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PPAR γ and RXR α Represses the *TGF β 1* Gene via PTEN-Mediated S6K1 Inhibition: Role for Zf9 Dephosphorylation

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Running Title: TGF β 1 Repression by PPAR γ -RXR α Heterodimer

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Abbreviations: AP-1, activator protein-1; CA, constitutively active mutant; DN, dominant negative mutant; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGJ₂, 15-deoxy- δ (12,14)-prostaglandin J₂; PI3-kinase, phosphatidylinositol 3-kinase; PPAR γ , peroxisome proliferator-activated receptor- γ ; PPRE, peroxisome proliferator-activated receptor response element; PI(3,4,5)P₃, phosphatidylinositol-3,4,5 phosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; RA, 9-*cis*-retinoic acid; RAR, retinoic acid A receptor; RT-PCR, reverse transcription-polymerase chain reaction; RSK1, p90 ribosomal S6 kinase-1; RXR, retinoic acid X receptor; S6K1, p70 ribosomal S6 kinase-1; TGF β 1, transforming growth factor- β 1; Zf9, zinc finger transcription factor-

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Peroxisome proliferator-activated receptor- γ (PPAR γ) and retinoic acid X-receptor (RXR) heterodimer regulates cell growth and differentiation. Zf9, whose phosphorylation promotes target genes, is a transcription factor essential for transactivation of the *transforming growth factor- β 1* (*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 to individual treatment alone, synergistically inhibited the *TGF β 1* gene expression, which was abrogated by PPAR γ antagonists. Similarly, 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 comprising up to -323 bp region, but lacking PPAR-responsive elements (PPREs), responded to PGJ₂+RA. PGJ₂+RA treatment inhibited the activity of p70 ribosomal S6 kinase-1 (S6K1), abolishing Zf9 phosphorylation at serine as did rapamycin (an 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. Consistently, *TGF β 1* gene repression by PGJ₂+RA was 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 PTEN, whose overexpression repressed the *TGF β 1* gene through S6K1 inhibition, decreasing ERK1/2-RSK1 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.

Introduction

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. In particular, transforming growth factor- β 1 (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 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 (A 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, promote 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-kinase (PI3-

kinase)-mTOR pathway, plays as a multifunctional kinase for the phosphorylation of ribosomal S6 protein (Jeno et al., 1988), CREM (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 never 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), and thus promotes 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 were tempted 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. Also, we tried to determine 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. Since phosphatase and tensin homolog deleted on chromosome (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 upregulation in the S6K1 inhibition for *TGF β 1* gene repression by PGJ₂+RA.

Materials and Methods

Materials. PGJ₂ were purchased from Biomol Research Labs (St. Louis, MO). Pioglitazone and rosiglitazone were supplied from Dong-A Pharmaceutical Co. (Shingal, Korea). RA, rapamycin and anti-phosphoserine antibody were purchased from Sigma Chemical (St. Louis, MO). Anti-NF1 antibody, anti-SP1 antibody, and anti-Zf9 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies directed against S6 protein, phosphorylated S6 protein, and PTEN were supplied from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit and rabbit anti-goat IgGs were purchased from Zymed Laboratories (San Francisco, CA). A series of deletion constructs of pGL3-TGFβ1 containing the human *TGFβ1* promoter region were kindly provided from Dr. S.J. Kim (NCI, Bethesda, MD). The expression construct encoding mouse PPARγ1 (pCMX-mPPARγ1) was supplied from Dr. C.K. Glass (University of California, San Diego, CA). The human RXRα expression plasmid (PECE-RXRα) 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 (NIH, Bethesda, MD).

Cell Culture. L929 a mouse fibroblast cell line was obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/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 dimethylsulfoxide, for the indicated time period at 37°C.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Real-Time RT-PCR. 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 semi-quantitative 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'-CTTCAGCTCCACAGAGAAGAACTGC-3', antisense primer: 5'-CACGATCATGTTGGACAACCTGCTCC-3', 298 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes (sense: 5'-TCGTGGAGTCTACTGGCGT-3', antisense: 5'-GCCTGCTTCACCACCTTCT-3', 510 bp). PCRs were carried out for 26-29 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. In some experiments, real-time PCRs were performed in a LightCycler 1.5 (Roche, Mannheim, Germany) using LightCycler 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 TGF β 1 activity, we used the dual-luciferase reporter assay system (Promega, Madison, WI). Briefly, L929 cells (7×10^5 cells/well) were re-plated in six-well plates overnight, serum-starved for 12 h, and transiently transfected with pGL3-TGF β 1 promoter-luciferase construct and 0.3 μ g of pCMV- β -galactosidase plasmid (Invitrogen, Carlsbad, CA) in the presence of Lipofectamine[®] Reagent (Life Technologies, Gaithersburg, MD) 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 (Life Technologies, Gaithersburg, MD) 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 μ g of cell lysates was added to the solution containing 0.88 mg/ml *o*-nitrophenyl- β -D-galactopyranoside, 100 μ M 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 PPAR γ and/or RXR α overexpression, cells were cotransfected with pCMX-mPPAR γ 1 and/or PECE-RXR α 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). Briefly, cells were centrifuged at 2,300g 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 μ l hypotonic buffer. The lysates were incubated for 10 min on ice and then centrifuged at 7,200g for 5 min at 4°C. Pellets containing crude nuclei were resuspended in 50 μ l 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 μ g) was immunoprecipitated, and the samples were washed three times in lysis buffer, and once in the kinase buffer containing Tris-HCl (25 mM, pH 7.4), 10 mM MgCl₂, 25 mM β -glycerophosphate, 1 mM Na₃VO₄, 2 mM dithiothreitol, 1 mM PMSF, 1 μ g/ml leupeptin and 200 μ M ATP. Kinase reaction was initiated by adding S6 substrate peptide (5 μ g per assay) and 2 μ Ci of [γ -³²P]ATP to a 20 μ l 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, Waltham, MA). 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 β -counter (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 \times Laemmli buffer and boiled. Samples were resolved in 7.5% SDS-PAGE and then transferred to nitrocellulose membranes. The samples were immunoblotted with anti-phosphoserine antibody. The

bands were developed using an ECL chemiluminescence detection kit.

Transient Transfection. Cells (5×10^5 cells/well) were re-plated in six-well plates overnight, serum-starved for 6 h, and transiently transfected with pCMX-mPPAR γ 1 and/or PECE-RXR α (0.5 μ g 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 μ g) with or without empty plasmid to adjust the total amount of plasmids transfected to 1 μ g.

Immunoblot Analysis. SDS-PAGE 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 & Analysis System (Alpha-Innotech Corporation, San Leandro, CA). One way analysis of variance (ANOVA) 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 *TGF β 1* Gene by PGJ₂+RA. To examine the role of PPAR γ activation in TGF β 1 expression, we first assessed the dose-dependent effects of PGJ₂, an endogenous PPAR γ agonist, or RA, a RXR agonist, on TGF β 1 expression in L929 cells (Fig. 1A). Semi-quantitative RT-PCR analysis showed that PGJ₂, at the concentration of 100 nM or 1000 nM 20-40% inhibited TGF β 1 expression 12 h after treatment, indicating that PGJ₂ at the relatively high concentrations weakly inhibited the gene expression. Treatment of the cells with RA (30-100 nM) for 12 h also repressed the level of TGF β 1 mRNA by 30-40% (Fig. 1A). RA at 1000 nM blocked TGF β 1 expression by >50%. Data showed that either PGJ₂ or RA alone moderately decreased the expression of *TGF β 1* gene in L929 fibroblasts.

PPAR γ heterodimerizes with RXR for activation, and the PPAR γ -RXR heterodimers are widely expressed in major organs (Dubuquoy et al., 2002). Next, we determined whether PGJ₂+RA inhibited

TGF β 1 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. When compared to the individual treatment, combination treatment of PGJ₂ and RA at the equal molar concentrations synergistically down-regulated TGF β 1. TGF β 1 expression was 40% inhibited by treatment of the cells with PGJ₂+RA as low as at 10 nM each (Fig. 1B). PGJ₂+RA at the concentrations of 30-100 nM 70-80% inhibited TGF β 1 expression. A time-course study using 30 nM each of PGJ₂ and RA showed that the TGF β 1 mRNA levels were markedly decreased 12-24 h after treatment (Fig. 1C). Real-time RT-PCR analysis using SYBR Green I confirmed the synergistic repression of *TGF β 1* gene expression by PGJ₂+RA treatment compared to PGJ₂ or RA treatment alone (Fig. 1D), implying that PPAR γ activation in combination with that of RXR efficaciously down-regulates the *TGF β 1* gene.

PPAR γ -Dependent Repression of *TGF β 1* Gene by PGJ₂+RA. The role of PPAR γ activation in TGF β 1 repression was examined by the experiments using thiazolidinedione PPAR γ agonists. Treatment of L929 cells with either rosiglitazone (10 μ M) or pioglitazone (10 μ M) for 12 h significantly decreased the expression of TGF β 1 mRNA (Fig. 2A). Such as result of 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 PGJ₂+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 PGJ₂+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 PGJ₂+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 *TGFβ1* gene transactivation that is regulated by the proximal DNA response elements were examined as an effort to identify the molecular basis of TGFβ1 repression by PGJ₂+RA. The potential regulatory sites responsible for the *TGFβ1* 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 *TGFβ1* gene downstream of the -1.36 kb 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 PGJ₂+RA (30 nM each) resulted in a 35% decrease in the luciferase activity (Fig. 3B). PGJ₂+RA decreased the relative luciferase-inducible activity by 50% in cells transfected with pGL3-731, compared to vehicle-treated control. TGFβ1 repression by PGJ₂+RA was unaffected by deletion of the promoter containing the region down to -453 bp. Luciferase activity was down-regulated by PGJ₂+RA treatment in cells transfected with pGL3-453 that contains no putative PPREs, indicating that the putative PPRE sites located at the upstream region were not functional for the gene repression. We further examined whether PGJ₂+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 bp and -323 bp. Although the constitutive luciferase expression was largely decreased by deletion of the AP-1 containing region, PGJ₂+RA still inhibited luciferase expression from pGL3-323. Such promoter deletion analyses indicate that PGJ₂+RA significantly inhibits luciferase reporter activity in cells transfected with the *TGFβ1* promoter, suggesting that neither the putative PPREs nor the AP-1 binding sites be directly regulated by PGJ₂+RA for the gene repression.

Inhibition of S6K1 by PGJ₂+RA. Because S6K1 has been implicated in the regulation of fibrogenesis (Zhu et al., 1999), we sought to determine the effects of PGJ₂+RA on the activity of S6K1 in association with *TGFβ1* gene repression. The S6K1 is a physiological kinase that phosphorylates 40S ribosomal S6 protein in cells (Chung et al., 1992). PGJ₂+RA decreased phosphorylation of S6 protein 3-12 h after treatment (Fig 4A, left). The inhibition of S6 protein phosphorylation sustained at least up to 24 h (data not shown). A concentration-response study indicated that S6 protein phosphorylation was

decreased by PGJ₂+RA at the concentrations of 10 nM each or above (Fig 4A, right). Further, we measured the kinase activity of S6K1 immunoprecipitated in the lysates of cells treated PGJ₂+RA. Consistently, PGJ₂+RA treatment decreased the immune complex kinase activity in a time- and concentration-dependent manner (Fig 4B).

Role of S6K1 Inhibition by PGJ₂+RA in Zf9 Dephosphorylation. The transcription factors that interact with the known DNA binding sites on the region downstream within the –323 bp of the *TGFβ1* gene include Zf9, NF1 and SP1 (Fig. 3A). In view of the previous observations that Zf9 is crucial as a transcription factor for TGFβ1 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 *TGFβ1* gene repression by PGJ₂+RA might have resulted from dephosphorylation of Zf9.

Given the inhibition of S6K1 activity by PGJ₂+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 PGJ₂+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 PGJ₂+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 to that in mock-transfected cells, which may have resulted from saturation of Zf9 phosphorylation in L929 cells due to 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 completely 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 inhibit

TGFβ1 gene transcription via Zf9 dephosphorylation as a consequence 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). Apparently, the basal *TGFβ1* reporter gene activity was rather increased by CA-S6K1 transfection alone. As expected, DN-S6K1 transfection inhibited luciferase expression 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 completely abolished luciferase expression from pGL3-323, the extent of which was comparable to 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. Further, 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. 7B). As anticipated, pGL3-323 *TGFβ1* gene repression by PPARγ and RXRα 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 PPARγ-RXRα results in the inhibition of S6K1, and that the S6K1 inhibition was responsible for the *TGFβ1* 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 PPARγ 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 *TGFβ1* gene by PGJ₂+RA, we determined whether ligand activation of PPARγ-RXRα 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 *TGFβ1* gene expression. S6 protein phosphorylation notably decreased after PTEN induction presumably through decrease in the formation of PI(3,4,5)P₃, whose production is catalyzed by PI3-kinase (Fig. 8B). *TGFβ1* gene was also repressed by PTEN expression (Fig. 8C). To verify the antagonism of PI3-kinase activity against TGFβ1 repression by PGJ₂+RA, PGJ₂+RA-dependent luciferase gene expression was measured in cells transfected the plasmid encoding p110, the catalytic subunit of PI3-kinase. The basal *TGFβ1* 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. Taken together, these data indicate that the induction of PTEN by PGJ₂+RA may result in *TGFβ1* 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 *TGFβ1* gene and the molecular interactions of ligand-activated nuclear receptors for the activation of responsible transcription factor(s) bring insights into the transcriptional control mechanism. In the present study, we demonstrated that either PPARγ or RXR agonist alone at relatively high concentrations down-regulated the *TGFβ1* gene, whereas concomitant treatment with both PPARγ and RXR agonists synergistically repressed the gene. PGJ₂ at low concentrations serves as an agonist of PPARγ. 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 TGFβ1 because activated PPARγ requires additional binding of ligand-bound RXR for the formation of a PPARγ-RXR heterodimer. This is consistent with the current observation that RA potentiated *TGFβ1* gene repression by PGJ₂ although

RA alone weakly repressed the gene. Enhanced TGF β 1 repression by PGJ₂+RA, compared to 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 is 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 MAP 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 bp and -323 bp, play a crucial role in TGF β 1 up-regulation. The cell signaling pathways involving PKC 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 (Lee et al., 2006; Kim et al., 1990; Zhang et al., 1992). 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 (Weigert et al., 2003; Salbert et al., 1993). Such a result 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 *TGFβ1* promoter luciferase activity (supplemental data #2). This in conjunction with a substantial decrease in pGL-323 activity compared to AP-1 box-containing pGL3-453 (Fig. 3) renders us to infer that the target molecule altered by PPARγ-RXRα-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 PPARγ and RXRα expression clearly indicates that the *TGFβ1* gene repression may have not resulted from direct inhibition of AP-1, but other mechanistic basis.

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 PPARγ1 in combination with RXRα strongly inhibited S6K1 activity supports PPARγ-RXRα heterodimer as a target for S6K1 inhibition.

Zf9 as a transcription factor plays a crucial role for the induction of TGFβ1 (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 TGFβ1 luciferase activity by PGJ₂+RA paralleled decrease in the level of serine-phosphorylated Zf9. On the other hand, 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 *TGFβ1* gene was unaffected (data not shown), implying that TGFβ1 repression by PGJ₂+RA might result from a

change in transactivating protein complex formation such as recruitment of corepressor presumably due to 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 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 PI(3,4,5)P₃ 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). PPAR γ activation upregulates PTEN, which has been implicated in tumor-inhibitory or anti-inflammatory actions of PPAR γ (Patel et al., 2001; Lee et al., 2005). Our significant finding that PGJ₂+RA induced PTEN during the time period of S6K1 inhibition gives credence to the role of PPAR γ -RXR-mediated PTEN expression in S6K1 inhibition. These data along with the observation that PTEN overexpression inhibits S6K1 activity with *TGF β 1* repression render us to conclude that PPAR γ -RXR α heterodimer leads to the inhibition of S6K1 activity as a result of PPRE-mediated PTEN induction, which appeared to be mediated by the pathways of ERK1/2-RSK1 and Akt-mTOR (Fig. 9).

In summary, PPAR γ -RXR α heterodimer, which upregulates PTEN, represses the *TGF β 1* gene by inhibiting the activity of S6K1 that catalyzes Zf9 phosphorylation. Phosphorylated Zf9 may serve as an essential component that recruits coactivator, which is an important question to answer in the future.

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Footnotes

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Figure Legends

FIG. 1. The effects of PGJ₂ and/or RA on *TGFβ1* gene expression. (A) RT-PCR analysis of the TGFβ1 mRNA levels. Semi-quantitative RT-PCR analyses were performed in the total RNA prepared from L929 cells treated with 30-1000 nM PGJ₂ or RA for 12 h. The GAPDH mRNA levels were monitored as controls. The change in TGFβ1 mRNA relative to that of GAPDH was assessed by scanning densitometry of the band intensities. (B) The effect of PGJ₂+RA on TGFβ1 mRNA expression. Representative RT-PCR analysis shows the levels of TGFβ1 mRNA in cells treated with PGJ₂+RA at the concentrations of 1-100 nM each for 12 h. (C) The relative TGFβ1 mRNA levels in cells treated with PGJ₂+RA (30 nM each) for 6-24 h. (D) Real-time RT-PCR analysis. Real-time RT-PCR analysis was performed in the total RNA prepared from cells treated with PGJ₂ or RA alone, or in combination for 12 h. Data represent the mean ± S.D. with 3 separate experiments (significant as compared to control, *p<0.05, **p<0.01; TGFβ1 mRNA level in control, 100%).

FIG. 2. PPARγ-dependent repression of the *TGFβ1* gene by PGJ₂+RA. (A) Repression of the TGFβ1 mRNA expression by thiazolidinediones. TGFβ1 expression was measured in L929 cells treated with 10 μM rosiglitazone or pioglitazone for 12 h. (B) The effect of the PPARγ antagonists on TGFβ1 repression by PGJ₂+RA. Cells were pretreated with BADGE (10 μM) or GW9662 (1 μM) for 1 h and subsequently exposed to PGJ₂+RA (30 nM each) for 12 h in the continuing presence of BADGE or GW9662. Data represent the mean ± S.D. with 3 separate experiments (significant as compared to control, *p<0.05, **p<0.01; TGFβ1 mRNA level in control, 100%).

FIG. 3. Promoter deletion analysis of the *TGFβ1* gene. (A) The promoter region of the *TGFβ1* gene construct. The promoter region of *TGFβ1* gene containing up to -1362 bp shows the FSE, putative PPREs, AP-1, NF1, ZI9 and SP1 DNA binding elements at multiple locations. (B) Repression of luciferase activity by PGJ₂+RA. Luciferase reporter assays were performed in the lysates of L929 cells transfected with the TGFβ1 luciferase reporter construct and subsequently exposed to PGJ₂+RA (30 nM each) for 12 h. Data represented the mean ± S.D. with 3 separate experiments (significant as compared to vehicle for the respective construct transfection, *p<0.05, **p<0.01) (luciferase activity in pGL3-175-transfected cells treated with vehicle, 100%).

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FIG. 4. S6K1 inhibition by PGJ₂+RA. (A) The 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 1-12 h or those treated with 1-100 nM PGJ₂+RA for 6 h. (B) The 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 3 separate experiments (significant as compared to control, *p<0.05, **p<0.01; S6K1 activity in control, 1).

FIG. 5. The 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 to that in mock-transfected cells. Results were confirmed by three independent experiments.

FIG. 6. The role of S6K1 inhibition in TGFβ1 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 µg) 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 µg). Data represented the mean ± S.D. with 3 separate experiments (significant as compared to mock-transfected control, *p<0.05, **p<0.01)(luciferase activity in vehicle-treated mock-transfected cells, 100%). N.S., not significant.

FIG. 7. The role of S6K1 inhibition in TGFβ1 repression by PPARγ1-RXRα heterodimer. (A) *TGFβ1* gene

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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 in the legend to panel 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 3 separate experiments (significant as compared to mock-transfected control, * p <0.05, ** p <0.01)(luciferase activity in mock transfection, 100%). N.S., not significant.

FIG. 8. The role of PTEN in TGF β 1 repression by PGJ₂+RA. (A) The effect of PGJ₂+RA on PTEN expression. PTEN was immunoblotted in the lysates of L929 cells treated with PGJ₂+RA (30 nM each) for 1–12 h. (B) The 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 μ g). Data represented the mean \pm S.D. with 3 separate experiments (significant as compared to mock-transfected vehicle-treated control; * p <0.05, ** p <0.01, luciferase activity in vehicle-treated control, 100%). N.S., not significant.

FIG. 9. The 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, Beverly, MA) in the lysates (30 μ g each for ERK1/2 or RSK1) or Akt-, mTOR- or S6K1-immunoprecipitates prepared from lysates (200 μ g

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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) The basal untreated condition. (b) The effects of PGJ₂+RA.

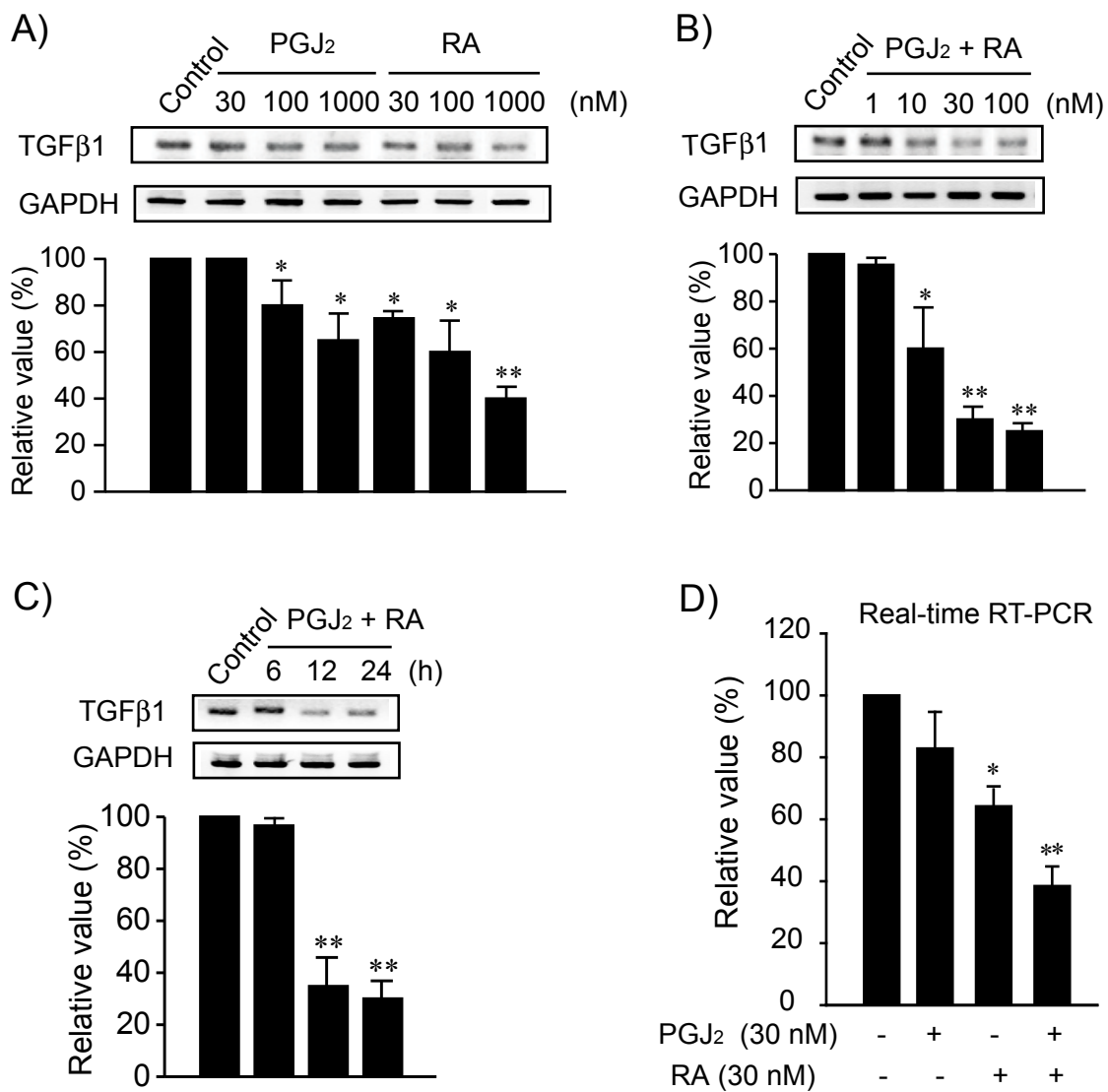


Fig.1

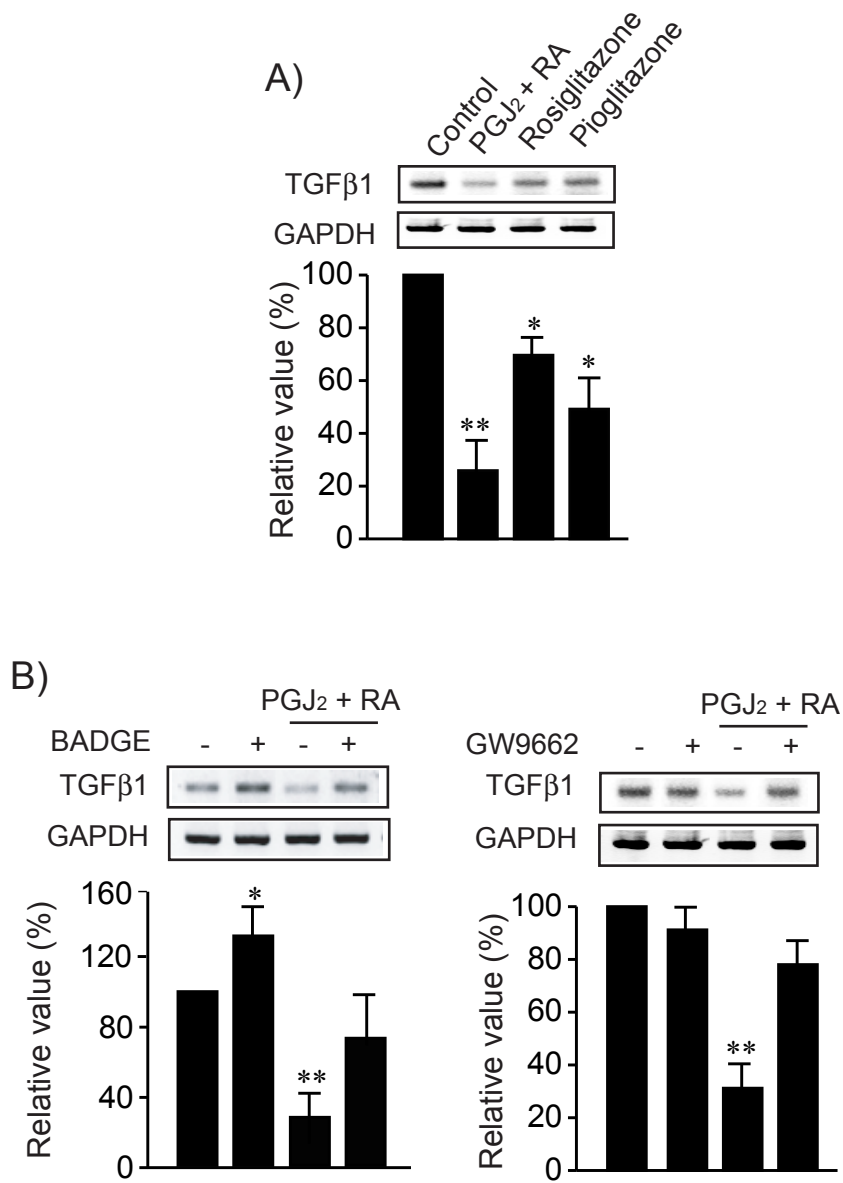


Fig.2

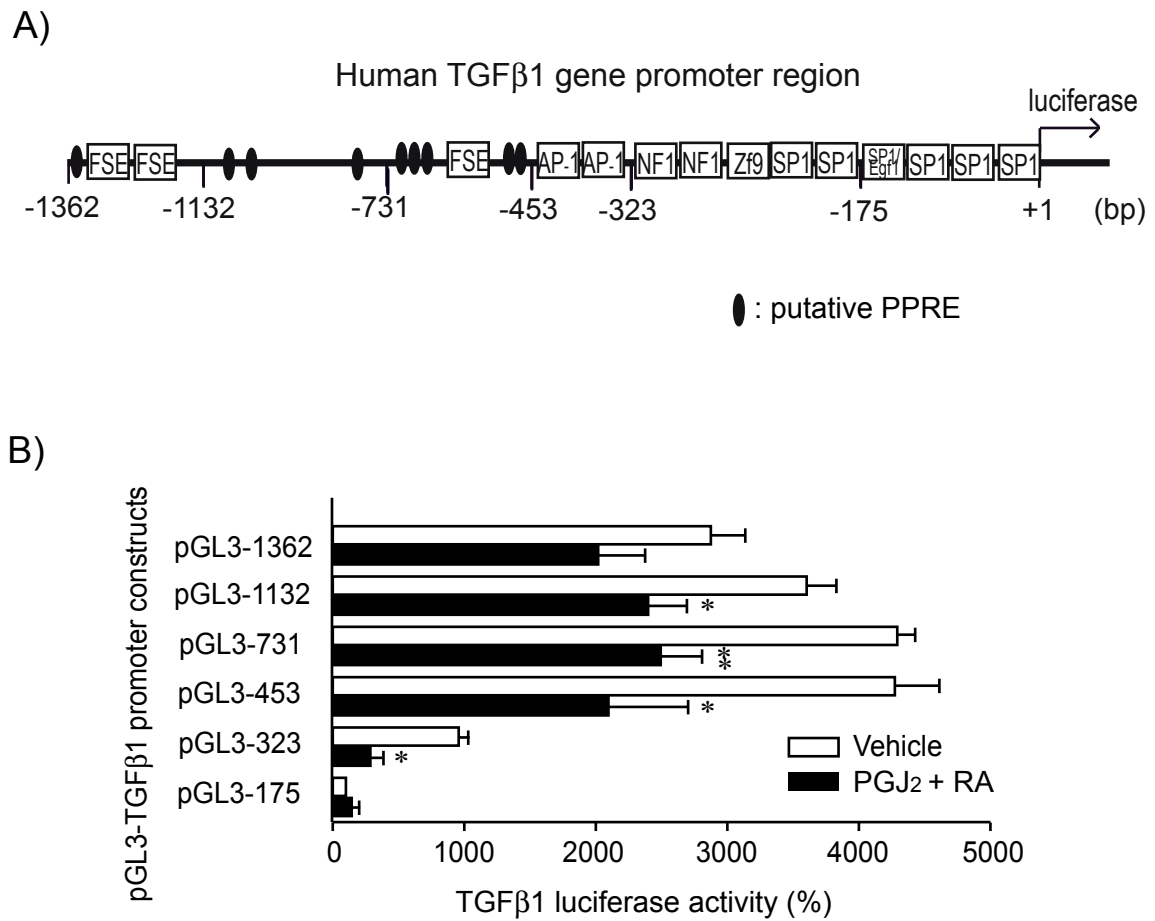


Fig.3

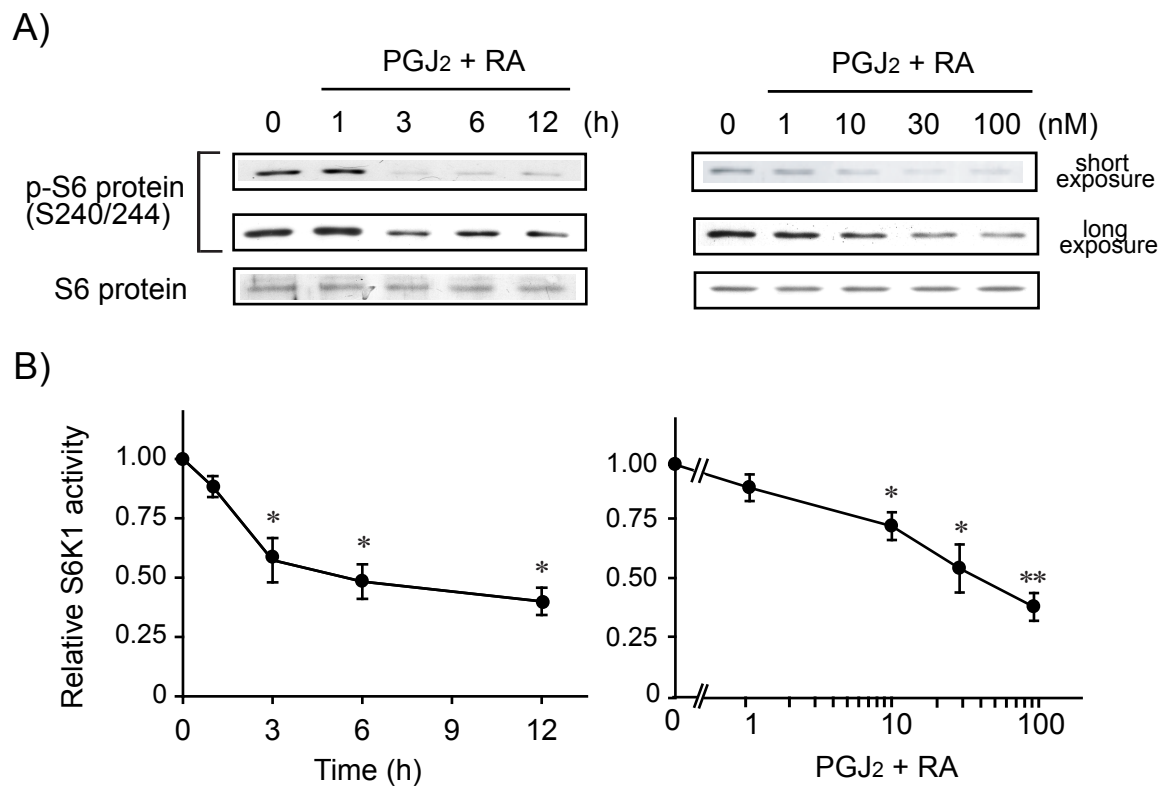
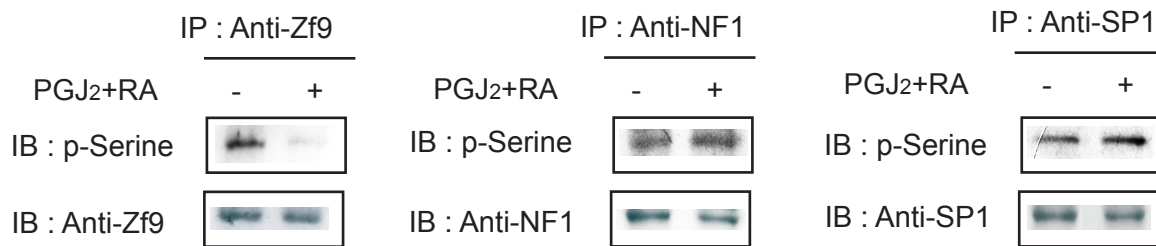
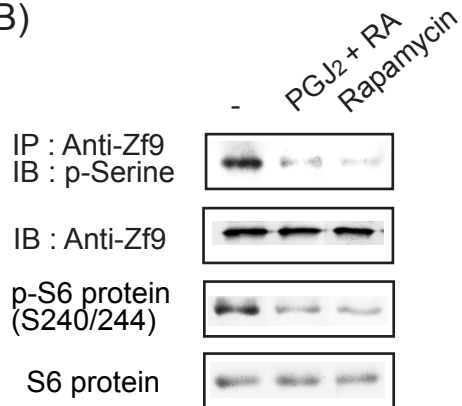


Fig.4

A)



B)



C)

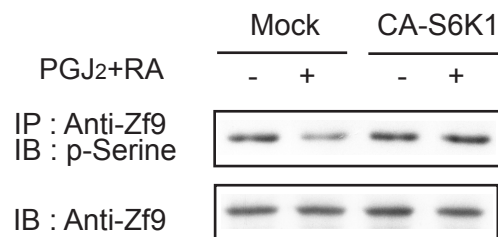


Fig.5

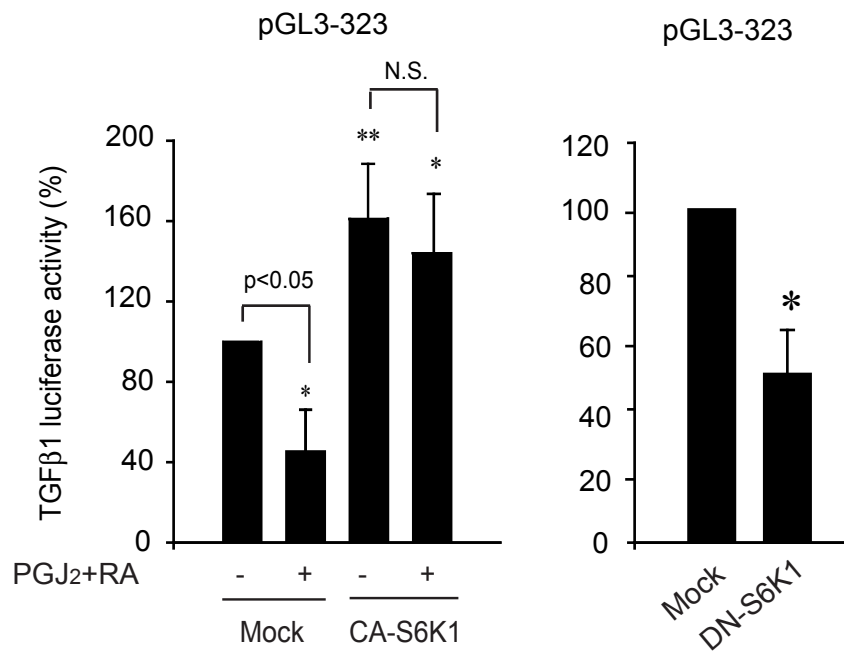


Fig.6

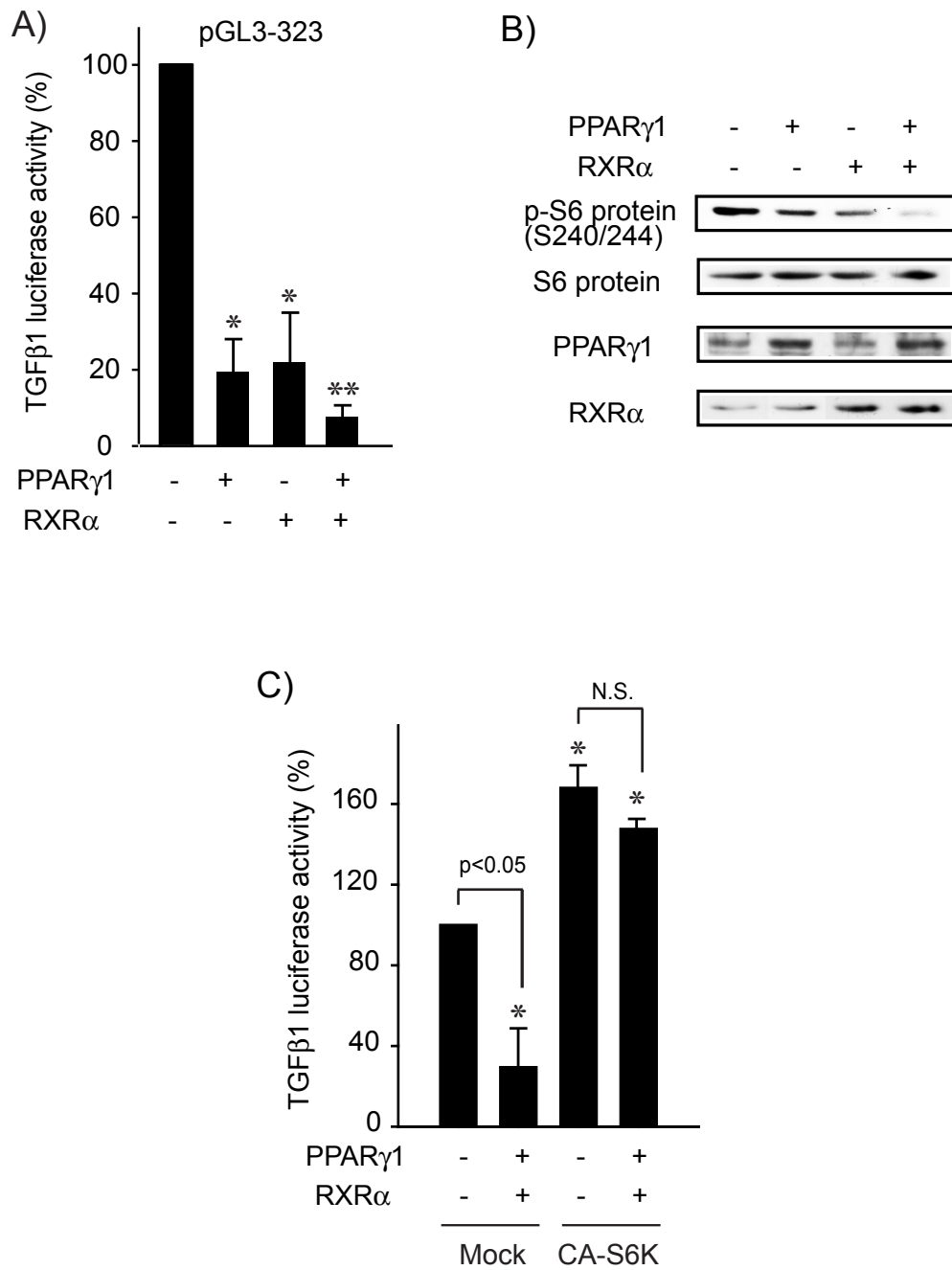


Fig.7

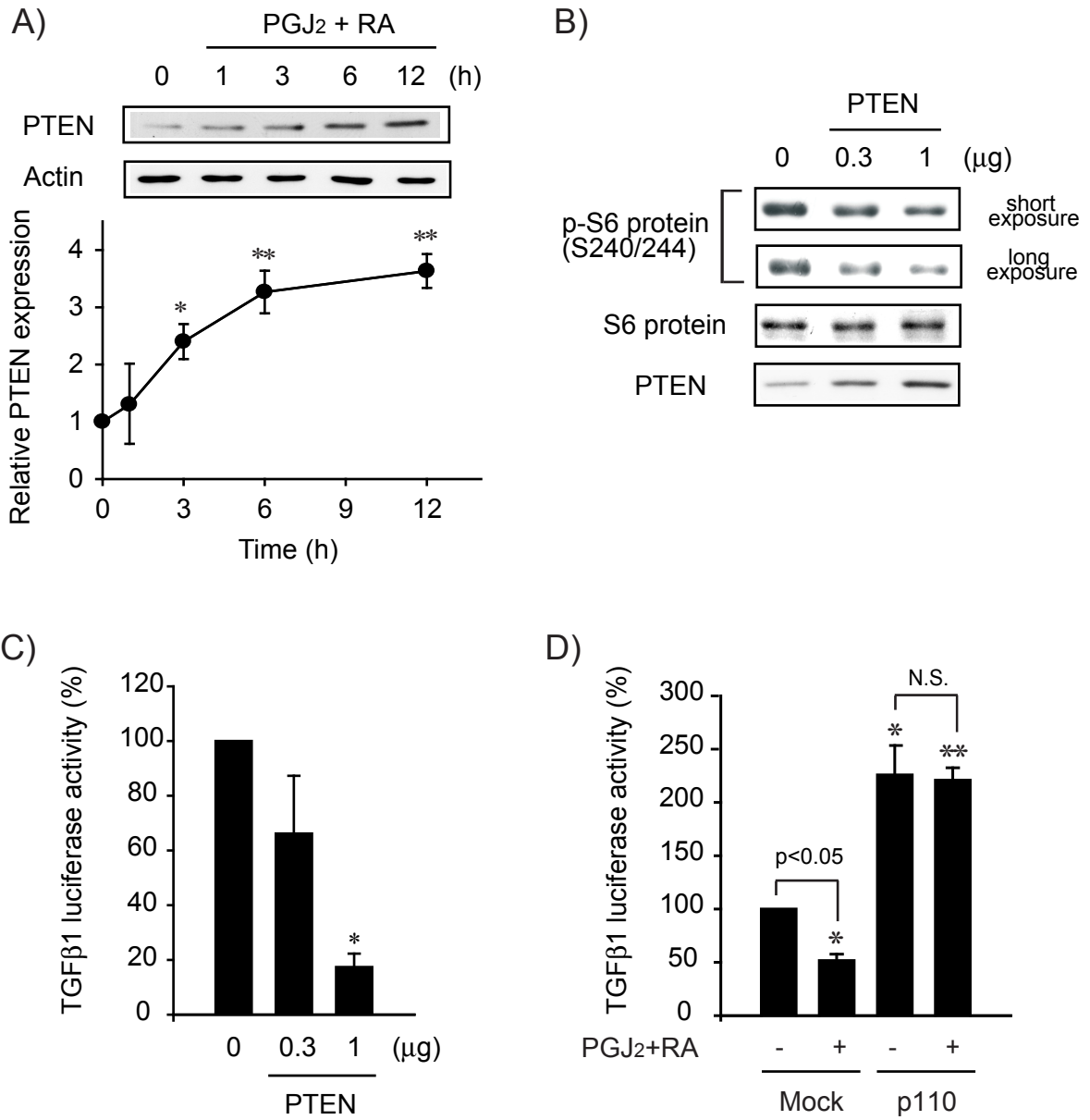


Fig.8

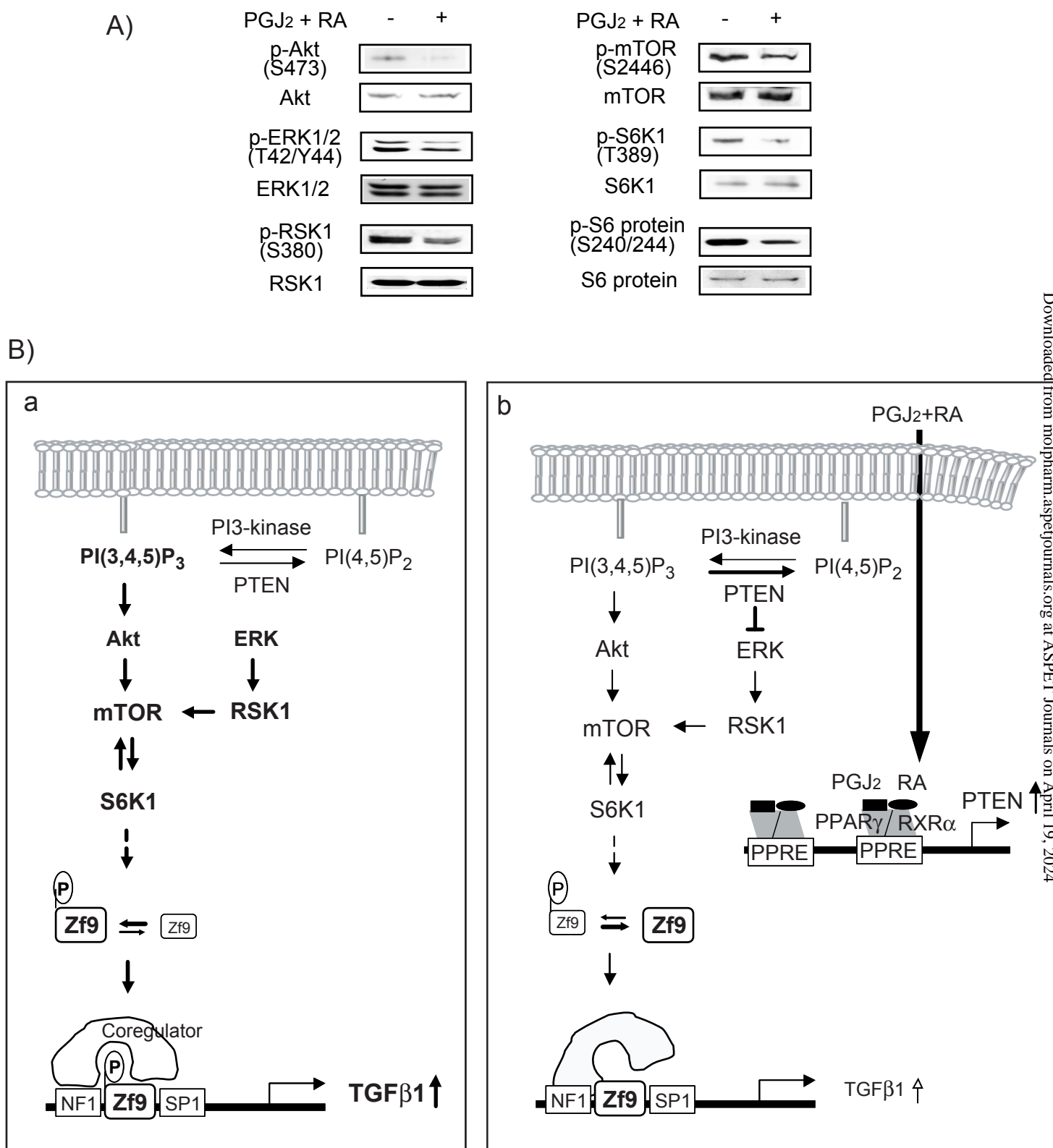


Fig. 9