression as a consequence of S6K1 inhibition.

Effects on Cellular Kinases Downstream of PTEN. Finally, we observed that PGJ₂ + RA treatment inhibited phosphorylations of the major cellular kinases (Akt, ERK1/2, RSK1, and mTOR) downstream of PTEN (Fig. 9A). The results indicate that PTEN induction by PGJ₂ + RA leads to S6K1 inhibition via the pathways of ERK1/2-RSK1 as well as Akt-mTOR (Fig. 9B).

Discussion

Studies on the regulation of the *TGF β 1* gene and the molecular interactions of ligand-activated nuclear receptors for the activation of responsible transcription factor(s) provide insight into the transcriptional control mechanism. In the

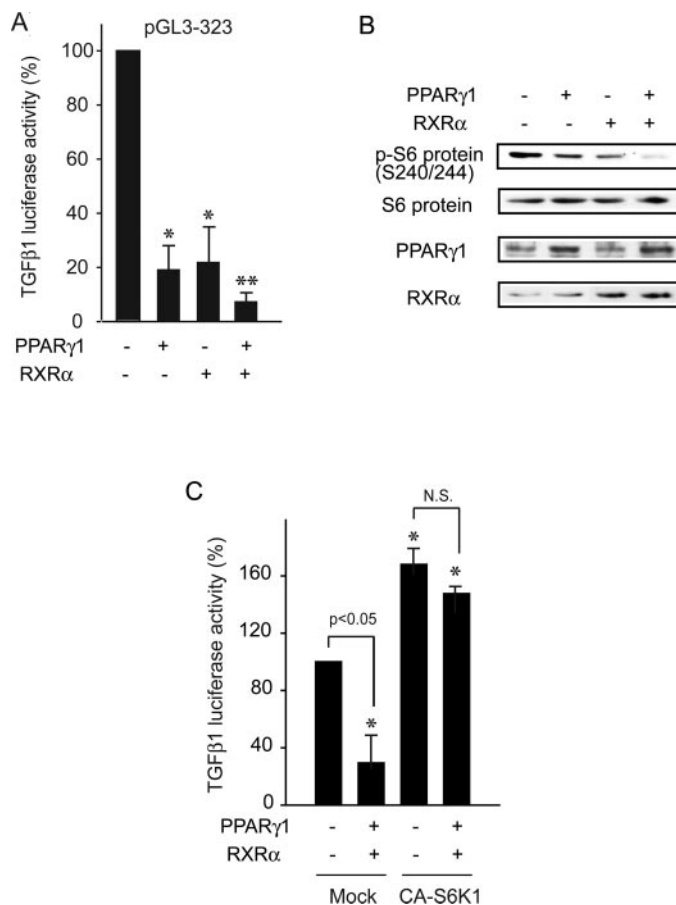


Fig. 7. Role of S6K1 inhibition in *TGF β 1* repression by PPAR γ 1-RXR α heterodimer. **A**, *TGF β 1* gene repression by PPAR γ 1 and RXR α heterodimer. Cells were transfected with pGL3-323 (1 μ g) in combination with an empty vector or with the PPAR γ 1 or/and RXR α plasmids (0.5 μ g each) in the presence of LipofectAMINE for 3 h and incubated in the medium containing 1% FBS for 12 h. Luciferase activity from pGL3-323 was measured in cell lysates. **B**, inhibition of S6 protein phosphorylation by PPAR γ 1-RXR α heterodimer. Serine-phosphorylated S6 protein was determined by immunoblotting in the lysates of cells transfected with the PPAR γ 1 or/and RXR α plasmids, as described in **A**. **C**, *TGF β 1* luciferase activity. Luciferase activity from pGL3-323 was determined in the lysates prepared of cells transfected with the PPAR γ 1 and RXR α plasmids (0.5 μ g each) with or without an empty plasmid (mock) or the plasmid encoding CA-S6K1 (0.5 μ g). The total amount of plasmids transfected was identical in each sample (1.5 μ g). Data represented the mean \pm S.D. with three separate experiments (significant compared with mock-transfected control: *, $p < 0.05$, **, $p < 0.01$) (luciferase activity in mock transfection, 100%).

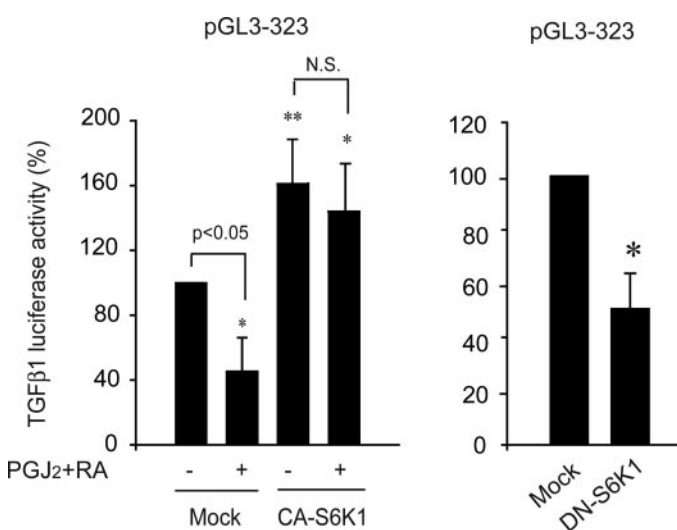


Fig. 6. Role of S6K1 inhibition in *TGF β 1* repression by PGJ₂ + RA. Cells were transfected with pGL3-323 in combination with the empty plasmid (mock) or the plasmid encoding CA-S6K1 (0.5 μ g) for 3 h and further incubated in the medium containing 1% FBS for 16 h. Luciferase activity was determined in the lysates prepared from cells treated with PGJ₂ + RA for 12 h. Luciferase activity from pGL3-323 was also assayed in cells transfected with DN-S6K1 (0.5 μ g). Data represented the mean \pm S.D. with three separate experiments (significant compared with mock-transfected control: *, $p < 0.05$, **, $p < 0.01$) (luciferase activity in vehicle-treated mock-transfected cells, 100%).

present study, we demonstrated that either PPAR γ or RXR agonist alone at relatively high concentrations down-regulated the *TGF β 1* gene, whereas concomitant treatment with both PPAR γ and RXR agonists synergistically repressed the gene. PGJ₂ at low concentrations serves as an agonist of PPAR γ . RXRs are modular proteins with a highly conserved central DNA binding domain and a less conserved ligand binding domain (Holmbeck et al., 1998). PGJ₂ alone at low nanomolar concentrations is a weak repressor of *TGF β 1* because activated PPAR γ requires additional binding of ligand-bound RXR for the formation of a PPAR γ -RXR heterodimer. This is consistent with the current observation that RA potentiated *TGF β 1* gene repression by PGJ₂, although RA alone weakly repressed the gene. Enhanced *TGF β 1* repression by PGJ₂ + RA, compared with that by each agent alone, implies that PPAR γ and RXR heterodimer activation contributes to the gene regulation. Our hypothesis was strongly supported by the observation that ectopic expression of PPAR γ 1 and RXR α almost completely inhibited luciferase expression from pGL3-323. The role of PPAR γ in the repression of the *TGF β 1* gene was further evidenced by the repressing effect of its glitazone ligand and also by the reversal of *TGF β 1* repression by PPAR γ antagonists. Our results presented here identify the novel aspect that PPAR γ activation contributes to *TGF β 1* gene down-regulation and that ligand activation of RXR α is necessary for the full responsiveness in the gene repression by PPAR γ activator.

Such a finding showing PGJ₂ + RA-mediated *TGF β 1* gene repression with deletion of the promoter region comprising the putative PPRES lends support to the conclusion that the putative binding sites for PPAR γ -RXR in the promoter region are neither active nor responsible for the gene repression by

activated PPAR γ and RXR heterodimer. The promoter region of human *TGF β 1* gene contains two AP-1 binding sites that mediate up-regulation of the gene in response to the conditions of mitogen-activated protein kinase activation such as phorbol esters or hyperglycemia (Kim et al., 1989b; Weigert et al., 2000). The studies showed that the AP-1 binding sites, located at between -453 and -323 bp, play a crucial role in *TGF β 1* up-regulation. The cell signaling pathways involving protein kinase C and p38 kinase enhance AP-1 binding to its DNA binding elements predominantly to the proximal AP-1 box in the *TGF β 1* promoter (Weigert et al., 2000). The proteins bound with the AP-1 binding elements in cells involve c-Jun, JunD, and c-Fos (Kim et al., 1990; Zhang et al., 1992; Lee et al., 2006). AP-1 interacts with CBP/p300 coactivator after complex formation with DNA, which is essential for AP-1-mediated gene transactivation (Kamei et al., 1996).

The effects of either PPAR γ or retinoid ligands on *TGF β 1* gene expression have been claimed to be mediated in part by AP-1 inhibition (Salbert et al., 1993; Weigert et al., 2003). That deletion of the DNA region containing both AP-1 sites still had the capability to repress the gene by PGJ₂ + RA (Fig. 3) provides evidence that the AP-1 binding sites may not be a major regulatory target in the *TGF β 1* gene repression. In addition, we found that PGJ₂ + RA (30 nM each) did not alter the AP-1 promoter or DNA binding activity (Supplemental Data 1), suggesting that PPAR γ -RXR activation does not affect AP-1. However, it should be noted here that specific mutation of the proximal AP-1 element (Weigert et al., 2000), primarily recognized by AP-1 complex, abolished the repressing effect of PGJ₂ + RA on *TGF β 1* promoter luciferase activity (Supplemental Data 2). This in conjunction with a substantial decrease in pGL-323 activity compared with AP-1

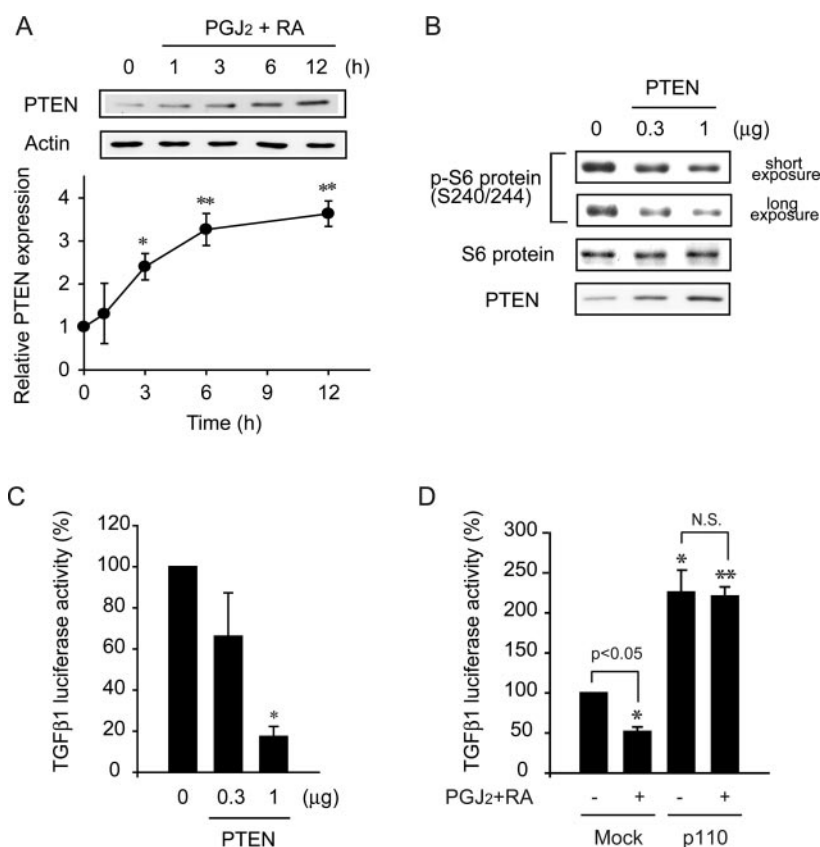


Fig. 8. Role of PTEN in *TGF β 1* repression by PGJ₂ + RA. **A**, effect of PGJ₂ + RA on PTEN expression. PTEN was immunoblotted in the lysates of L929 cells treated with PGJ₂ + RA (30 nM each) for 1 to 12 h. **B**, effect of PTEN overexpression on S6 protein phosphorylation. Phosphorylated S6 protein was measured in cells transfected with a construct encoding PTEN. **C**, repression of *TGF β 1* luciferase activity by PTEN overexpression. Cells were transfected with pGL3-323 in combination with an empty plasmid or the plasmid encoding PTEN. **D**, *TGF β 1* luciferase activity. Luciferase activity from pGL3-323 was determined in the lysates prepared from cells treated with PGJ₂ + RA (30 nM each) for 12 h after transfection with an empty plasmid (mock) or the plasmid encoding p110 (0.5 μg). Data represented the mean \pm S.D. with three separate experiments (significant compared with mock-transfected vehicle-treated control: *, $p < 0.05$, **, $p < 0.01$) (luciferase activity in vehicle-treated control, 100%).

box-containing pGL3-453 (Fig. 3) allows us to infer that the target molecule altered by PPAR γ -RXR α -activated cell signal may be involved in the interaction with the protein recruited on the AP-1 DNA complex. Nonetheless, our observation that substantial repression of pGL3-323 lacking the AP-1 binding sites and putative PPREs by ectopic PPAR γ and RXR α expression clearly indicates that the TGF β 1 gene repression may have not resulted from direct inhibition of AP-1 but by other mechanistic bases.

S6K1, a ubiquitous serine/threonine kinase, controls the translational efficiency by phosphorylating ribosomal S6 protein (Jeno et al., 1988). Rapamycin inhibits S6K1 activity via mTOR inhibition. Yet, other pharmacological agents that modulate S6K1 activity, especially in association with Zf9 dephosphorylation, have not been reported. Our data presented here identify the efficacy of PGJ₂ + RA in suppressing S6K1 activity. The finding that S6K1 inhibition by PGJ₂ + RA was rapid and sustained suggests that the proposed signaling pathway may serve as a pharmacological molecular target. Our result showing that ectopic expression of PPAR γ 1 in combination with RXR α strongly inhibited S6K1 activity supports PPAR γ -RXR α heterodimer as a target for S6K1 inhibition.

Zf9 as a transcription factor plays a crucial role for the

induction of TGF β 1 (Kim et al., 1998). Studies have shown that Zf9 phosphorylation enhances its nuclear localization and transcriptional activity (Slavin et al., 2004). Thus, phosphorylation status of Zf9 contributes to the promotion of its target gene expression (Warke et al., 2003). In the present study, PGJ₂ + RA treatment repressed the luciferase activity of pGL3-323, whose promoter region comprises the DNA binding sites for Zf9, NF1, and SP1. Repression in TGF β 1 luciferase activity by PGJ₂ + RA paralleled decrease in the level of serine-phosphorylated Zf9. In contrast, PGJ₂ + RA treatment did not change phosphorylation of other transcription factors, NF1 and SP1. Thus, our studies here suggest that decrease in Zf9 phosphorylation contributes to the gene repression. Additional gel shift and chromatin immunoprecipitation experiments indicated that Zf9 (or phosphorylated Zf9) binding activity to its DNA binding site in the TGF β 1 gene was unaffected (data not shown), implying that TGF β 1 repression by PGJ₂ + RA might result from a change in transactivating protein complex formation, such as recruitment of corepressor, presumably because of Zf9 dephosphorylation, but not a decrease in Zf9 DNA binding activity. As expected, we found that the NF1 and SP1 DNA binding activities were unchanged by PGJ₂ + RA treatment (data

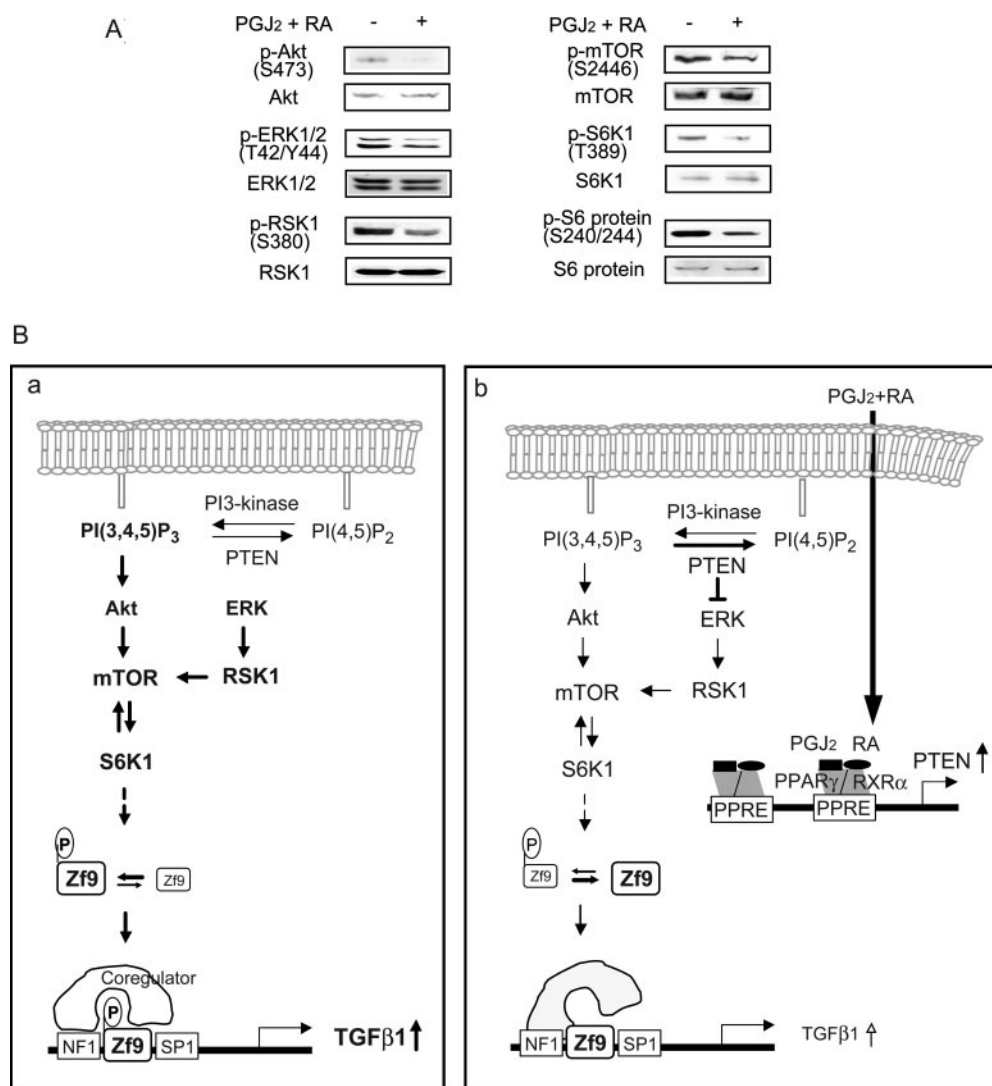


Fig. 9. Effects of PGJ₂ + RA treatment on the phosphorylations of cellular kinases downstream of PTEN. A, immunoblot analyses of Akt, ERK1/2, RSK1, mTOR, and S6K1 phosphorylated at regulatory sites. Phosphorylated forms of Akt (S473), ERK1/2 (T202/Y204), RSK1 (S380), mTOR (S2448), and S6K1 (T389) were immunoblotted by using their specific antibodies (Cell Signaling Technology Inc.) in the lysates (30 μ g each for ERK1/2 or RSK1) or Akt, mTOR, or S6K1 immunoprecipitates prepared from lysates (200 μ g each) of cells treated with vehicle or PGJ₂ + RA (30 nM each; 3 h). B, schematic diagram illustrating the proposed mechanism, by which activation of the PPAR γ and RXR heterodimer represses the TGF β 1 gene. a, basal untreated condition. b, effects of PGJ₂ + RA.

not shown). Identification of the partners of Zf9 or phosphorylated Zf9 for the gene regulation and their molecular interactions, which is beyond the scope of this study, constitutes an important question to answer.

The signaling pathway and the kinases responsible for Zf9 phosphorylation have not been elucidated. Either PMA/A23187 treatment or hypoxia, which has been implicated in the cell signaling pathway of S6K1, increased Zf9 phosphorylation (Warke et al., 2003; Skinner et al., 2004). An important finding of this study is that the pathway involving S6K1 mediates Zf9 phosphorylation. This was supported first by the observation that Zf9 phosphorylation was inhibited in cells treated with rapamycin, an mTOR and S6K1 inhibitor, and further strengthened by the finding that CA-S6K1 abolished Zf9 dephosphorylation by PGJ₂ + RA. Our results demonstrate that S6K1 (if not, another kinase downstream of S6K1) mediates Zf9 phosphorylation. The constitutive Zf9 phosphorylation by S6K1 highlights the important role of S6K1 as a multifunctional kinase for transcription factor regulation (de Groot et al., 1994; Harada et al., 2001; Wang et al., 2001).

Our finding that CA-S6K1 transfection enhanced *TGFβ1* gene transactivation, whereas that with DN-S6K1 transfection inhibited it, provides compelling evidence that S6K1 activity is directly associated with the gene regulation. Furthermore, the result of reporter gene analysis showing that *TGFβ1* gene repression by PGJ₂ + RA was completely reversed by CA-S6K1 lends support to the conclusion that S6K1 inhibition by PGJ₂ + RA is responsible for *TGFβ1* gene regulation. In addition to the ligand activation of nuclear receptors, we were able to show that ectopic expression of PPARγ1 or RXRα alone, or in combination, was capable of repressing *TGFβ1* gene with S6K1 inhibition. Furthermore, CA-S6K1 transfection completely reversed *TGFβ1* gene inhibition by PPARγ + RXRα. These observations led us to conclude that PPARγ-RXR heterodimer activation results in *TGFβ1* gene repression via S6K1 inhibition. Inhibition of S6K1 activity provides a central mechanism, by which PPARγ-RXR regulates Zf9-dependent *TGFβ1* gene expression.

One of the target genes, whose promoter region contains PPRE(s) for PPARγ-RXRα-dependent gene induction, is PTEN (Patel et al., 2001). PTEN serves as a phosphatidylinositol-(3,4,5)-trisphosphate lipid phosphatase, which antagonizes PI3-kinase-mediated signaling cascade. Thus, PTEN expression inhibited cell signals such as mTOR-S6K1 activity downstream of PI3-kinase (Liu et al., 2005). PPARγ activation up-regulates PTEN, which has been implicated in tumor-inhibitory or anti-inflammatory actions of PPARγ (Patel et al., 2001; Lee et al., 2005). Our significant finding that PGJ₂ + RA induced PTEN during the period of S6K1 inhibition gives credence to the role of PPARγ-RXR-mediated PTEN expression in S6K1 inhibition. These data along with the observation that PTEN overexpression inhibits S6K1 activity with *TGFβ1* repression render us to conclude that PPARγ-RXRα heterodimer leads to the inhibition of S6K1 activity as a result of PPRE-mediated PTEN induction, which seemed to be mediated by the pathways of ERK1/2-RSK1 and Akt-mTOR (Fig. 9).

In summary, PPARγ-RXRα heterodimer, which up-regulates PTEN, represses the *TGFβ1* gene by inhibiting the activity of S6K1 that catalyzes Zf9 phosphorylation. Phos-

phorylated Zf9 may serve as an essential component that recruits coactivator, which is an important question to answer in the future.

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