

15-Deoxy- $\Delta^{12,14}$ -prostaglandin J₂ Up-regulates KLF4 Expression Independently of PPAR γ by Activating the MEK/ERK Signal Transduction Pathway in HT-29 Colon Cancer Cells

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ABBREVIATIONS: 15d-PGJ₂, 15-deoxy- $\Delta^{12,14}$ prostaglandin J₂; PPAR γ , peroxisome proliferators-activated receptor γ ; PPRE, PPAR response element; KLF4, Krüppel-like factor 4; CHX, cycloheximide; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PI-3 kinase, phosphoinositide 3-kinase; STAT, signal transducers and activators of transcription; AOX, acyl-CoA oxidase; FACS, fluorescence-activated cell sorter; siRNA, small interference RNA.

ABSTRACT

15-deoxy- $\Delta^{12,14}$ prostaglandin J₂ (15d-PGJ₂) is a natural ligand for the peroxisome proliferators-activated receptor γ (PPAR γ) 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-PGJ₂. Treatment of HT-29 cells with 15d-PGJ₂ 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-PGJ₂-mediated G₁ phase arrest, suggesting that KLF4 mediated function of 15d-PGJ₂. The effect of 15d-PGJ₂ on KLF4 expression appears not to involve its nuclear receptor PPAR γ , as our data show that: 1) KLF4 gene promoter does not contain putative PPRE sequence; 2) 15d-PGJ₂ rapidly activates ERK and induces KLF4 mRNA expression; 3) KLF4 is induced by 15d-PGJ₂ but not by rosiglitazone, a synthetic PPAR γ ligand; and 4) 15d-PGJ₂ is unable to stimulate PPAR-dependent promoter activity in the absence of co-transfected PPAR γ . Moreover, 15d-PGJ₂-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-PGJ₂ was occurred only in STAT1-expressing but not in STAT1-knockout cells. Together, these results suggest that 15d-PGJ₂-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 PPAR γ receptor, but may involve in MEK/ERK pathway, and is STAT1-dependent.

Introduction

Peroxisome proliferators-activated receptor- γ (PPAR γ) 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 (Kliwer et al., 1997), PPAR γ is also expressed at high levels in colonic epithelial cells and colon cancer cells (Brockman et al., 1998; Sarraf et al., 1998). PPAR γ 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 (Kliwer et al., 1992; Lemberger et al., 1996). J₂ series cyclopentenone prostaglandins (cyPGs), especially 15-deoxy- $\Delta^{12,14}$ prostaglandin J₂ (15d-PGJ₂), have recently received increasing attention because they functioned as potential regulators of diverse processes including cell growth, differentiation, and inflammation. 15d-PGJ₂, a natural ligand for PPAR γ , has been shown to possess an anti-tumorigenic activity. For example, activation of PPAR γ by 15d-PGJ₂ 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-PGJ₂ possess similar anti-proliferative activity, and that KLF4 could be one of the mediators of 15d-PGJ₂ in colon cancer cells.

In this study, we examined the effect of 15d-PGJ₂ on KLF4 expression in HT-29 colon cancer cells. Our results show that that 15d-PGJ₂ inhibits proliferation of HT-29 cells and induces up-regulation of KLF4 mRNA and protein levels. The induction of KLF4 by 15d-PGJ₂ appears not to involve in nuclear receptor PPAR γ , but is dependent on the activation of MEK/ERK pathway. These data may provide a novel mechanism governing the anti-proliferative property of 15d-PGJ₂ in colon cancer cells.

Materials and Methods

Reagents. 15-deoxy- Δ 12,14 prostaglandin J₂ (15d-PGJ₂) and rosiglitazone were purchased from Cayman Chemicals (Ann Arbor, MI). PD98059, U0126, wortmannin, and LY294002 were obtained from Calbiochem (La Jolla, CA). [α -³²P]dCTP and [³H] 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% CO₂ at 37 °C. Cells in exponential growth were plated before serum starvation for 24 h. Serum-starved cells were then exposed to 15d-PGJ₂ 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-PGJ₂ (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 [³H]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 [³H]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 phospho-ERK1/2, ERK1/2, phospho-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™ 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-PstI* fragment of the human KLF4 DNA that was radiolabeled with [³²P]-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 PPAR γ or PPAR δ cDNA probe. PPAR γ and PPAR δ 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 μ L) 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 (GenBankTM accession number XM_047517). The sequences of the 21-nucleotide (nt) sense and antisense RNA are as follows: 5'-GACCGAGGAGUUCAACGAUUU-3' (sense) and 5'-

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 performed 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 \pm SEM, and a 2-way ANOVA with Dunnett's post-test (InStat 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-PGJ₂ 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-PGJ₂ on cell growth and DNA synthesis. HT-29 cells were cultured in the medium containing 10% FBS in the presence or absence of 15d-PGJ₂ for the indicated intervals, and cell growth was measured by MTT assay. For comparison, rosiglitazone, a synthetic TZD PPAR γ ligand, was also used in this experiment. Fig. 1A showed that the growth of HT-29 cells was inhibited by 15d-PGJ₂ in a time-dependent manner, whereas rosiglitazone had no

significant effect. To confirm that this inhibitory effect is specific for 15d-PGJ₂, we examined the effect of 15d-PGJ₂ on cell growth and DNA synthesis under the serum-free condition by MTT and [³H]-thymidine incorporation assays. As shown in Fig. 1B-1E, treatment of HT-29 cells with 15d-PGJ₂, but not rosiglitazone, resulted in markedly inhibition of both cell growth and DNA synthesis. The growth inhibitory effect of 15d-PGJ₂ was also dose-dependent (Fig. 1D, and 1E). These results indicate that the effect of 15d-PGJ₂ on cell growth is not mediated through other serum factors.

15d-PGJ₂ 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-PGJ₂-mediated growth inhibition, KLF4 mRNA and protein levels were examined in 15d-PGJ₂-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-PGJ₂ 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-PGJ₂ 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-PGJ₂ treatment. Moreover, treatment with 15d-PGJ₂ also led to a time-dependent accumulation of KLF4 protein, with the level peaked at 4 h (Fig. 2D).

Previous studies have shown that 15d-PGJ₂ was a potent activator of nuclear PPAR γ receptor and many of its functions appeared to be mediated through this receptor. To determine the role of PPAR γ in 15d-PGJ₂-induced KLF4 up-regulation, PPAR γ mRNA levels were examined with a human PPAR γ cDNA probe. Unexpectedly, the effect of 15d-PGJ₂ on PPAR γ expression is opposite to what observed with KLF4 (Fig. 2A and 2B). Treatment with 15d-PGJ₂ reduced PPAR γ mRNA expression in time- and dose-dependent manners, with the maximal effects seen at 4 h and at 20 μ M, respectively. On the contrary, 15d-PGJ₂-treatment up-regulated the expression levels of PPAR δ 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-PGJ₂-mediated up-regulation of KLF4 mRNA was not associated with increased levels of the nuclear receptor PPAR γ mRNA.

The Induction of KLF4 mRNA by 15d-PGJ₂ is Independent of PPAR γ 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 PPAR γ response element (PPRE) in this region (Biology Workbench, version 3.2; <http://workbench.sdsc.edu>). These data suggest that 15d-PGJ₂-induced KLF4 up-regulation is mediated most likely through a PPAR γ -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-PGJ₂. HT-29 cells were

pre-incubated with CHX (100 $\mu\text{g/ml}$) for 30 min, and then treated with 15d-PGJ₂. As shown in Fig. 3A, CHX alone has no effect on KLF4 mRNA level. In contrast, the combination of 15d-PGJ₂ and CHX resulted in a greater increase in KLF4 mRNA level than that observed following treatment with 15d-PGJ₂ 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-PGJ₂-induced KLF4 up-regulation. Next, we compared the effect of 15d-PGJ₂, rosiglitazone and Wy-14,643, a ligand of PPAR α , 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-PGJ₂, but not with either rosiglitazone or Wy-14,643, suggesting a different signaling mechanism involved in these ligands.

“Loss-of-function” mutations in PPAR γ have recently been identified in a subset of colorectal tumors (Sarraf et al., 1999). To examine whether the endogenous PPAR γ in HT-29 cells is responsive to 15d-PGJ₂, 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 PPAR γ , treatment with 15d-PGJ₂ has no effect on PPRE reporter activity. However, when pAOXPPREluc were co-transfected with PPAR γ expression vector into HT29 cells, a significant increase in PPRE reporter activity was detected and the activity was increased further after treatment with 15d-PGJ₂ (Fig. 3D). These results suggest that endogenous PPAR γ in HT-29 cells may not be functional or is transcriptionally inactive, even though high level of its expression in these cancer cells

has been reported (Sarraf et al., 1998). These data further support our hypothesis that 15d-PGJ₂-induced KLF4 expression is likely mediated through a PPAR γ -independent pathway in HT-29 cells.

15d-PGJ₂ 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-PGJ₂-induced KLF4 expression, we examined the effect of 15d-PGJ₂ on the activity and expression ERK1/2, the downstream substrates of MEK1/2, in HT-29 cells. Cells were treated with 15d-PGJ₂ (10 μ M) 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-PGJ₂, 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-PGJ₂ 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 μ M) of 15d-PGJ₂ 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 phosphatase, is induced by many stimuli that activate ERK1/2, and subsequently inactivate ERK1/2. To confirm above

findings, the level of MKP1 expression in HT-29 cells was evaluated after treatment with 15d-PGJ₂. As illustrated in Fig. 4D, the MKP1 was rapidly induced by 15d-PGJ₂ in a time-dependent manner, supporting the involvement of ERK1/2 activation and inactivation in 15d-PGJ₂-mediated effects.

MEK/ERK, But not PI-3 Kinase Pathway is Involved in 15d-PGJ₂-mediated KLF4 mRNA Expression

To determine whether the MEK/ERK signaling pathway is involved in 15d-PGJ₂-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-PGJ₂ 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-PGJ₂-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-PGJ₂.

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-PGJ₂-treatment. As shown in Fig. 6A, Akt was activated by 15d-PGJ₂ in a time-dependent manner, with the highest level observed at 5 min. However, pretreatment of HT-29 cells with increasing concentrations of two

different PI-3 kinase inhibitors, wortmannin (10-1000 nM) or LY294002 (0.25-25 μ M), has no effect on 15d-PGJ₂-mediated KLF4 mRNA expression (Fig. 6B and 6C). These data indicate that PI-3 kinase is activated by 15d-PGJ₂ in HT-29 cells, but its activation may not involve in 15d-PGJ₂-induced KLF4 mRNA expression.

15d-PGJ₂-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-PGJ₂-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-PGJ₂ 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-PGJ₂-mediated KLF4 mRNA up-regulation. Moreover, the effect of 15d-PGJ₂ on STAT1 tyrosine phosphorylation was examined by Western blot analysis. HT-29 cells were treated with 15d-PGJ₂ (10 μ M) 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-PGJ₂ also induced tyrosine phosphorylation of STAT1 in HT-29 cells.

Down-regulation of KLF4 Expression by siRNA Reduces 15d-PGJ₂-mediated G₁ 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-PGJ₂-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-PGJ₂ induced a significant increase in cells arrested at G₁ phase and this effect was markedly attenuated by KLF4 siRNA,. The percentage of cell arrested at G₀/G₁ after 15d-PGJ₂ 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-PGJ₂, one of the well-defined cyclopentenone prostaglandins, has been recognized as a natural ligand with high affinity to PPAR γ receptor (Forman et al., 1995). PPAR γ 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 PPAR γ , 15d-PGJ₂ 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 PPAR γ can be achieved by PPAR γ -independent manner (Rossi et al., 2000; Straus et al., 2000). In this report, we show that 15d-PGJ₂ 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-PGJ₂ is not depend on other serum factors, and may not require activation of PPAR γ receptor. Our studies also demonstrate that the expression of KLF4, a transcription factor involved in growth arrest, is induced by 15d-PGJ₂ in both time-and dose-dependent manners. When endogenous KLF4 protein is reduced by siRNA transfection, the growth arrest properties of 15d-PGJ₂ was attenuated. These results suggest that the inhibitory effect of 15d-PGJ₂ on cell growth is mediated, at least in part, through up-regulation of KLF4 expression. The induction of KLF 4 mRNA expression by 15d-PGJ₂, 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-PGJ₂ induces

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-PGJ₂ is not affected by wortmannin and LY294002, suggesting that PI3-kinase pathway may not involve in up-regulation of KLF4 by 15d-PGJ₂. 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-PGJ₂ induces rapid tyrosine phosphorylation of STAT1 in HT-29 cells, and that induction of KLF4 expression by 15d-PGJ₂ 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-PGJ₂-mediated effects, although detail mechanisms remain to be addressed.

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

troglitazone but not other PPAR γ ligands induces early growth response-1 (Erg-1) gene expression independently of PPAR γ in HCT116 colon cells (Baek et al., 2003). In the present study, our data also indicate that PPAR γ may not be required in 15d-PGJ₂-induced KLF4 expression. These data include: 1) KLF4 does not contain putative PPRE sequences in its promoter region; 2) 15d-PGJ₂ 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-PGJ₂ but not rosiglitazone, another specific ligand for PPAR γ ; and 4) 15d-PGJ₂ can not stimulate PPAR-dependent promoter activity in the absence of cotransfected PPAR γ . These results are consistent with report from Takeda et al., showing that the effect of 15d-PGJ₂ on *c-fos* gene expression in vascular smooth cells is mediated through a nongenomic or a PPAR γ -independent mechanism (Takeda et al., 2001). Although the possibility of a PPAR γ -dependent induction of KLF4 by 15d-PGJ₂ in other cells can not be excluded, our current study indicate that at least in HT-29 cells, the effect of 15d-PGJ₂ on KLF4 expression does not involve its nuclear receptor PPAR γ .

The interaction between KLF4 and PPAR γ has not been explored previously. As illustrated in Fig. 2, the induction of KLF4 by 15d-PGJ₂ was peaked at 4 h, and the effect was then gradually decreased. In contrast, 15d-PGJ₂-treatment in HT-29 cells resulted in transient decreases in PPAR γ levels between 2-8 h, and these decreases in PPAR γ expression is correspondent to the increases in KLF4 concentration in these cells. These data suggest that KLF4 may exhibit an inhibitory effect on PPAR γ expression.

Interestingly, the level of PPAR δ mRNA also increased when the expression of PPAR γ was inhibited (Fig. 2A and 2B). Recently, PPAR δ has been shown to function as a potent

inhibitor of transcriptional activity of PPAR γ 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-PGJ₂ in HT-29 colon cancer cells. 15d-PGJ₂ induces the expression of KLF4 in both time- and dose-dependent manners, and through a PPAR γ -independent mechanism that requires ERK signaling, and is STAT1-dependent. Recently, several studies have reported that cyclopentenone prostaglandins, especially 15d-PGJ₂, 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-PGJ₂ required to induce growth inhibition and KLF4 expression in colon cancer cells was significantly higher than the concentration of intracellular 15d-PGJ₂ formed under physiological condition, as reported by Bell-Parikh et al (Bell-Parikh et al., 2003). However, the level of 15d-PGJ₂ 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-PGJ₂.

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FIGURE LEGENDS

Fig. 1. Effect of PPAR γ ligand 15d-PGJ₂ or rosiglitazone on the growth of HT-29

cells. A, a time course of the effect of 15d-PGJ₂ or rosiglitazone on the growth of HT-29 cells in the presence of serum. Cells were seeded at a density of 5×10^4 cell/well (12-well plates) in McCoy's medium with 10% FBS. After 24 h, the medium was replaced with fresh medium, and 15d-PGJ₂ (10 μ M) or rosiglitazone (10 μ M) 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 \pm 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-PGJ₂ or rosiglitazone on cell growth and DNA synthesis in HT-29 cells in the absence of serum. Cells were seeded at a density of 5×10^4 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-PGJ₂ (10 μ M) or rosiglitazone (10 μ M). After 24 h, cell viability and DNA synthesis were measured by MTT and [³H]thymidine incorporation assay as described under "Materials and Methods". Each value represents the mean \pm 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-PGJ₂ 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 μ M 15d-PGJ₂ (P) for indicated time (0-24 h). Total RNA was prepared, and the expression of KLF4, PPAR γ , and PPAR δ 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-PGJ₂ for 4 h. The expression of KLF4, PPAR γ , and PPAR δ 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-PGJ₂. D, HT-29 cells were incubated with either vehicle alone (-) or with 10 μM 15d-PGJ₂ (+) 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-PGJ₂ is independent of PPAR γ in HT-29 cells. A and B, induction of 15d-PGJ₂-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-PGJ₂ 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-PGJ₂ (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 ³²p-labeled KLF4 probe (B). C, 15d-PGJ₂ induced specifically expression of KLF4 mRNA in HT-29 cells. Cells were serum-starved for 18 h and treated with vehicle (Me₂SO, 0.2%), 15d-PGJ₂ (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 PPAR γ receptor does not antagonize 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-PGJ₂ for 4 h. Total RNA was isolated and levels of KLF4 RNA expression were determined by Northern blot analysis. D, cotransfection with PPAR γ expression vector is necessary to observe pAOXPPREluc activity in 15d-PGJ₂-stimulated HT-29 cells. HT-29 cells were transfected with pAOXBluc or pAOXPPREluc, or together with PPAR γ expression vector, as shown, 15d-PGJ₂ (10 μ M) 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 \pm S.E.M. and were normalized to untreated cells transfected with the respective reporter gene construct alone.

Fig. 4. 15d-PGJ₂ 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-PGJ₂ (A) or rosiglitazone (B) for different times (2.5-60 min), or with increasing concentrations of 15d-PGJ₂ (1.0-10 μ M) 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 μ M 15d-PGJ₂ 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

right, represented means \pm 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-PGJ₂-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-PGJ₂ (10 μ M) 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 μ M) 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 \pm 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-PGJ₂ but without PD98059. In C and D, *, $P < 0.05$; **, $P < 0.01$ compared to cells treated with 15d-PGJ₂ but without U0126.

Fig. 6. PI3-kinase is not responsible for 15d-PGJ₂-induced KLF mRNA expression.

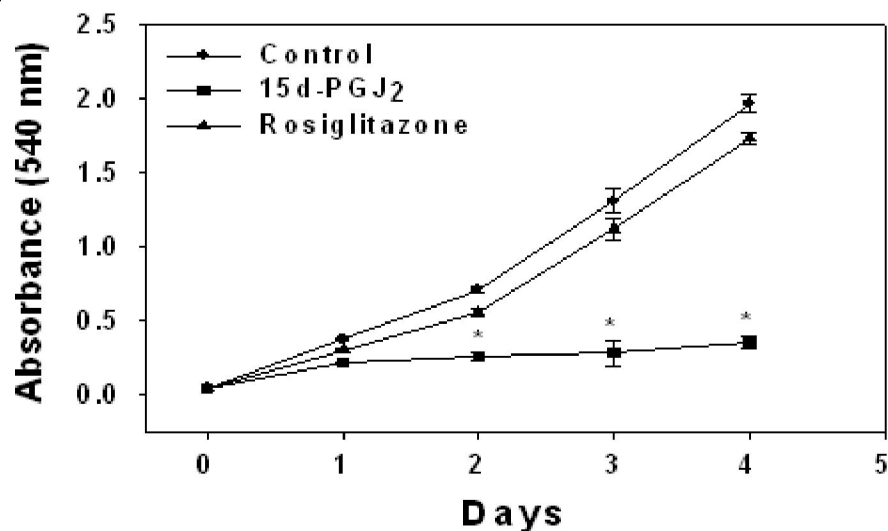
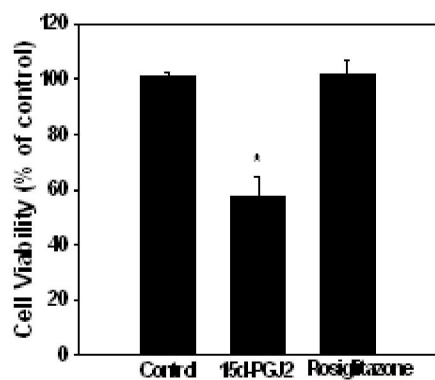
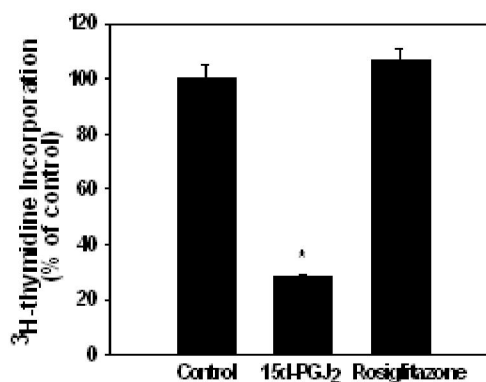
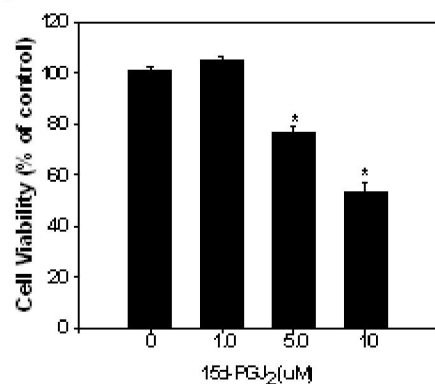
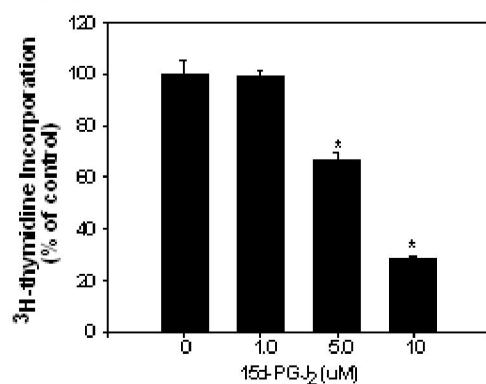
A, HT-29 cells were serum-starved for 18 h and stimulated with 15d-PGJ₂ (10 μ M) for the indicated times. Total lysates were isolated and phosphorylated Akt (*p-Akt*) and total Akt (*Akt*) were determined by Western blot analysis. B and C, the serum-starved cells were preincubated with increasing concentrations of wortmannin (10-1000 nM) or LY294002 (0.25-25 μ M) for 30 min and then treated with 15d-PGJ₂ (10 μ M) for 4 h. The

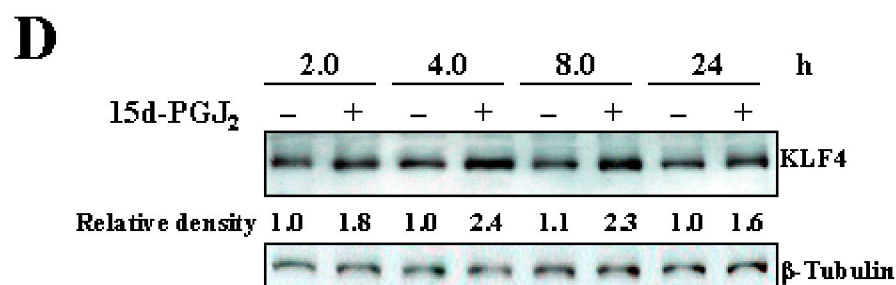
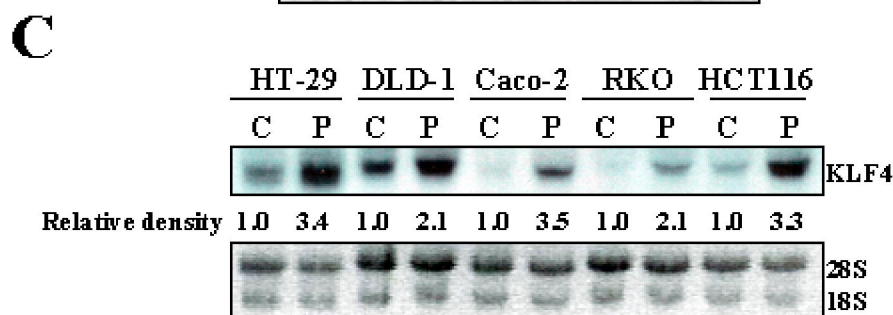
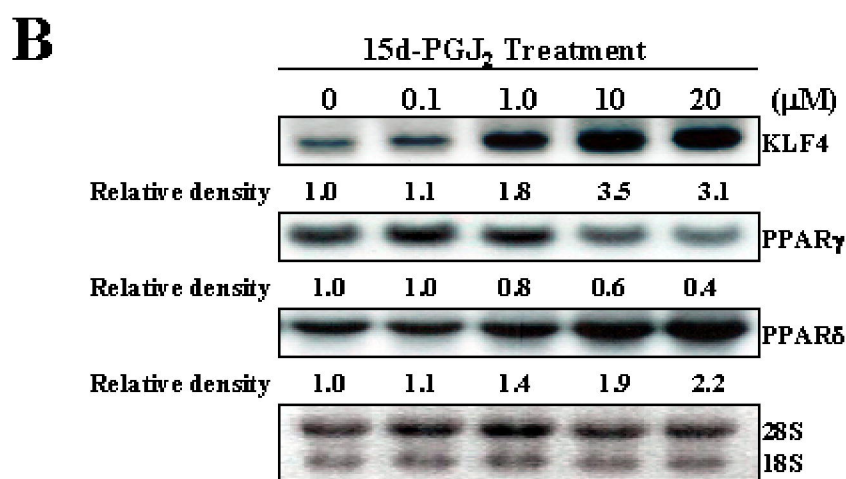
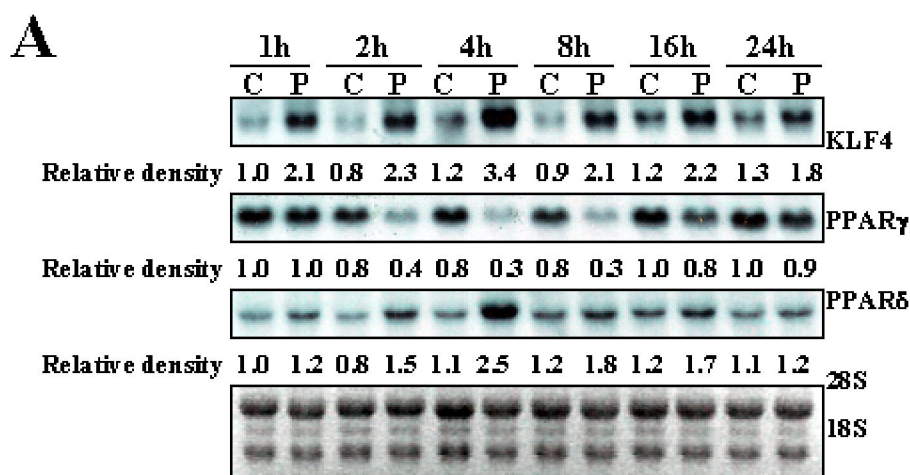
total RNA was isolated and expression of KLF4 mRNA was determined by Northern blot analysis. Quantitative data shown on the right, represented means \pm S.E.M. for five replicate determinations, and expressed as fold induction over control. *, $P < 0.05$ versus control.

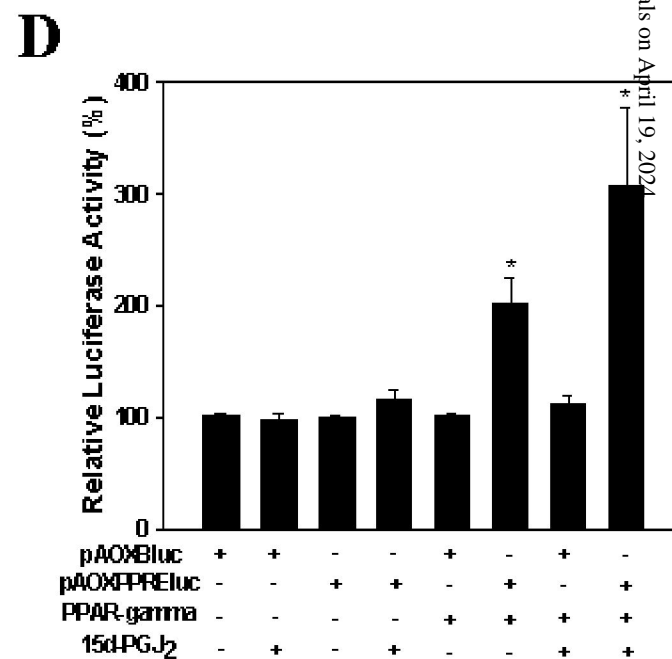
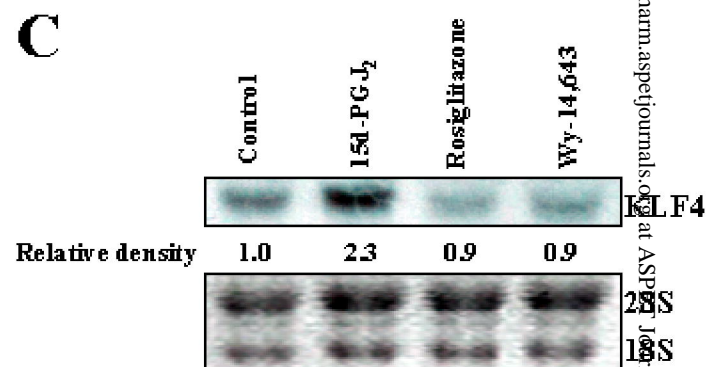
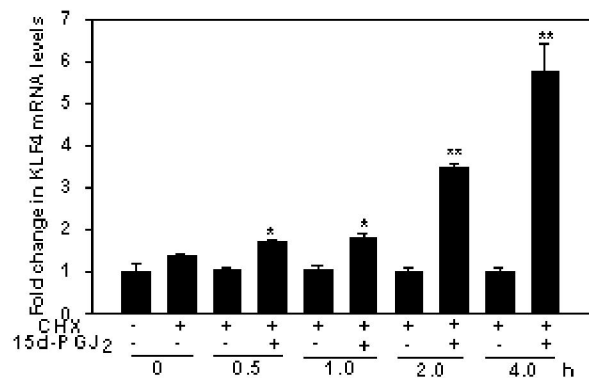
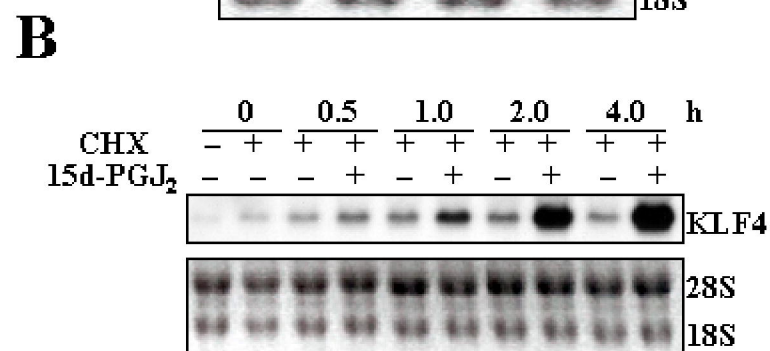
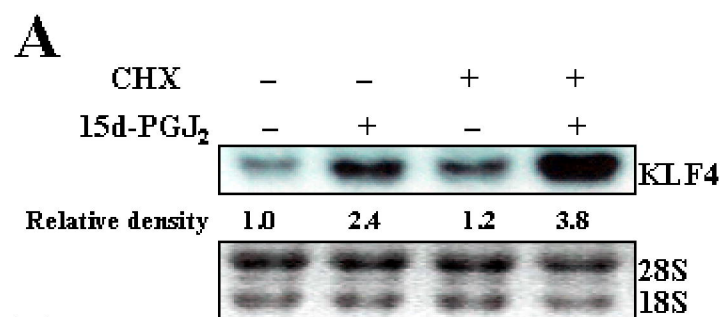
Fig. 7. 15d-PGJ₂-mediated KLF4 mRNA expression requires STAT1 and its phosphorylation. A, STAT1 is necessary for 15d-PGJ₂-mediated KLF4 mRNA up-regulation. STAT1^{+/+} and STAT1^{-/-} cells, derived from the wild-type and the STAT1 knockout mice, respectively, were incubated with 15d-PGJ₂ (10 μ M) for the indicated times and levels of KLF4 mRNA expression were determined by Northern blot analysis. B and C, 15d-PGJ₂ induces STAT1 phosphorylation on tyrosine. HT-29 cells were grown in the absence of serum for 18 h and stimulated with 15d-PGJ₂ (10 μ M) 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 \pm S.E.M. for five replicate determinations, and expressed as fold induction over control. *, $P < 0.05$ versus control.

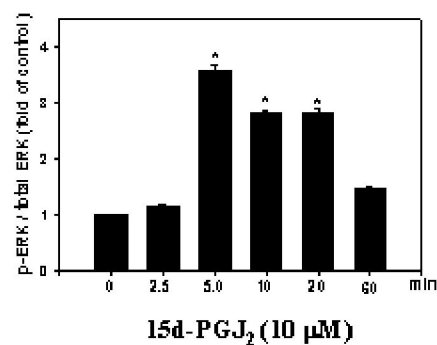
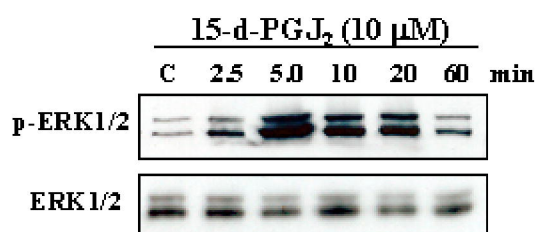
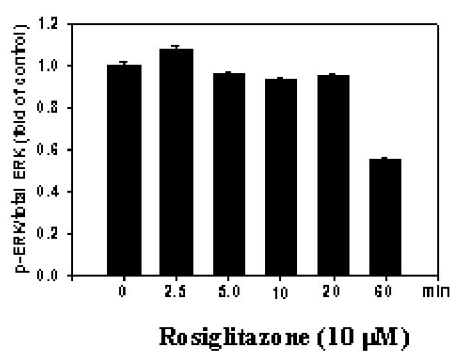
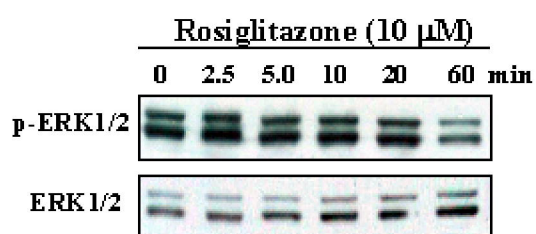
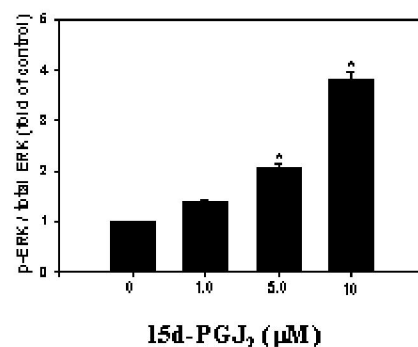
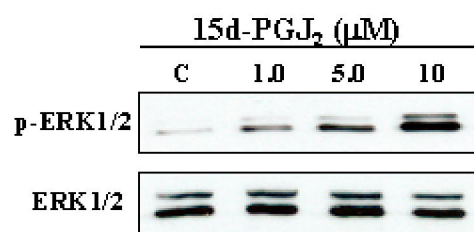
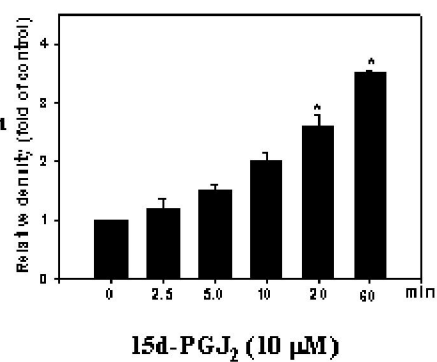
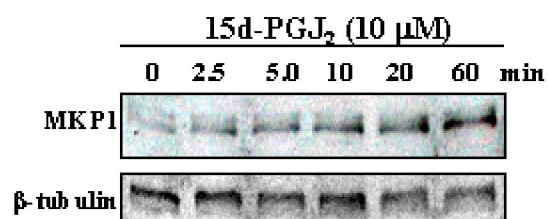
Fig. 8. Down-regulation of KLF4 expression by siRNA reduces 15d-PGJ₂-mediated G₁ phase arrest in HT-29 cells. Effect of KLF4 siRNA on the expression of KLF4 protein in HT-29 cells. Cells were transfected with KLF4 siRNA or control siRNA, and

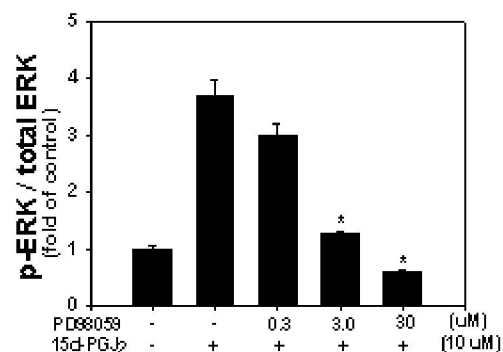
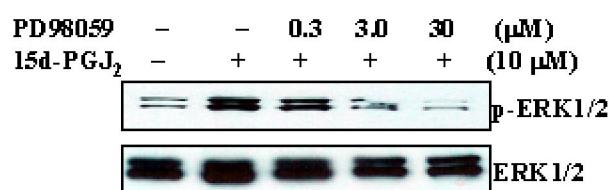
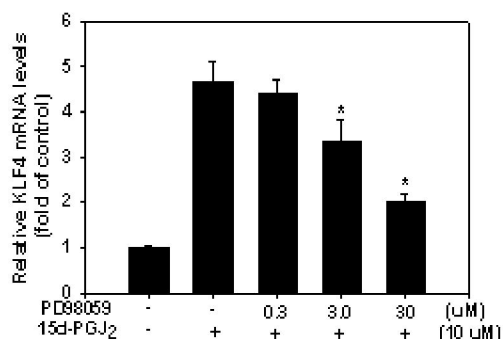
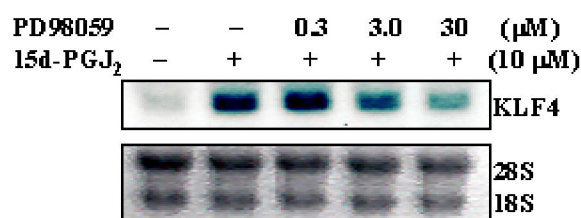
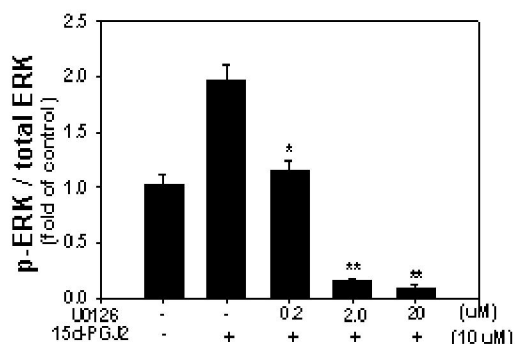
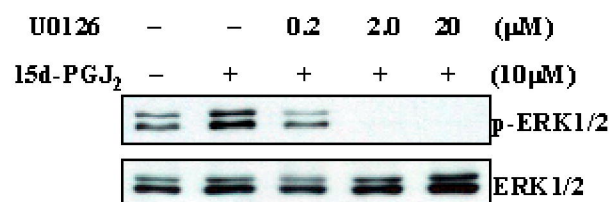
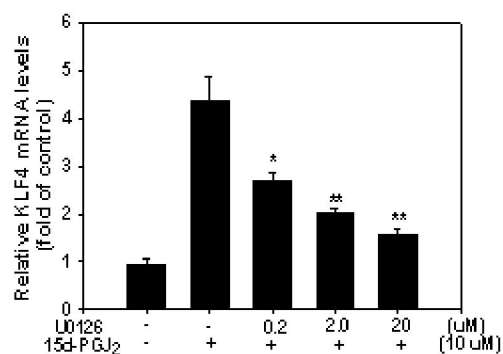
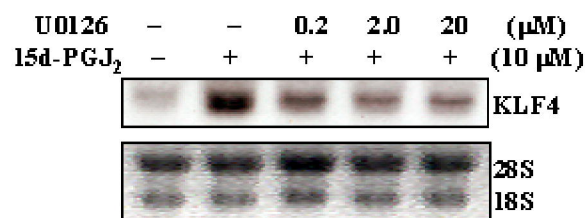
cell lysates were analyzed for the level of KLF4 protein by Western Blot analysis as described under “*Materials and Methods*”. A, a representative result from three independent experiments is shown. B, the density of each band was scanned and quantified as indicated. Results are means \pm S.E.M. for three replicate determinations, *, $P < 0.05$ compared with control. C, effect of KLF4 siRNA on 15d-PGJ₂-mediated cell cycle progression in HT-29 cells. HT-29 cells were treated with Me₂SO (control) or with 15d-PGJ₂, or transfected with KLF4 siRNA followed by treatment with 15d-PGJ₂, and the percent of distribution of cells in G₁, S, and G₂/M were determined by FACS analysis as described under “*Materials and Methods*”. Similar results were observed in a duplicate analysis.

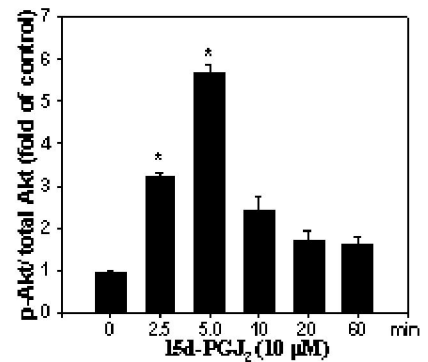
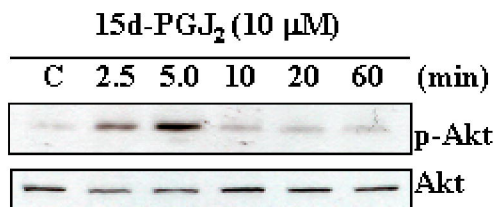
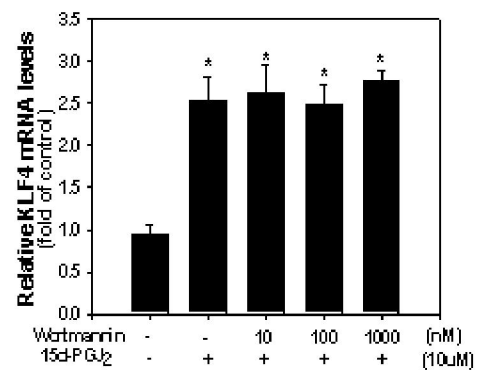
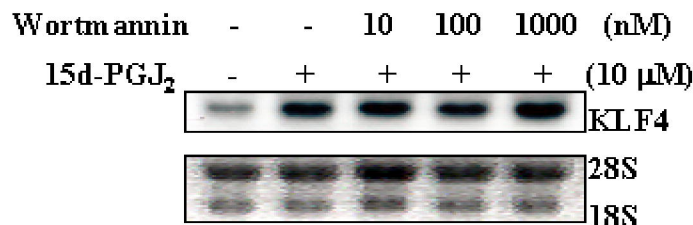
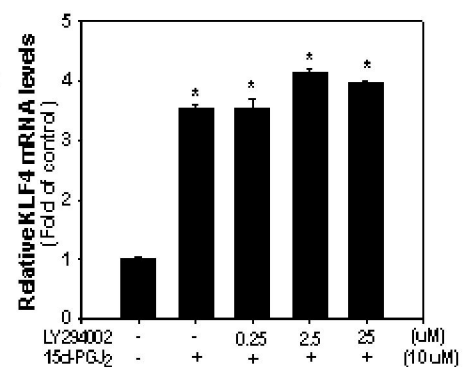
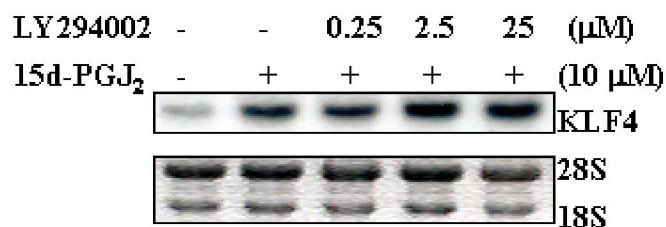
A**B****C****D****E**

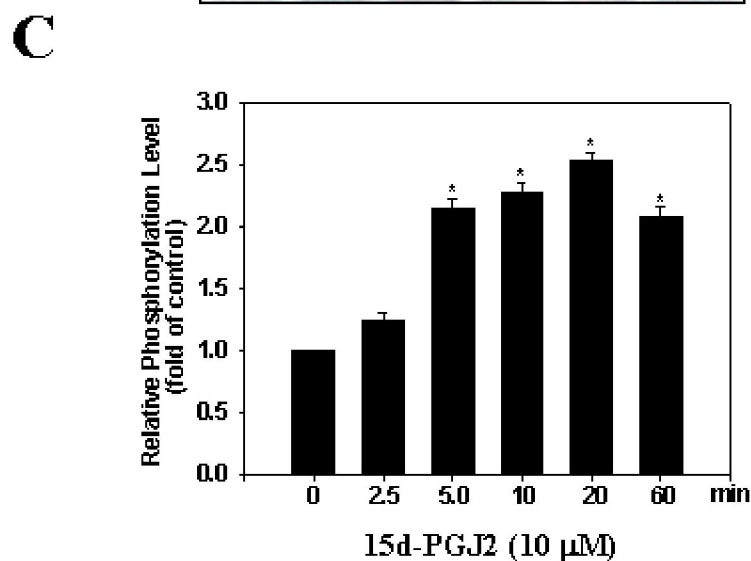
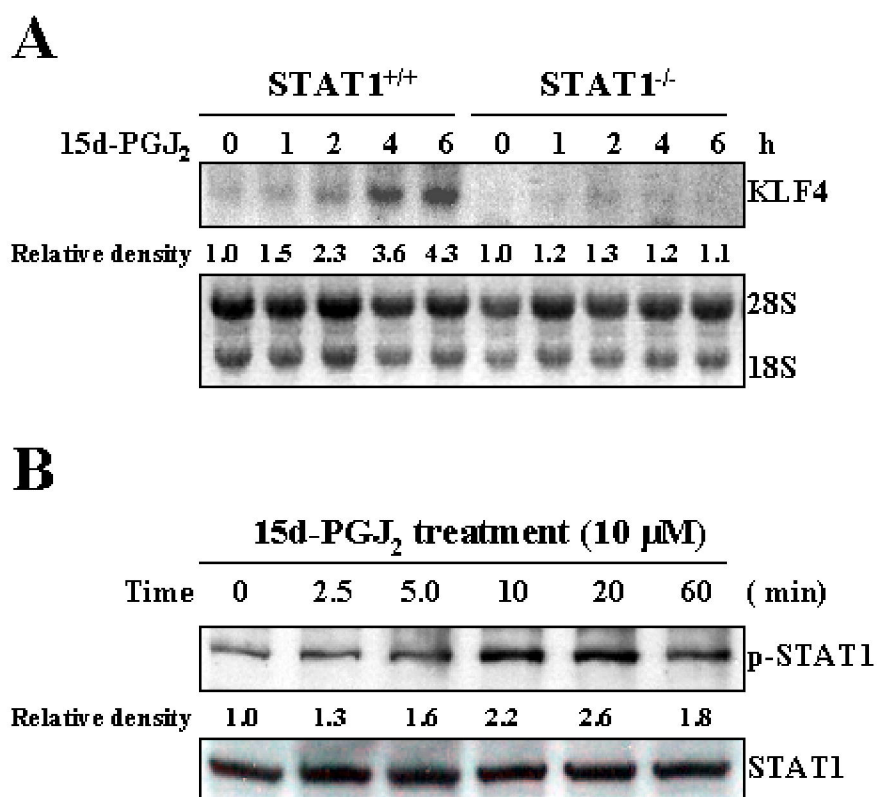




A**B****C****D**

A**B****C****D**

A**B****C**



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