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15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 Up-regulates KLF4 Expression Independently of PPARγ by Activating the MEK/ERK Signal Transduction Pathway in HT-29 Colon Cancer Cells

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Running title: Regulation of KLF4 Expression by 15d-PGJ_2

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ABBREVIATIONS: 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2; PPARγ, peroxisome proliferators-activated receptor γ; PPRE, PPAR response element; KLF4, Krüppel-like factor 4; CHX, cycloheximide; MTT, 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl-tetrazolium bromide; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PI-3 knase, phosphoinositide 3-kinase; STAT, signal transducers and activators of transcription; AOX, acyl-CoA oxidase; FACS, fluorescence-activated cell sorter; siRNA, small interference RNA.

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ABSTRACT

15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2) is a natural ligand for the peroxisome proliferators-activated receptor γ (PPARγ) that exhibits anti-proliferative activity in colon cancer cells, but its mechanism of action is still poorly understood. Here we show that Krüppel-like factor 4 (KLF4) is one of the down-stream effectors of 15d-PGJ_2. Treatment of HT-29 cells with 15d-PGJ_2 resulted in up-regulation of both KLF4 mRNA and protein expression, and these increases were also observed in other colon cancer cell lines. Down-regulation of KLF4 expression by small interfering RNA (siRNA) targeting KLF4 reduced 15d-PGJ_2-mediated G_1 phase arrest, suggesting that KLF4 mediated function of 15d-PGJ_2. The effect of 15d-PGJ_2 on KLF4 expression appears not to involve its nuclear receptor PPARγ, as out data show that: 1) KLF4 gene promoter does not contain putative PPRE sequence; 2) 15d-PGJ_2 rapidly activates ERK and induces KLF4 mRNA expression; 3) KLF4 is induced by 15d-PGJ_2 but not by rosiglitazone, a synthetic PPARγ ligand; and 4) 15d-PGJ_2 is unable to stimulate PPAR-dependent promoter activity in the absence of co-transfected PPARγ. Moreover, 15d-PGJ_2-mediated KLF4 mRNA expression was blocked by PD98059 or U0126, two ERK kinase inhibitors, whereas PI3-kinase inhibitors, wortmannin or LY294002, had no such effect. Furthermore, KLF4 induction by 15d-PGJ_2 was occurred only in STAT1-expressing but not in STAT1-knockout cells. Together, these results suggest that 15d-PGJ_2-induced growth inhibition of colon cancer cells is mediated, at least in part, through up-regulation of KLF4 expression. This induction is unlikely mediated through PPARγ receptor, but may involve in MEK/ERK pathway, and is STAT1-dependent.

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Introduction

Peroxisome proliferators-activated receptor-γ (PPARγ) is a member of the nuclear receptor superfamily of ligand-dependent transcriptional factors (Evans, 1988; Schoonjans et al., 1997). Besides being highly expressed in adipocytes and involved in fatty acid metabolism and adipocyte differentiation (Kliewer et al., 1997), PPARγ is also expressed at high levels in colonic epithelial cells and colon cancer cells (Brockman et al., 1998; Sarraf et al., 1998). PPARγ is activated by its ligands, and then forms a heterodimer with retinoid X receptor, binds to a specific DNA sequence, PPAR response element (PPRE), and stimulates transcription of target genes (Kliewer et al., 1992; Lemberger et al., 1996). J_2 series cyclopentenone prostaglandins (cyPGs), especially 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2), have recently received increasing attention because they functioned as potential regulators of diverse processes including cell growth, differentiation, and inflammation. 15d-PGJ_2, a natural ligand for PPARγ, has been shown to possess an anti-tumorigenic activity. For example, activation of PPARγ by 15d-PGJ_2 significantly inhibits cell growth and induces apoptosis in several types of cancer cells, including colorectal (Chen et al., 2003), gastric (Shimada and Terano, 2002), breast (Clay et al., 2001), and hepatic (Li et al., 2001). However, the molecular mechanisms of its action are still poorly understood.

KLF4, also known as gut-enriched Krüppel-like factor (GKLF), is a member of the Krüppel-like zinc finger transcription factor family, and expresses extensively in the epithelial cells of the gastrointestinal tract (Bieker, 2001; Dang et al., 2000; Shie et al., 2000b). In cultured fibroblasts, the levels of KLF4 mRNA expression were associated with the growth state of cells. Serum deprivation or contact inhibition increases KLF4

expression, and its level decreased in response to serum stimulation (Shields et al., 1996). Forced expression of KLF4 in colon cancer cells resulted in inhibition of DNA synthesis and cell growth (Chen et al., 2000; Dang et al., 2003). These effects appear to be mediated through activating of *p21* $^{WAF1/Cip1}$ expression and/or suppressing of *cyclin D1* or *ODC* (ornithine decarboxylase) gene promoter activity (Chen et al., 2002a; Shie et al., 2000a; Zhang et al., 2000). More recently, the importance of KLF4 in controlling the G1/S cell cycle checkpoint and preventing mitotic entry following DNA damage is further demonstrated by using small interfering RNA (siRNA) (Yoon et al., 2003). Altogether, these results indicate that both KLF4 and 15d-PGJ_2 possess similar anti-proliferative activity, and that KLF4 could be one of the mediators of 15d-PGJ_2 in colon cancer cells.

In this study, we examined the effect of 15d-PGJ_2 on KLF4 expression in HT-29 colon cancer cells. Our results show that that 15d-PGJ_2 inhibits proliferation of HT-29 cells and induces up-regulation of KLF4 mRNA and protein levels. The induction of KLF4 by 15d-PGJ_2 appears not to involve in nuclear receptor PPARγ, but is dependent on the activation of MEK/ERK pathway. These data may provide a novel mechanism governing the anti-proliferative property of 15d-PGJ_2 in colon cancer cells.

Materials and Methods

Reagents. 15-deoxy-Δ12,14 prostaglandin J_2 (15d-PGJ_2) and rosiglitazone were purchased from Cayman Chemicals (Ann Arbor, MI). PD98059, U0126, wortmannin, and LY294002 were obtained form Calbiochem (La Jolla, CA). [α-^{32}P]dCTP and [^{3}H] thymidine were purchased from PerkinElmer Life Sciences (Boston, MA). Other

chemical reagents were obtained from Sigma (St. Louis, MO) unless mentioned specifically.

Cell Culture and Treatment. The human colon carcinoma cell lines, HT-29, DLD-1, Caco-2, RKO, and HCT116, were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in McCoy's growth medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 U/ml penicillin (Invitrogen, Carlsbad, CA) in an atmosphere of 95% air and 5% CO_2 at 37 °C. Cells in exponential growth were plated before serum starvation for 24 h. Serum-starved cells were then exposed to 15d-PGJ_2 for the indicated times in serum-free media.

Mouse fibroblasts CD+ and CD− cell lines, derived from wild-type and STAT1 knockout mice, respectively, were kindly provided by Dr. David Levy (New York University Medical Center, NY).

Cell Viability Assay and Measurement of DNA Synthesis. The cells were treated with 15d-PGJ_2 (1-10 μM) in serum free media after starving for 18 h. Cell viability was determined by tetrazolium salt 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, and was presented as % of control. For the assay, 20 μl of MTT dye was directly added to cell culture. After 2 h, media was removed and cells were lysed with 800 μl of dimethylsuloxide (DMSO). The absorbance at 540 nm was read with a spectrophotometer. For DNA synthesis assay, the cells were labeled with [^{3}H]thymidine during the final 2 h. After labeling, the cells were washed with cold phosphate-buffered saline (PBS), fixed in 10% trichloroacetic acid. The cells were lysed in 0.5 N NaOH, and incorporated [^{3}H]thymidine was measured by a liquid scintillation counter and presented as % of control.

Western Blot Analysis. After treatment, cells were washed twice with ice-cold PBS, scraped, and pelleted by centrifugation (200 x g). Cell pellets were then lysed in the standard RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors. Protein concentrations were determined by Bio-Rad assays and 25-50 μg of protein from each sample was separated on the 10% SDS-polyacrylamide gel. Following electrophoresis, the proteins were transferred to nitrocellulose membranes. The membrane was probed with antibodies against phosphor-ERK1/2, ERK1/2, phosphor-Akt, Akt, and MKP1. Polyclonal KLF4 antibody was used at 1:500 dilution. Protein levels were detected using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) following the manufacturer's instruction (Amersham, Arlington Heights, IL). The concentration of each protein was quantified by scanning blot images, using Scion Image software (Scion Image Co.), and expressed as relative density (fold increase) to that of the control (designated as 1.0). The expression level of ERK1/2 kinase was calculated from at least five independent experiments.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated by the STAT-60^{TM} method following the manufacturer's instructions (Leedo Medical Laboratories, INC. Houston, TX). RNA samples (20 μg) were denatured and size fractionated by electrophoresis on 1.1% agarose-formaldehyde gels, and transferred onto Hybound-N nylon membranes (Amersham Biosciences, Piscataway, NJ). Hybridization was then performed overnight at 42°C using a 450-bp *Apa-Pst1* fragment of the human KLF4 DNA that was radiolabeled with [^{32}p]-dCTP (Random primer labeling kit from Boehringer Mannheim, Indianapolis, Ind.). Blots were washed with 2 x SSPE/0.1% SDS,

followed by 0.1 x SSPE/0.1% SDS. All blots were stripped and reprobed with PPARγ or PPARδ cDNA probe. PPARγ and PPARδ expression plasmids were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University). The blots were also stained with ethidium bromide to verify RNA loading. The level of mRNA expression was quantified by scanning blot images, using Scion Image software (Scion Image Co.), and expressed as relative density (fold increase) to that of the control (designated as 1.0). The results were calculated from 4-5 independent experiments.

Plasmids, Transfections and Luciferase Assays. The reporter plasmids, pAOXPPREluc containing the PPRE of the rat acyl-CoA oxidase gene, and pAOXBluc containing only the basal promoter of the gene, were kindly provided by Dr. Takashi Osumi (Himeji Institute of Technology, Department of life science, Graduate School of Science, Hyogo, Japan). All transfection experiments were performed using LipofectAMINE reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

For luciferase assay, the cell lysate (100 μL) was first mixed with the luciferase substrate solution, and luciferase activity was measured using a luminometer with automatic injection. With each experiment, luciferase activity was determined in triplicate and normalized with β-galactosidase activity for each sample.

Preparation of siRNA and Transfection. KLF4 siRNA was synthesized by Dharmacon (Boulder, CO) and targeted the coding region 86-92 relative to the start codon of human KLF4 gene (GenBank™ accession number XM_047517). The sequences of the 21-nucleotide (nt) sense and antisense RNA are as follows: 5'-GACCGAGGAGUUCAACGAUUU-3' (sense) and 5'-

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AUCGUUGAACUCCUCGGUCUU-3' (antisense). The control siRNA duplexe was also generated based on the sequence of an unrelated protein. For transient transfections, HT-29 cells were seeded at a density of 50 to 70 % in 60-mm-diameter plates in McCoy's medium containing 10% FBS. On the following day, transfections were perfomed by using LipofectAMINE Plus Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's recommended protocol. Cells were harvested 48 h after transfection by manual scraping in lysis buffer. The relative expression of endogenous KLF4 was monitored by Western blot analysis, using a polyclonal antibody against KLF4, as described previously.

Statistical Methods. Results are expressed as means ± SEM, and a 2-way ANOVA with Dunnett's post-test (InSat software, GraphPad software) was performed. Differences between group means are analyzed by Student's *t* test, and are considered significant at $P < 0.05$.

Results

15d-PGJ_2 Inhibits Cell Growth and Reduces DNA Synthesis in HT-29 Cells

HT-29 cells are a well characterized cell culture model for human colon cancer. Thus, in this study we used this cell line to examine the effect of 15d-PGJ_2 on cell growth and DNA synthesis. HT-29 cells were cultured in the medium containing 10% FBS in the presence or absence of 15d-PGJ_2 for the indicated intervals, and cell growth was measured by MTT assay. For comparison, rosiglitazone, a synthetic TZD PPARγ ligand, was also used in this experiment. Fig. 1A showed that the growth of HT-29 cells was inhibited by 15d-PGJ_2 in a time-dependent manner, whereas rosiglitazone had no

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significant effect. To confirm that this inhibitory effect is specific for 15d-PGJ_2, we examined the effect of 15d-PGJ_2 on cell growth and DNA synthesis under the serum-free condition by MTT and [^{3}H]-thymidine incorporation assays. As shown in Fig. 1B-1E, treatment of HT-29 cells with 15d-PGJ_2, but not rosiglitazone, resulted in markedly inhibition of both cell growth and DNA synthesis. The growth inhibitory effect of 15d-PGJ_2 was also dose-dependent (Fig. 1D, and 1E). These results indicate that the effect of 15d-PGJ_2 on cell growth is not mediated through other serum factors.

15d-PGJ_2 Up-regulates Levels of KLF4 mRNA and Protein Expression in HT-29 and Other Colon Cancer Cells

KLF4 has recently been shown to function as a negative regulator of cell proliferation. To explore whether KLF4 is involved in the 15d-PGJ_2-mediated growth inhibition, KLF4 mRNA and protein levels were examined in 15d-PGJ_2-treated or untreated cells by Northern and Western blot analysis. As shown in Fig. 2A and 2B, expression of KLF4 mRNA was induced rapidly by 15d-PGJ_2 in time- and dose-dependent manners. The increase in KLF4 mRNA levels was first detected as early as 1 h after treatment, and the maximal effect was observed at 4 h and then the KLF4 concentration was gradually decreased. To determine whether the effects of 15d-PGJ_2 on KLF4 expression could be observed in other colon cancer cells, KLF4 mRNA levels in additional cell lines were examined. As illustrated in Fig. 2C, increases in KLF4 mRNA levels were observed not only in HT-29, but also in DLD1, CaCo2, RKO, and HCT116 cells after 15d-PGJ_2 treatment. Moreover, treatment with 15d-PGJ_2 also led to a time-dependent accumulation of KLF4 protein, with the level peaked at 4 h (Fig. 2D).

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Previous studies have shown that 15d-PGJ_2 was a potent activator of nuclear PPARγ receptor and many of its functions appeared to be mediated through this receptor. To determine the role of PPARγ in 15d-PGJ_2-induced KLF4 up-regulation, PPARγ mRNA levels were examined with a human PPARγ cDNA probe. Unexpectedly, the effect of 15d-PGJ_2 on PPARγ expression is opposite to what observed with KLF4 (Fig. 2A and 2B). Treatment with 15d-PGJ_2 reduced PPARγ mRNA expression in time-and dose-dependent manners, with the maximal effects seen at 4 h and at 20 μM, respectively. On the contrary, 15d-PGJ_2-treatment up-regulated the expression levels of PPARδ mRNA, another member of the nuclear hormone receptor superfamily of ligand-activated transcription factors and these increases were similar to those of KLF4 (Fig. 2A and 2B). These results showed that in HT-29 cells, 15d-PGJ_2-mediated up-regulation of KLF4 mRNA was not associated with increased levels of the nuclear receptor PPARγ mRNA.

The Induction of KLF4 mRNA by 15d-PGJ_2 is Independent of PPARγ in HT-29 Cells

By analyzing its promoter sequence (1,200 bp upstream from the transcription starting site), KLF4 does not appear to contain a potential conserved consensus PPARγ response element (PPRE) in this region (Biology Workbench, version 3.2; http://workbench.sdsc.edu). These data suggest that 15d-PGJ_2-induced KLF4 up-regulation is mediated most likely though a PPARγ-independent mechanism. To test this idea, we examined the effect of cycloheximide (CHX), an inhibitor of *de novo* protein synthesis, on the expression of KLF4 mRNA in presence of 15d-PGJ_2. HT-29 cells were

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pre-incubated with CHX (100 μg/ml) for 30 min, and then treated with 15d-PGJ_2. As shown in Fig. 3A, CHX alone has no effect on KLF4 mRNA level. In contrast, the combination of 15d-PGJ_2 and CHX resulted in a greater increase in KLF4 mRNA level than that observed following treatment with 15d-PGJ_2 alone. In addition, these increases are also time-dependent (Fig. 3B, *upper* and *lower panels*). These data indicate that early *de novo* protein synthesis may not be required for 15d-PGJ_2-induced KLF4 up-regulation. Next, we compared the effect of 15d-PGJ_2, rosiglitazone and Wy-14,643, a ligand of PPARα, on KLF4 mRNA induction. HT-29 cells were treated with these ligands for 4 h, and then KLF4 mRNA levels were determined by Northern Blot analysis. As shown in Fig. 3C, KLF4 mRNA levels increased significantly by treatment with 15d-PGJ_2, but not with either rosiglitazone or Wy-14,643, suggesting a different signaling mechanism involved in these ligands.

"Loss-of-function" mutations in PPARγ have recently been identified in a subset of colorectal tumors (Sarraf et al., 1999). To examine whether the endogenous PPARγ in HT-29 cells is responsive to 15d-PGJ_2, we performed transient transfection experiments. A report construct containing the PPRE of the rat acyl-CoA oxidase gene (pAOXPPREluc) or a control plasmid (pAOXBluc) was transfected into HT-29 cells. As illustrated in Fig. 3D, in the absence of PPARγ, treatment with 15d-PGJ_2 has no effect on PPRE reporter activity. However, when pAOXPPREluc were co-transfected with PPARγ expression vector into HT29 cells, a significant increase in PPRE reporter activity was detected and the activity was increased further after treatment with 15d-PGJ_2 (Fig. 3D). These results suggest that endogenous PPARγ in HT-29 cells may not be functional or is transcriptionally inactive, even though high level of its expression in these cancer cells

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has been reported (Sarraf et al., 1998). These data further support our hypothesis that 15d-PGJ_2-induced KLF4 expression is likely mediated through a PPARγ-independent pathway in HT-29 cells.

15d-PGJ_2 Induces a Rapid and a Transient Activation of ERK1/2 in HT-29 Cells

Activation of the ERK1/2 is generally related to growth stimulating actions of many growth factors. However, recent studies have indicated that growth inhibition could also result from ERK1/2 activation (Lahlou et al., 2003; Tsukada et al., 2001). To understand the signaling mechanisms involving in 15d-PGJ_2-induced KLF4 expression, we examined the effect of 15d-PGJ_2 on the activity and expression ERK1/2, the downstream substrates of MEK1/2, in HT-29 cells. Cells were treated with 15d-PGJ_2 (10 μM) in a serum-free medium for 2,5 –60 min, the levels of phosphorylated ERK1/2 and total ERK1/2 proteins were determined by using antibodies specific to the phosphorylated ERK1/2 (activated forms) and to total ERK1/2. As shown in Fig., 4A and 4B, 15d-PGJ_2, but not rosiglitazone, markedly induced a transient activation of p-ERK1/2. The increase in phosphorylated ERK1/2 was first observed at 2.5 min after addition of 15d-PGJ_2 and reached a peak (a 3.5-fold increase) at 5 min before returning to basal levels at 60 min. Treatment of HT-29 cells with increasing concentrations (1.0-10 μM) of 15d-PGJ_2 for 5 min also resulted in a dose-dependent activation of p-ERK1/2 (Fig. 5C). No change in total ERK1/2 protein level was detected in these studies.

The activity of ERK1/2 is tightly regulated by phosphorylation and dephosphorylation processes. MKP1, a dual specific phosphotase, is induced by many stimuli that activate ERK1/2, and subsequently inactivate ERK1/2. To confirm above

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findings, the level of MKP1 expression in HT-29 cells was evaluated after treatment with 15d-PGJ_2. As illustrated in Fig. 4D, the MKP1 was rapidly induced by15d-PGJ_2 in a time-dependent manner, supporting the involvement of ERK1/2 activation and inactivation in 15d-PGJ_2–mediated effects.

MEK/ERK, But not PI-3 Kinase Pathway is Involved in 15d-PGJ_2-mediated KLF4 mRNA Expression

To determine whether the MEK/ERK signaling pathway is involved in 15d-PGJ_2-induced KLF4 mRNA expression, the effects of specific inhibitors (PD98059 and U0126) of MRK, an upstream kinase of ERK, on KLF4 mRNA levels were further tested. HT-29 cells were pretreated with increasing concentrations of PD98059 (0.3-30 μM) or U0126 (0.2-20 μM) for 30 min followed by 4 h of 15d-PGJ_2 stimulation, ERK1/2 activities as well as KLF4 mRNA levels were determined by Western and Northern blot analysis. As shown in Fig. 5A-5D, pretreatment with PD98059 or U0126 dose-dependently inhibited 15d-PGJ_2-induced ERK1/2 phosphorylation and KLF4 mRNA up-regulation. These results suggest that MEK/ERK pathway is engaged in the induction of KLF4 mRNA by 15d-PGJ_2.

It has been reported that the activation of the MEK/ERK signaling pathway is regulated by PI-3 kinase (Pandey et al., 1999). To examine the involvement of PI-3 kinase, we examined the expression level of phosphorylated Akt, one of the downstream targets of PI3-kinase, in response to 15d-PGJ_2-treatment. As shown in Fig. 6A, Akt was activated by 15d-PGJ_2 in a time-dependent manner, with the highest level observed at 5 min. However, pretreatment of HT-29 cells with increasing concentrations of two

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different PI-3 kinase inhibitors, wortmannin (10-1000 nM) or LY294002 (0.25-25 μM), has no effect on 15d-PGJ_2-mediated KLF4 mRNA expression (Fig. 6B and 6C). These data indicate that PI-3 kinase is activated by 15d-PGJ_2 in HT-29 cells, but its activation may not involve in 15d-PGJ_2-induced KLF4 mRNA expression.

15d-PGJ_2-Mediated KLF4 mRNA Expression Requires STAT1 and Its Phosphorylation

STAT1, a member of the signal transducers and activators of transcription (STAT) family, has been shown to play a role in IFN-γ-induced KLF4 expression (Chen et al., 2002b). To define the role of STAT1 in 15d-PGJ_2-mediated KLF4 activation, we examined KLF4 mRNA levels in two mouse fibrosarcoma cell lines: CD+ ($STAT1^{+/+}$), and CD– ($STAT1^{-/-}$), derived from wild-type and STAT1 knockout mice, respectively. As shown in Fig. 7A, 15d-PGJ_2 induced a time-dependently increase in KLF4 mRNA levels in the STAT1- expressing ($STAT1^{+/+}$), but not in STAT1-knockout ($STAT1^{-/-}$) cells. These data suggest that STAT1 is necessary for 15d-PGJ_2-mediated KLF4 mRNA up-regulation. Moreover, the effect of 15d-PGJ_2 on STAT1 tyrosine phosphorylation was examined by Western blot analysis. HT-29 cells were treated with 15d-PGJ_2 (10 μM) for various periods of times (0-60 min), cell lysates were collected and analyzed for the presence of phosphorylated STAT1 using a mouse monoclonal antibody that specifically recognized Tyr-701 phosphorylated STAT1 (p-STAT1). As demonstrated in Fig. 7B and 7C, 15d-PGJ_2 also induced tyrosine phosphorylation of STAT1 in HT-29 cells.

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Down-regulation of KLF4 Expression by siRNA Reduces 15d-PGJ$_2$-mediated G$_1$ Phase Arrest in HT-29 Cells

Small interfering RNA (siRNA) targeted endogenous genes has been used to suppress intracellular expression of a specific genes in mammalian cells (Elbashir et al., 2001). To determine whether KLF4 mediates 15d-PGJ$_2$-induced growth inhibition of HT-29 cells, a sequence-specific duplex of 21 nucleotides targeted to KLF4 mRNA (KLF4 siRNA) was synthesized. The results illustrated in Fig. 8A showed that transfection of KLF4 siRNA in HT-29 cells reduced the expression levels of KLF4 protein in a dose-dependent manner, and about 50% of endogenous KLF4 protein was suppressed by 200 nM of KLF4 siRNA (Fig 8B). No change in KLF4 level was observed when control non-specific siRNA was used, demonstrating the specificity of the KLF4 siRNA. The effect of KLF4 siRNA on cell cycle progression of HT-29 cells was further examined. As shown in Fig. 8C, 15d-PGJ$_2$ induced a significant increase in cells arrested at G$_1$ phase and this effect was markedly attenuated by KLF4 siRNA,. The percentage of cell arrested at G$_0$/G$_1$ after15d-PGJ$_2$ treatment decreased from 86 ± 5 % to 61± 4 % ($p < 0.05$) when KLF4 siRNA was transfected, but the cells at S phase increased from 9.0 ± 3 % to 27± 8 % ($p < 0.01$).

Discussion

15d-PGJ$_2$, one of the well-defined cyclopentenone prostanglandins, has been recognized as a natural ligand with high affinity to PPARγ receptor (Forman et al., 1995). PPARγ is expressed at high level in human colonic epithelia cells and colorectal cancer cells (Brockman et al., 1998; Sarraf et al., 1998). As an activator of PPARγ, 15d-PGJ$_2$ exhibits many of its activities such as anti-proliferative, anti-tumorigenic, pro-apoptotic,

and anti-inflammatory through this receptor. However, several recent reported have indicated that anti-proliferative and growth inhibitory effects of PPARγ can be achieved by PPARγ-independent manner (Rossi et al., 2000; Straus et al., 2000). In this report, we show that 15d-PGJ_2 but not rosiglitazone reduces DNA synthesis and induces growth inhibition of HT-29 cells either in the presence or absence of serum. These data suggest that the growth inhibitory effect of 15d-PGJ_2 is not depend on other serum factors, and may not require activation of PPARγ receptor. Our studies also demonstrate that the expression of KLF4, a transcription factor involved in growth arrest, is induced by 15d-PGJ_2 in both time-and dose-dependent manners. When endogenous KLF4 protein is reduced by siRNA transfection, the growth arrest properties of15d-PGJ_2 was attenuated. These results suggest that the inhibitory effect of 15d-PGJ_2 on cell growth is mediated, at least in part, through up-regulation of KLF4 expression. The induction of KLF 4 mRNA expression by 15d-PGJ_2, observed in 5 different colon cancer cell lines, also supports the physiological significance of this effect.

The MAP kinase superfamily is composed of several subfamilies including ERK, c-Jun N-terminal kinase, and p38. Their activation is regulated by two upstream protein kinases: a MAP kinase kinase kinase, such as Raf and a MAP kinase kinase. The MAP kinase kinase that regulates ERK is called MEK or ERK kinase (Chang and Karin, 2001). Many different stimuli can activate the protein kinase cascade that activates ERK. Although the activation of ERK signaling pathway is linked to cell proliferation and tumorigenic activity, several recent studies have indicated that ERK activation can also lead to growth arrest by induction of $p21^{WAF/Cip1}$ or $p27^{kip1}$ cyclin-dependent kinase inhibitor (Hu et al., 1999; Pages et al., 1999). In the present study, 15d-PGJ_2 induces

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rapid and transient activation of ERK1/2 and triggers expression of MKP1, which may form an inhibitory feedback loop to fine tune the activity of ERK1/2, resulting in a net transient activation of the ERK1/2 signal. Moreover, activation of ERK1/2 signal appears to be essential for up-regulation of KLF4 mRNA expression, because two specific MEK inhibitors, PD98059 and U0126, attenuate KLF4 mRNA induction in a dose-dependent manner. Although PI3-kinase has also been reported to activate the ERK pathway by several stimuli, our data show that the induction of KLF4 by 15d-PGJ_2 is not affected by wortmannin and LY294002, suggesting that PI3-kinase pathway may not involve in up-regulation of KLF4 by 15d-PGJ_2. Further studies are required to identify the exact molecular mechanism by which ERK activation induces the expression of KLF4 mRNA.

The transcription factor STAT1 plays an important role in growth arrest. It has been reported that STAT1 can directly induce expression of many key proteins including $p21^{WAF/Cip1}$ involved in controlling the cellular processes of growth arrest (Chin et al., 1996). Our previous studies have demonstrated that up-regulation of KLF4 expression by IFN-γ is dependent on STAT1 and its phosphorylation (Chen et al., 2002b). In this study, we find that 15d-PGJ_2 induces rapid tyrosine phosphorylation of STAT1 in HT-29 cells, and that induction of KLF4 expression by 15d-PGJ_2 is only observed in the STAT1-expressing ($STAT1^{+/+}$), but not in STAT1-knockout ($STAT1^{-/-}$) cells. These data suggest that STAT1 and its phosphorylation are essential for 15d-PGJ_2-mediated effects, although detail mechanisms remain to be addressed.

PPARγ ligands regulate the expression of several genes, associated with cell growth and differentiation, in a ligand- and cell type-specific manner. Both PPARγ-dependent and PPARγ-independent mechanisms have been reported. For example,

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troglitazone but not other PPARγ ligands induces early growth response-1 (Erg-1) gene expression independently of PPARγ in HCT116 colon cells (Baek et al., 2003). In the present study, our data also indicate that PPARγ may not be required in 15d-PGJ_2-induced KLF4 expression. These data include: 1) KLF4 does not contain putative PPRE sequences in its promoter region; 2) 15d-PGJ_2 rapidly activated ERK and induced KLF4 mRNA expression, which does not require new protein synthesis (Clay et al., 2001; Gupta et al., 2003); 3) KLF4 expression is selectively induced by 15d-PGJ_2 but not rosiglitazone, another specific ligand for PPARγ; and 4) 15d-PGJ_2 can not stimulate PPAR-dependent promoter activity in the absence of cotransfected PPARγ. These results are consistent with report from Takeda et al., showing that the effect of 15d-PGJ_2 on *c-fos* gene expression in vascular smooth cells is mediated through a nongenomic or a PPARγ-independent mechanism (Takeda et al., 2001). Although the possibility of a PPARγ-dependent induction of KLF4 by 15d-PGJ_2 in other cells can not be excluded, our current study indicate that at least in HT-29 cells, the effect of 15d-PGJ_2 on KLF4 expression does not involve its nuclear receptor PPARγ.

The interaction between KLF4 and PPARγ has not been explored previously. As illustrated in Fig. 2, the induction of KLF4 by 15d-PGJ_2 was peaked at 4 h, and the effect was then gradually decreased. In contrast, 15d-PGJ_2-treatment in HT-29 cells resulted in transient decreases in PPARγ levels between 2-8 h, and these decreases in PPARγ expression is correspondent to the increases in KLF4 concentration in these cells. These data suggest that KLF4 may exhibit an inhibitory effect on PPARγ expression. Interestingly, the level of PPARδ mRNA also increased when the expression of PPARγ was inhibited (Fig. 2A and 2B). Recently, PPARδ has been shown to function as a potent

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inhibitor of transcriptional activity of PPARγ gene (Shi et al., 2002). More studies will be required to elucidate the association between KLF4 and the family of PPAR receptors.

In conclusion, we have demonstrated that KLF4 is one of the down-stream effectors of 15d-PGJ_2 in HT-29 colon cancer cells. 15d-PGJ_2 induces the expression of KLF4 in both time- and dose-dependent manners, and through a PPARγ-independent mechanism that requires ERK signaling, and is STAT1-dependent. Recently, several studies have reported that cyclopentenone prostaglandins, especially 15d-PGJ_2, are not formed in a biologically relevant level *in vivo* (Bell-Parikh et al., 2003; Powell, 2003). In the present study, the amount of 15d-PGJ_2 required to induce growth inhibition and KLF4 expression in colon cancer cells was significantly higher than the concentration of intracellular 15d-PGJ_2 formed under physiological condition, as reported by Bell-Parikh et al (Bell-Parikh et al., 2003). However, the level of 15d-PGJ_2 in the micro-environment of the colonic mucosa has not yet been determined. The physiological relevance of our current observation will, therefore, warrant further evaluation. Nevertheless, our data may provide a novel mechanism for the anti-tumorigenic properties of 15d-PGJ_2.

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FIGURE LEGENDS

Fig. 1. Effect of PPARγ ligand 15d-PGJ$_2$ or rosiglitazone on the growth of HT-29 cells. A, a time course of the effect of 15d-PGJ$_2$ or rosiglitazone on the growth of HT-29 cells in the presence of serum. Cells were seeded at a density of 5 x 10^4 cell/well (12-well plates) in McCoy's medium with 10% FBS. After 24 h, the medium was replaced with fresh medium, and 15d-PGJ$_2$ (10 μM) or rosiglitazone (10 μM) were added to cells. At the indicated time, cell viability was determined by MTT assay as described under "*Materials and Methods*". Each value represents the mean ± S.E.M. of triplicate measurements, and the same experiment has been repeated for 3 times with similar results. B-E, the effect of 15d-PGJ$_2$ or rosiglitazone on cell growth and DNA synthesis in HT-29 cells in the absence of serum. Cells were seeded at a density of 5 x 10^4 cell/well (12-well plates) and starved for 18 h in serum-free medium. The serum-deprived cells were then treated with vehicle (as a control), 15d-PGJ$_2$ (10 μM) or rosiglitazone (10 μM). After 24 h, cell viability and DNA synthesis were measured by MTT and [^{3}H]thymidine incorporation assay as described under "*Materials and Methods*". Each value represents the mean ± S.E.M. of triplicate measurements, and the same experiment has been repeated for at least 3 times with similar results. * $P < 0.05$, compared to control.

Fig. 2. 15d-PGJ$_2$ up-regulates levels of KLF4 mRNA and protein expression in HT-29 cells. A, HT-29 cells were grown in the absence of serum for 18 h and then incubated with vehicle alone (*C*) or with 10 μM 15d-PGJ$_2$ (*P*) for indicated time (0-24 h). Total RNA was prepared, and the expression of KLF4, PPARγ, and PPARδ mRNA was determined by Northern blot analysis. *B*, HT-29 cells were grown in the absence of serum

for 18 h and then incubated with increasing concentrations (0-20 μM) of 15d-PGJ_2 for 4 h. The expression of KLF4, PPARγ, and PPARδ mRNA was determined by Northern blot analysis. The blots stained with ethidium bromide, showing the presence of 18S and 28S RNA, were included to confirm equal RNA loading. C, Northern blot analysis of KLF4 mRNA expression in five human colon cancer cell lines in the presence (*P*) or absence (*C*) of 15d-PGJ_2. D, HT-29 cells were incubated with either vehicle alone (-) or with 10 μM 15d-PGJ_2 (+) for 2-24 h. The KLF4 and β-tubulin protein levels were examined by Western blot analysis as described under "*Materials and Methods*". The level of mRNA and protein expression was quantified, and expressed as relative density to the control (fold increase over control), and was shown on the bottom of each gel.

Fig. 3. The induction of KLF4 mRNA by 15d-PGJ_2 is independent of PPARγ in HT-29 cells. A and B, induction of 15d-PGJ_2-mediated KLF4 mRNA expression is independent of new protein synthesis. HT-29 cells were preincubated with (+) or without (-) cycloheximide (CHX) for 30 min and then treated with or without (-) 15d-PGJ_2 for 4 h. Total RNA was isolated, and expression of KLF4 mRNA was determined by northern blot analysis (A). HT-29 cells were preincubated with CHX for 30 min, and then treated with vehicle or 15d-PGJ_2 (10 μm) for the previous periods of time as indicated. The levels of KLF4 mRNA expression were examined by Northern blot analysis of 20 μg of total RNA with ^{32}p-labeled KLF4 probe (B). C, 15d-PGJ_2 induced specifically expression of KLF4 mRNA in HT-29 cells. Cells were serum-starved for 18 h and treated with vehicle (Me_2SO, 0.2%), 15d-PGJ_2 (10 μM), rosiglitazone (10 μM), or Wy-14,643 (10 μM) for 4 h. Total RNA was isolated and subjected to Northern blot analysis. D, blocking

of PPARγ receptor does not antoganize induction of KLF4 mRNA by 15d-PGJ2. HT-29 cells were preincubated with various concentrations of GW9662, as indicated, for 30 min and then treated with 15d-PGJ_2 for 4 h. Total RNA was isolated and levels of KLF4 RNA expression were determined by Northern blot analysis. D, cotransfection with PPARγ expression vector is necessary to observe pAOXPPREluc activity in 15d-PGJ_2-stimulated HT-29 cells. HT-29 cells were transfected with pAOXBluc or pAOXPPREluc, or together with PPARγ expression vector, as shown, 15d-PGJ_2 (10 μM) was then added to cells 24 h posttransfection. Luciferase activity was measured 48 h posttransfection. All values reported above are the average of three transfections carried in duplicate ± S.E.M. and were normalized to untreated cells transfected with the respective reporter gene construct alone.

Fig. 4. 15d-PGJ_2 induces a rapid and transient activation of ERK1/2 in HT-29 cells. HT-29 cells were grown in the absence of serum for 18 h and then stimulated with 15d-PGJ_2 (A) or rosiglitazone (B) for different times (2.5-60 min), or with increasing concentrations of 15d-PGJ_2 (1.0-10 μM) for 5 min (*C*). The cell lysates were isolated and phosphorylated ERK1/2 and total ERK1/2 were determined by Western blot analysis using antibodies specific to phosphorylated ERK1/2 and total ERK1/2. D, HT-29 cells were treated with 10 μM 15d-PGJ_2 for the indicated times. The cell lysates were isolated and MKP1 protein levels were determined by Western blot analysis. *Left*, a representative result from three independent experiments is shown. *Right*, the density of specific band was scanned and quantified. The ratio of phosphorylated ERK1/2 to total ERK1/2 or MKP1 to β-tubulin protein expression level is shown. Quantitative data shown on the

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right, represented means ± S.E.M. for three replicate determinations, and expressed as fold induction over control. *, $P < 0.05$ *versus* control.

Fig. 5. MEK/ERK pathway is involved in 15d-PGJ$_2$-mediated KLF4 mRNA expression. The serum-starved HT-29 cells were preincubated with increasing concentrations of PD98059 or U0126, as indicated, for 30 min. The cells were then stimulated with 15d-PGJ$_2$ (10 μM) for 5 min. The cell lysates were isolated and phosphorylated ERK1/2 (*p-ERK1/2*) and total ERK1/2 (*ERK1/2*) was determined by Western blot analysis (A and C). The serum-starved HT-29 cells were precultured in the presence of various concentrations of PD98059 or 0126, as indicated, for 30 min. The cells were then treated with 15d-PGJ2 (10 μM) for 4 h. The total RNA was isolated and subjected to Northern blot analysis of KLF4 mRNA expression (B and D). Quantitative data shown on the right, represented means ± S.E.M. of five replicate determinations, and expressed as fold induction over control. In A and B, *, $P < 0.05$ compared to cells treated with 15d-PGJ$_2$ but without PD98059. In C and D, *, $P < 0.05$; **, $P < 0.01$ compared to cells treated with 15d-PGJ$_2$ but without U0126.

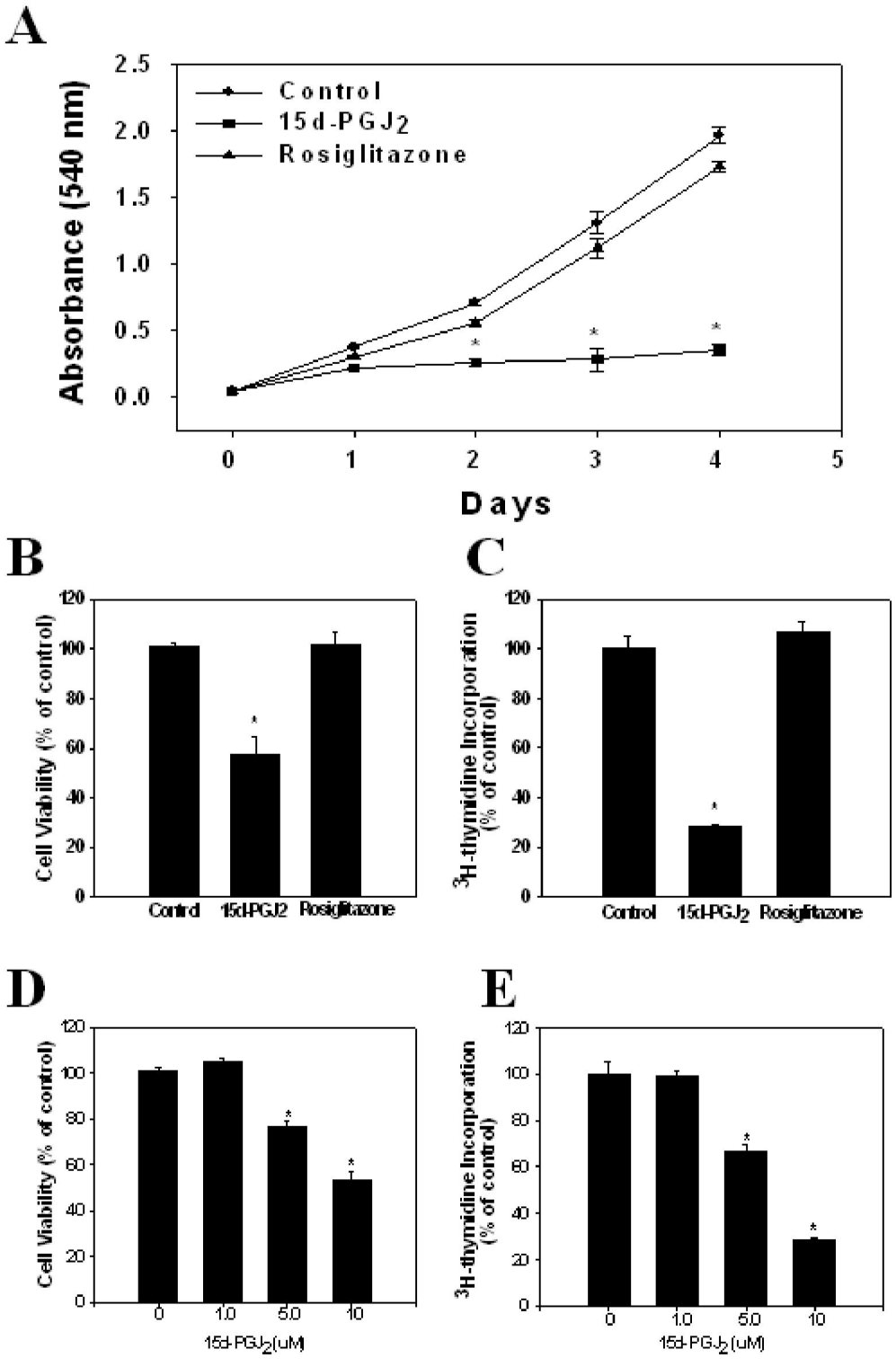
Fig. 6. PI3-kinase is not responsible for 15d-PGJ2-induced KLF mRNA expression. A, HT-29 cells were serum-starved for 18 h and stimulated with 15d-PGJ$_2$ (10 μM) for the indicated times. Total lysates were isolated and phosphorylated Akt (*p-Akt*) and total Akt (*Akt*) were determined by Western blot analysis. B and C, the serum-starved cells were preincubated with increasing concentrations of wortmannin (10-1000 nM) or LY294002 (0.25-25 μM) for 30 min and then treated with 15d-PGJ$_2$ (10 μM) for 4 h. The

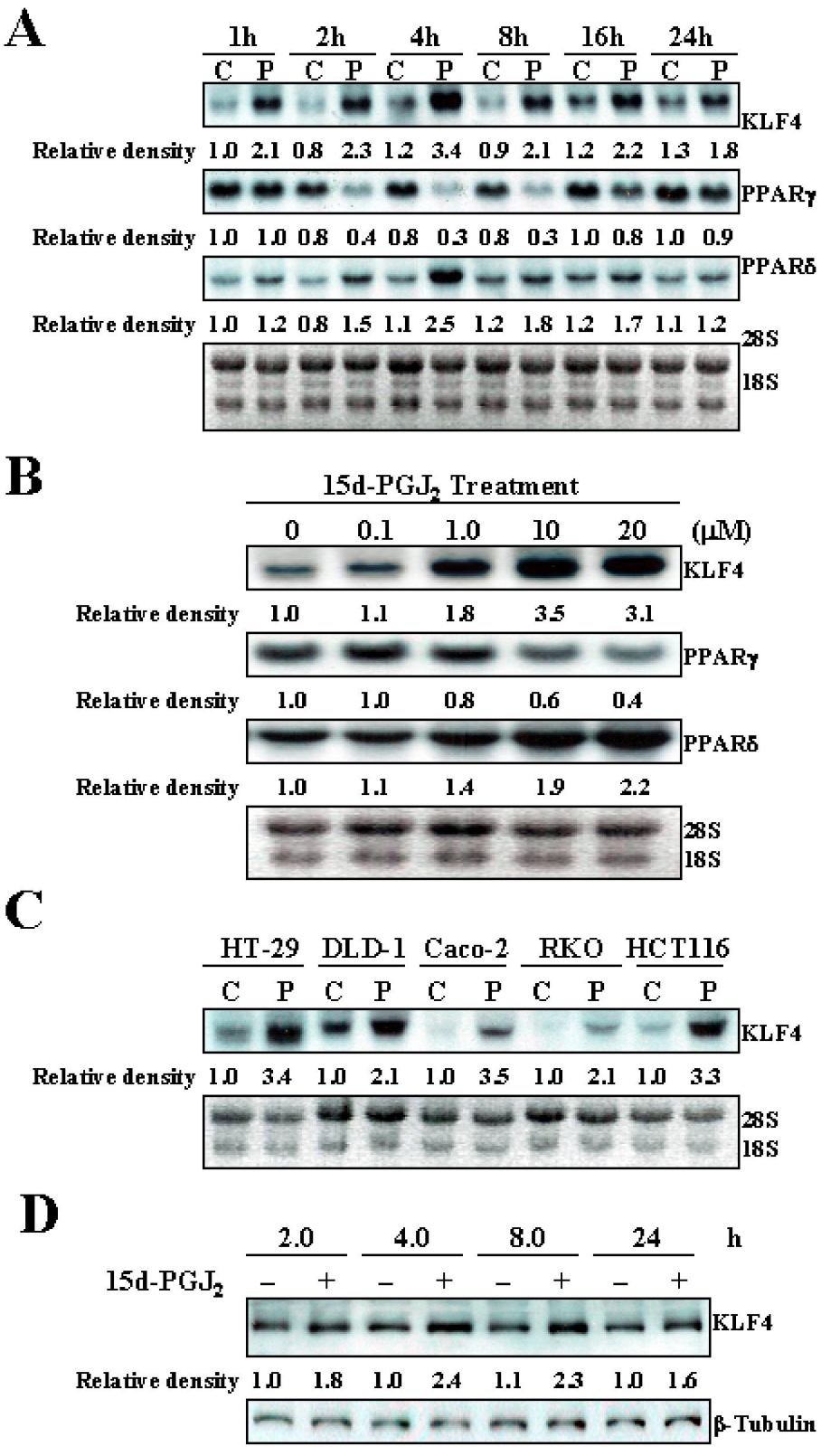
total RNA was isolated and expression of KLF4 mRNA was determined by Northern blot analysis. Quantitative data shown on the right, represented means ± S.E.M. for five replicate determinations, and expressed as fold induction over control. *, $P < 0.05$ *versus* control.

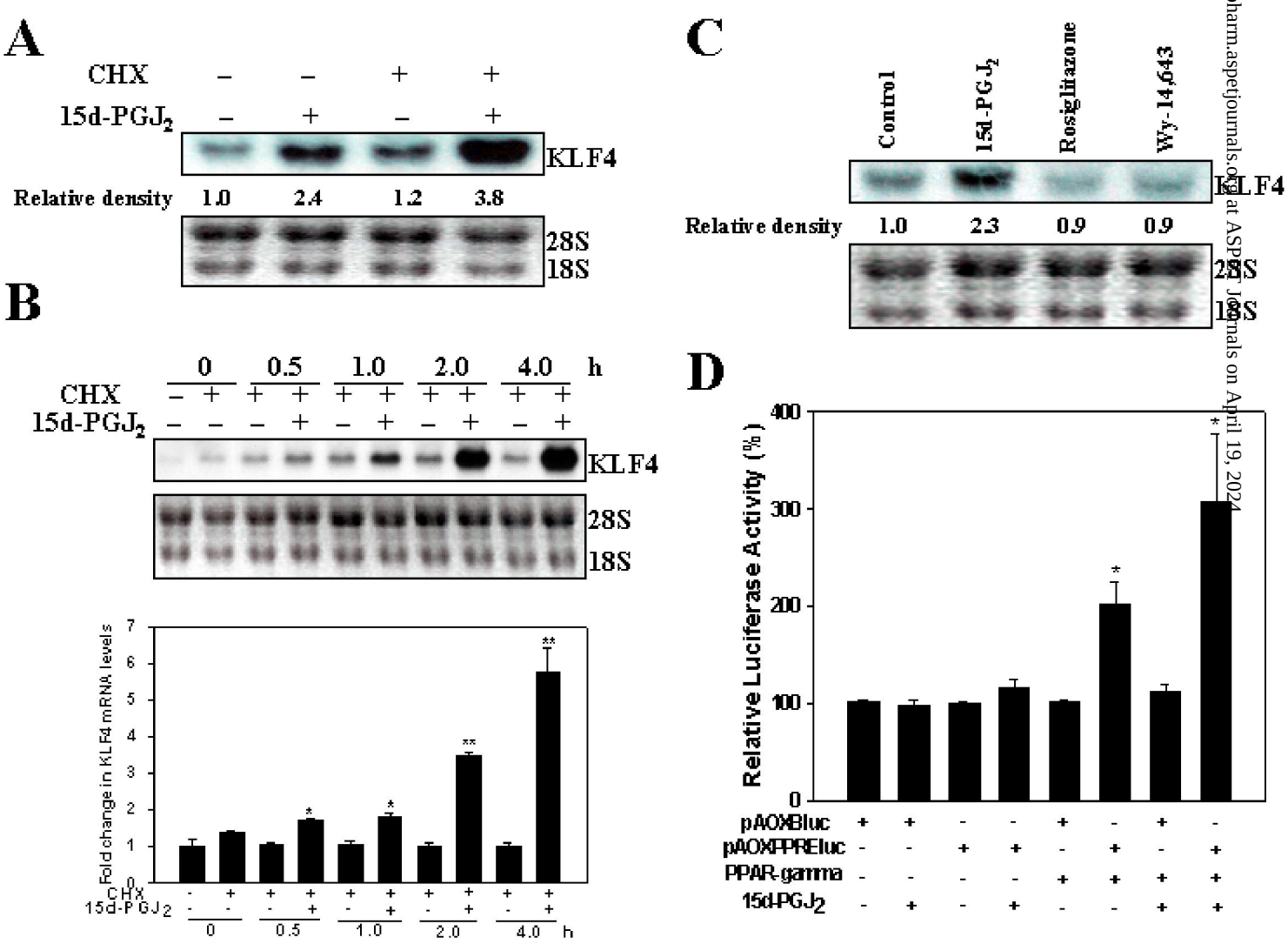
Fig. 7. 15d-PGJ_2-mediated KLF4 mRNA expression requires STAT1 and its phosphorylation. A, STAT1 is necessary for 15d-PGJ_2-mediated KLF4 mRNA up-regulation. STAT1+/ +/+ and STAT1-/- cells, derived from the wild-type and the STAT1 knockout mice, respectively, were incubated with 15d-PGJ_2 (10 μM) for the indicated times and levels of KLF4 mRNA expression were determined by Northern blot analysis. B and C, 15d-PGJ_2 induces STAT1 phosphorylation on tyrosine. HT-29 cells were grown in the absence of serum for 18 h and stimulated with 15d-PGJ_2 (10 μM) for the indicated times. The levels of STAT1 phosphorylated on Y701 (*p-STAT1*) and total STAT1 (*STAT1*) were detected by Western blot analysis using antibody specifically recognizing STAT1 phosphorylated on Y701 or total STAT1. Quantitative data of relative phosphorylation level as presented on blot A was shown on B, represented means ± S.E.M. for five replicate determinations, and expressed as fold induction over control. *, $P < 0.05$ *versus* control.

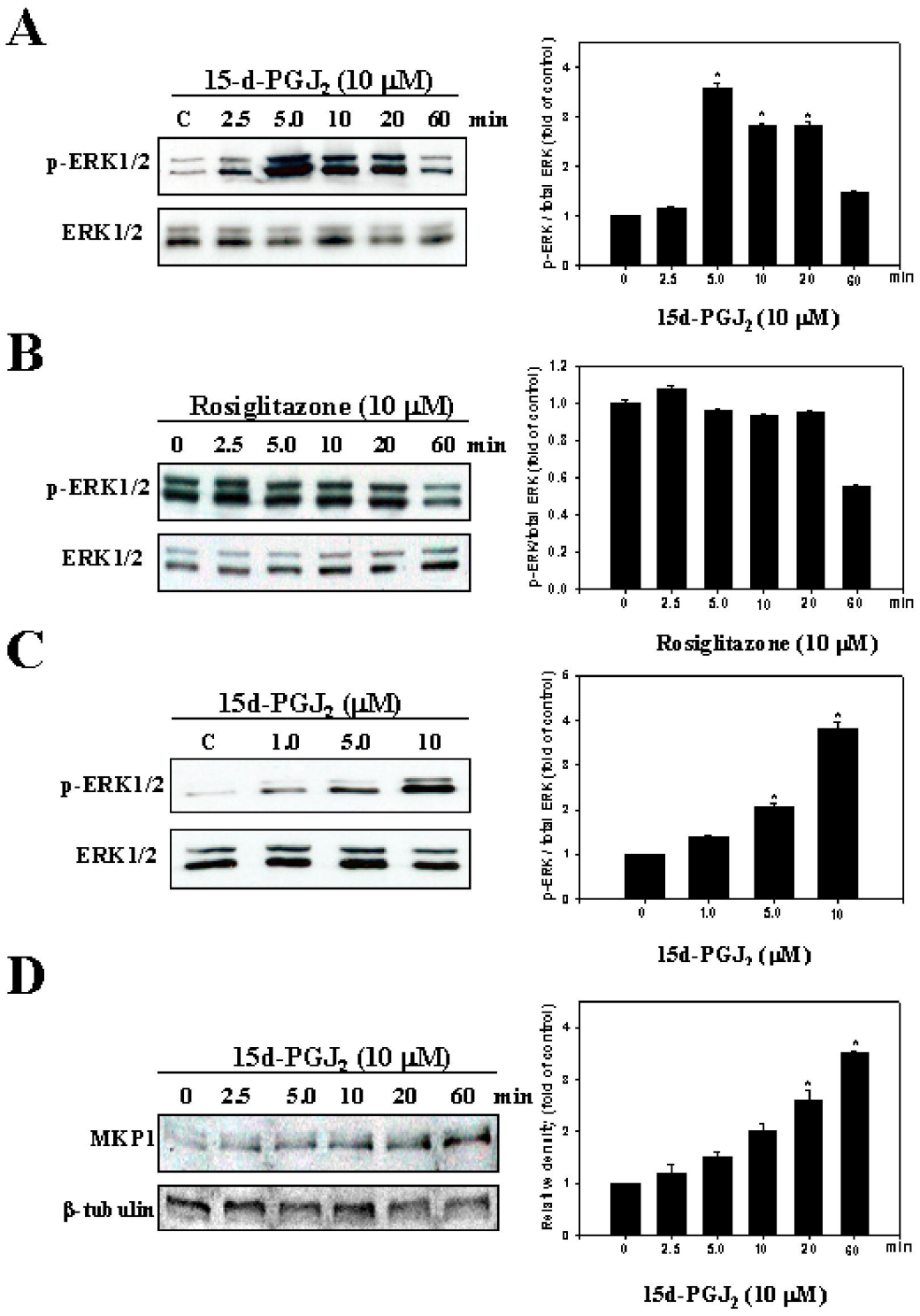
Fig. 8. Down-regulation of KLF4 expression by siRNA reduces 15d-PGJ_2-mediated G_1 phase arrest in HT-29 cells. Effect of KLF4 siRNA on the expression of KLF4 protein in HT-29 cells. Cells were transfected with KLF4 siRNA or control siRNA, and

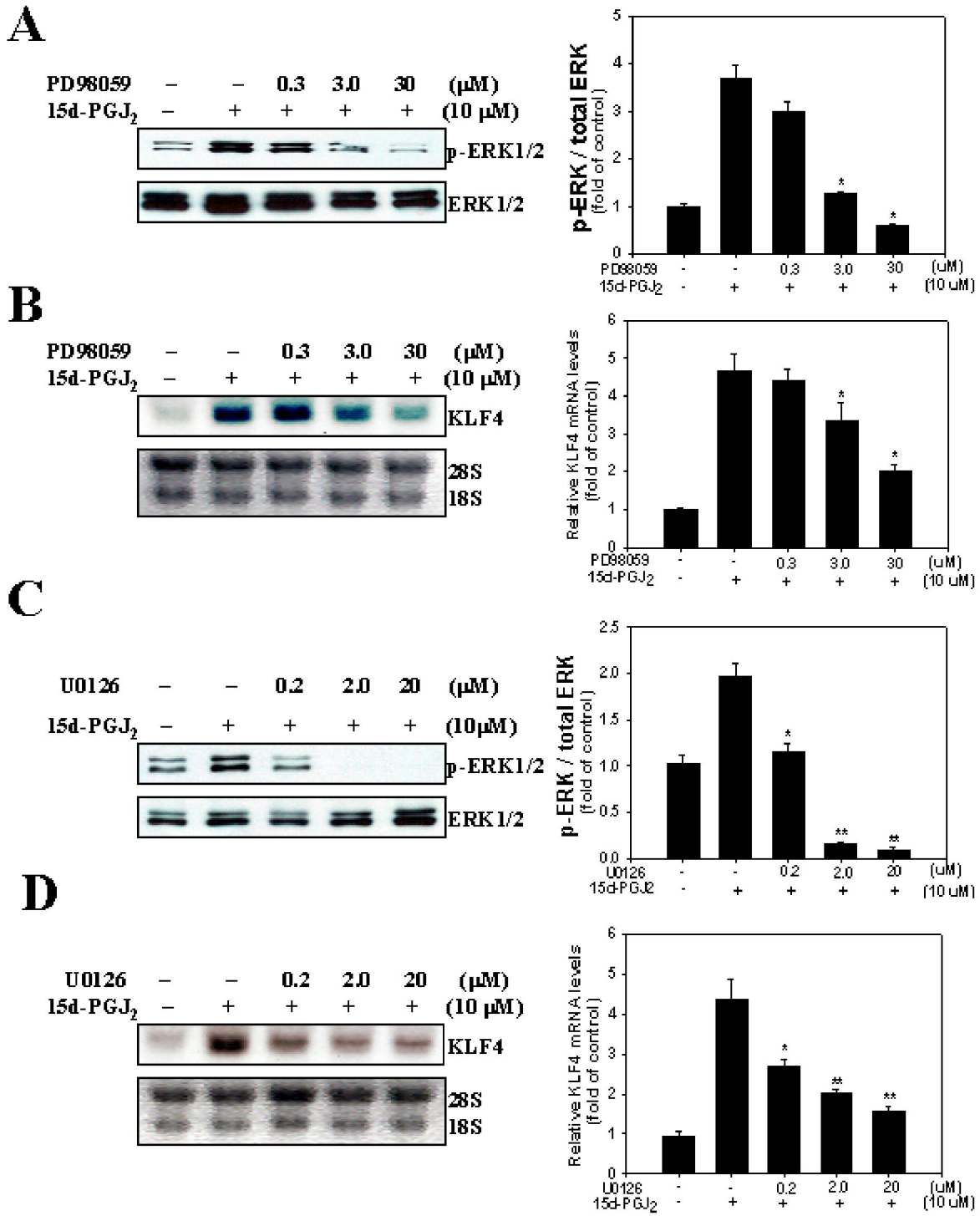
cell lysates were analyzed for the level of KLF4 protein by Western Blot analysis as described under "*Materials and Methods*". A, a representative result from three independent experiments is shown. B, the density of each band was scanned and quantified as indicated. Results are means ± S.E.M. for three replicate determinations, *, $P < 0.05$ compared with control. C, effect of KLF4 siRNA on 15d-PGJ_2-mediated cell cycle progression in HT-29 cells. HT-29 cells were treated with Me_2SO (control) or with 15d-PGJ_2, or transfected with KLF4 siRNA followed by treatment with 15d-PGJ_2, and the percent of distribution of cells in G_1, S, and G_2/M were determined by FACS analysis as described under "*Materials and Methods*". Similar results were observed in a duplicate analysis.

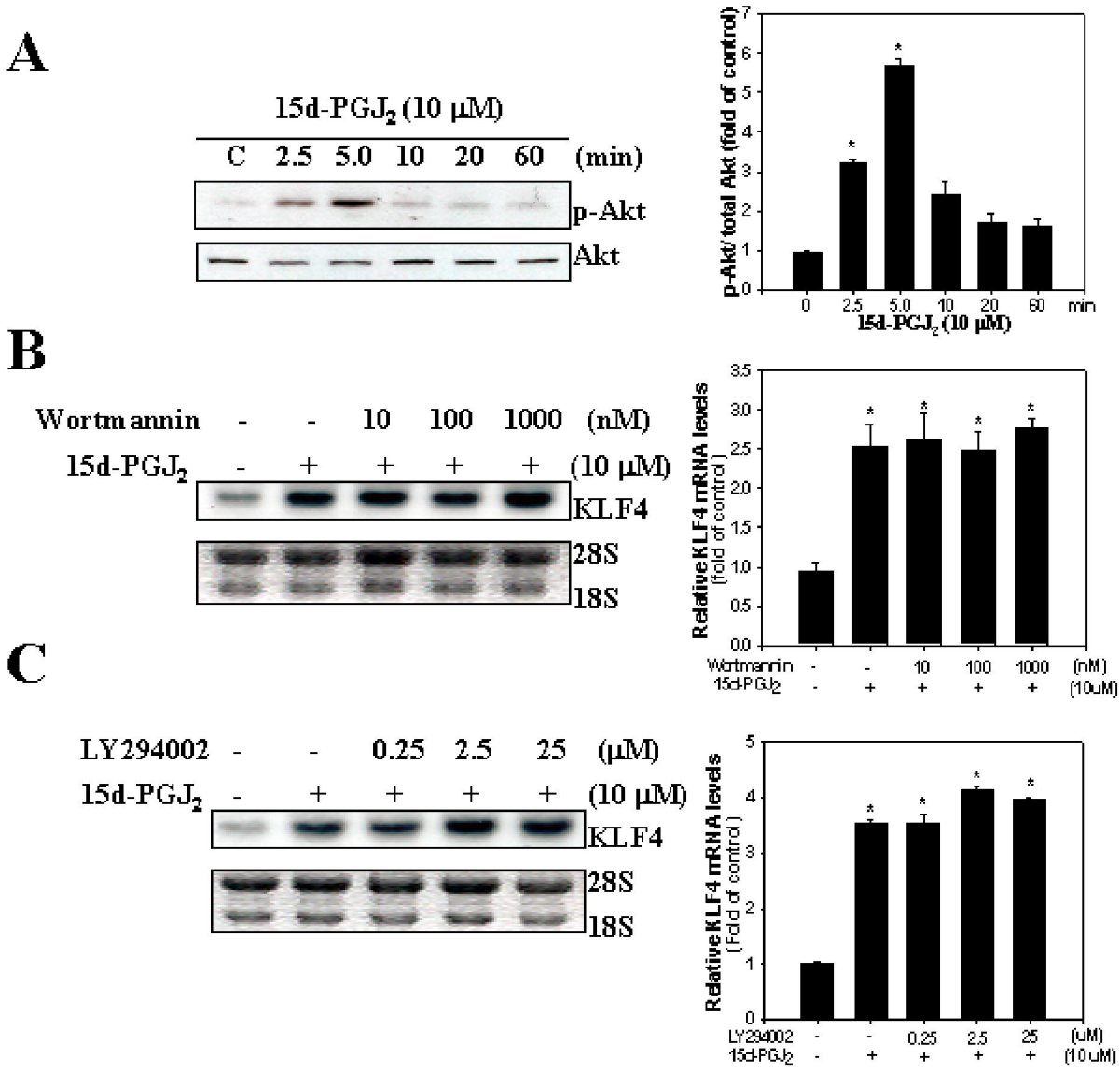

A
Absorbance (540 nm)
Control
15d-PGJ2
Rosiglitazone
Days
B
Cell Viability (% of control)
Contrd
15d-PGJ2
Rosiglitazone
C
3H-thymidine Incorporation (% of control)
Control
15d-PGJ2
Rosiglitazone
D
Cell Viability (% of control)
15d-PGJ2(uM)
E
3H-thymidine Incorporation (% of control)
15d-PGJ2(uM)

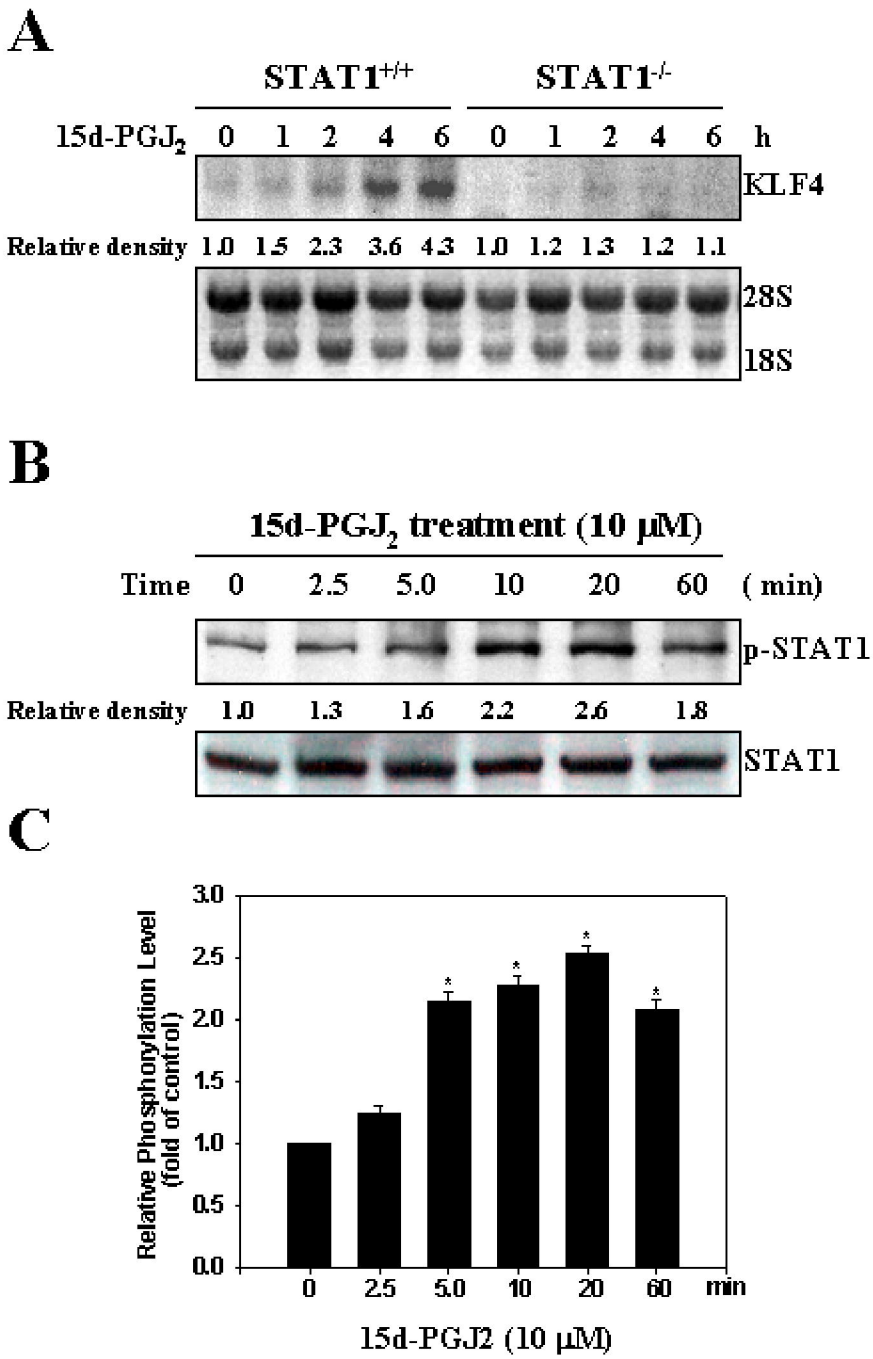

A
1h 2h 4h 8h 16h 24h
C P C P C P C P C P C P
KLF4
Relative density 1.0 2.1 0.8 2.3 1.2 3.4 0.9 2.1 1.2 2.2 1.3 1.8
PPARγ
Relative density 1.0 1.0 0.8 0.4 0.8 0.3 0.8 0.3 1.0 0.8 1.0 0.9
PPARδ
Relative density 1.0 1.2 0.8 1.5 1.1 2.5 1.2 1.8 1.2 1.7 1.1 1.2
28S
18S
B
15d-PGJ2 Treatment
0 0.1 1.0 10 20 (μM)
KLF4
Relative density 1.0 1.1 1.8 3.5 3.1
PPARγ
Relative density 1.0 1.0 0.8 0.6 0.4
PPARδ
Relative density 1.0 1.1 1.4 1.9 2.2
28S
18S
C
HT-29 DLD-1 Caco-2 RKO HCT116
C P C P C P C P C P
KLF4
Relative density 1.0 3.4 1.0 2.1 1.0 3.5 1.0 2.1 1.0 3.3
28S
18S
D
2.0 4.0 8.0 24 h
15d-PGJ2 – + – + – + – +
KLF4
Relative density 1.0 1.8 1.0 2.4 1.1 2.3 1.0 1.6
β-Tubulin




A
15-d-PGJ2 (10 μM)
C 2.5 5.0 10 20 60 min
p-ERK1/2
ERK1/2
p-ERK / total ERK (fold of control)
0 2.5 5.0 10 20 60 min
15d-PGJ2 (10 μM)
B
Rosiglitazone (10 μM)
0 2.5 5.0 10 20 60 min
p-ERK1/2
ERK1/2
p-ERK/total ERK (fold of control)
0 2.5 5.0 10 20 60 min
Rosiglitazone (10 μM)
C
15d-PGJ2 (μM)
C 1.0 5.0 10
p-ERK1/2
ERK1/2
p-ERK / total ERK (fold of control)
0 1.0 5.0 10
15d-PGJ2 (μM)
D
15d-PGJ2 (10 μM)
0 2.5 5.0 10 20 60 min
MKP1
β-tubulin
Relative density (fold of control)
0 2.5 5.0 10 20 60 min
15d-PGJ2 (10 μM)


A
PD98059 – – 0.3 3.0 30 (μM)
15d-PGJ2 – + + + + (10 μM)
p-ERK1/2
ERK1/2
p-ERK / total ERK (fold of control)
PD98059 - - 0.3 3.0 30 (uM)
15d-PGJ2 - + + + + (10 uM)
B
PD98059 – – 0.3 3.0 30 (μM)
15d-PGJ2 – + + + + (10 μM)
KLF4
28S
18S
Relative KLF4 mRNA levels (fold of control)
PD98059 - - 0.3 3.0 30 (uM)
15d-PGJ2 - + + + + (10 uM)
C
U0126 – – 0.2 2.0 20 (μM)
15d-PGJ2 – + + + + (10μM)
p-ERK1/2
ERK1/2
p-ERK / total ERK (fold of control)
U0126 - - 0.2 2.0 20 (uM)
15d-PGJ2 - + + + + (10 uM)
D
U0126 – – 0.2 2.0 20 (μM)
15d-PGJ2 – + + + + (10 μM)
KLF4
28S
18S
Relative KLF4 mRNA levels (fold of control)
U0126 - - 0.2 2.0 20 (uM)
15d-PGJ2 - + + + + (10 uM)


A
15d-PGJ2 (10 μM)
C 2.5 5.0 10 20 60 (min)
p-Akt
Akt
p-Akt/ total Akt (fold of control)
0 2.5 5.0 10 20 60 min
15d-PGJ2 (10 μM)
B
Wortmannin - - 10 100 1000 (nM)
15d-PGJ2 - + + + + (10 μM)
KLF4
28S
18S
Relative KLF4 mRNA levels (fold of control)
Wortmannin - - 10 100 1000 (nM)
15d-PGJ2 - + + + + (10uM)
C
LY294002 - - 0.25 2.5 25 (μM)
15d-PGJ2 - + + + + (10 μM)
KLF4
28S
18S
Relative KLF4 mRNA levels (Fold of control)
LY294002 - - 0.25 2.5 25 (uM)
15d-PGJ2 - + + + + (10uM)


A
STAT1+/+
STAT1-/-
15d-PGJ2 0 1 2 4 6 0 1 2 4 6 h
KLF4
Relative density 1.0 1.5 2.3 3.6 4.3 1.0 1.2 1.3 1.2 1.1
28S
18S
B
15d-PGJ2 treatment (10 μM)
Time 0 2.5 5.0 10 20 60 (min)
p-STAT1
Relative density 1.0 1.3 1.6 2.2 2.6 1.8
STAT1
C
Relative Phosphorylation Level (fold of control)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 2.5 5.0 10 20 60 min
15d-PGJ2 (10 μM)