

# Prostaglandin reductase-3 negatively modulates adipogenesis through regulation of PPAR $\gamma$ activity<sup>§</sup>

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**Abstract** Adipocyte differentiation is a multistep program under regulation by several factors. Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) serves as a master regulator of adipogenesis. However, the endogenous ligand for PPAR $\gamma$  remained elusive until 15-keto-PGE<sub>2</sub> was identified recently as an endogenous PPAR $\gamma$  ligand. In this study, we demonstrate that zinc-containing alcohol dehydrogenase 2 (ZADH2; here termed prostaglandin reductase-3, PTGR-3) is a new member of prostaglandin reductase family that converts 15-keto-PGE<sub>2</sub> to 13,14-dihydro-15-keto-PGE<sub>2</sub>. Adipogenesis is accelerated when endogenous PTGR-3 is silenced in 3T3-L1 preadipocytes, whereas forced expression of PTGR-3 significantly decreases adipogenesis. PTGR-3 expression decreased during adipocyte differentiation, accompanied by an increased level of 15-keto-PGE<sub>2</sub>. 15-keto-PGE<sub>2</sub> exerts a potent proadipogenic effect by enhancing PPAR $\gamma$  activity, whereas overexpression of PTGR-3 in 3T3-L1 preadipocytes markedly suppressed the proadipogenic effect of 15-keto-PGE<sub>2</sub> by repressing PPAR $\gamma$  activity. Taken together, these findings demonstrate for the first time that PTGR-3 is a novel 15-oxoprostaglandin- $\Delta^{13}$ -reductase and plays a critical role in modulation of normal adipocyte differentiation via regulation of PPAR $\gamma$  activity. ■ Thus, modulation of PTGR-3 might provide a novel avenue for treating obesity and related metabolic disorders.—Yu, Y.-H., Y.-C. Chang, T.-H. Su, J.-Y. Nong, C.-C. Li, and L.-M. Chuang. Prostaglandin reductase-3 negatively modulates adipogenesis through regulation of PPAR $\gamma$  activity. *J. Lipid Res.* 2013. 54: 2391–2399.

**Supplementary key words** adipocyte differentiation • nuclear receptor • ligand • eicosanoid

The conversion of preadipocytes to adipocytes is regulated by several transcription factors. These factors promote cell morphologic conversion, lipogenic gene expression, and triacylglycerol accumulation. Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) has been clearly demon-

strated to be a master regulator in adipogenesis. To activate PPAR $\gamma$ , a ligand is needed for binding its ligand-binding domain. Thiazolidinedione, an antidiabetic drug, has been known as a potent ligand for PPAR $\gamma$ . However, the natural ligands for PPAR $\gamma$  need to be elucidated.

Prostaglandins are eicosanoid lipid mediators derived from arachidonic acid that are involved in a variety of physiological functions. It has been demonstrated that prostaglandins are potential endogenous ligands for PPAR $\gamma$  that modulate adipocyte differentiation. Several candidates have been identified as potent endogenous ligands for PPAR $\gamma$ , including 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>) (1) and 15-keto-prostaglandin E<sub>2</sub> (15-keto-PGE<sub>2</sub>) (2). We previously reported that the prostaglandin reductase-2 (PTGR-2; previously known as zinc-binding alcohol dehydrogenase domain containing 1, ZADH1) is a 15-oxoprostaglandin- $\Delta^{13}$ -reductase that converts 15-keto-PGE<sub>2</sub> to inactive 13,14-dihydro-15-keto-PGE<sub>2</sub>, thereby suppressing PPAR $\gamma$  transcriptional activity and inhibiting adipogenesis (2). PTGR-2 belongs to the zinc-binding alcohol dehydrogenase (ZADH) gene family. In mammal, the ZADH family consists of three members: PTGR-1 (ZADH3), PTGR-2, and PTGR-3 (ZADH2). PTGR-2 is expressed at high levels in white adipose tissue with known 15-oxoprostaglandin- $\Delta^{13}$ -reductase activity (2). PTGR-1 is expressed at high levels in kidney and plays a role in preventing cancer growth in vitro and in vivo (3, 4). It has been demonstrated that PTGR-1 is a bifunctional enzyme capable of utilizing leukotriene B<sub>4</sub> and 15-keto-prostaglandins as substrates (5).

Abbreviations: aP2, adipocyte fatty acid-binding protein; COX, cyclooxygenase; DLK1, delta-like homologue 1; ERK, extracellular signal-regulated kinase; HFD, high-fat diet; LC, liquid chromatography; LTQ, linear trap quadrupole; MEK, mitogen-activated protein kinase; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; PPARE, PPAR response element; PREF1, preadipocyte factor 1; PTGR-3, prostaglandin reductase-3; SVF, stromal vesicular fraction; UHPLC, ultra high pressure liquid chromatography; UPLC, ultra performance liquid chromatography.

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The protein structure of PTGR-3 is computationally predicted to contain an oxidoreductase catalytic site located between amino acids 184 and 320 based on sequence homology and shares 23% amino acid homology to PTGR-2. However, the function of *PTGR-3* has not yet been investigated.

In this study, we demonstrate for first time that PTGR-3 is a 15-oxoprostaglandin- $\Delta^{13}$ -reductase that converts 15-keto-PGE<sub>2</sub> to inactive 13,14-dihydro-15-keto-PGE<sub>2</sub>, thereby decreasing adipogenesis through regulation of PPAR $\gamma$  transcriptional activity. PTGR-3 was highly expressed in 3T3-L1 preadipocytes, but it decreased during differentiation into adipocytes. Furthermore, PTGR-3 expression was reduced in white adipose tissue of murine models of obesity. These data indicate PTGR-3 is a critical modulator of adiposity.

## MATERIALS AND METHODS

### Cell culture

All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. The 3T3-L1 preadipocytes were cultured in Dulbecco modified Eagle's medium (DMEM) with 10% calf serum (CS) at 37°C in an atmosphere of 5% CO<sub>2</sub>. To induce adipocyte differentiation, confluent cells were cultured in induction medium (DMEM containing 10% FBS, 1  $\mu$ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 1  $\mu$ g/ml insulin). After 2 days, the cells were maintained in DMEM containing 10% FBS and 1  $\mu$ g/ml insulin. Two days later, cells were cultured in DMEM containing 10% FBS for 8 days, with a medium change every 2 day. After 8 days of culturing, cells on the plates were stained with Oil Red O to measure the degree of adipocyte differentiation. Total protein lysate was extracted to determine the protein levels of adipogenic genes whose expression increases during adipocyte differentiation: adipocyte fatty acid-binding protein (aP2) and adiponectin.

### Plasmids

Expression vector for GAL4-DBD fusion of PPAR $\gamma$ -LBD (CMX-GAL4-PPAR $\gamma$ ), CMX-GAL4, and UAS<sub>C</sub>×4-TK-LUC reporter plasmids were generous gifts from R. M. Evans (Howard Hughes Medical Institute, the Salk Institute for Biological Studies, La Jolla, CA). The Aox-TK-luciferase reporter plasmid, which contains three PPRE sites (PPRE-LUC), and the TK-LUC reporter plasmid were kindly provided by C. K. Glass (University of California San Diego, La Jolla, CA). Mouse PTGR-3 cDNA (Flag-PTGR-3) expression vector and control empty vector were purchased from OriGene (Rockville, MD).

### Lentiviral infection

Small hairpin RNA (shRNA) plasmids for the mouse PTGR-3 gene and the control vector (shLuc) were obtained from the National RNAi Core Facility of Academia Sinica in Taiwan. For PTGR-3 knockdown, confluent 3T3-L1 preadipocytes were infected with lentivirus carrying control shRNA and shRNA targeting PTGR-3, respectively. After lentiviral infection, cells were selected by antibiotic selection using puromycin for 2 days. Two days later, cells were isolated to culture cells for further propagation. For the PTGR-3 overexpression experiment, confluent 3T3-L1 preadipocytes were infected with lentivirus carrying empty vector (pLVX-IRES-neo, Clontech, Mountain View, CA) and PTGR-3, respectively. After lentiviral infection, cells were maintained in selective medium containing the G418 antibiotics. Cell lines stably expressing empty vector or PTGR-3 were established by

antibiotic selection. The selection was continued for at least 1 month. Individual colonies were isolated to culture cells for further propagation. Detailed protocols for lentivirus production and infection of cells were performed following the procedures of the National RNAi Core Facility of Academia Sinica in Taiwan.

### In vitro enzymatic reaction

All reagents were purchased from Sigma-Aldrich (St. Louis, MO). Colorimetric method was used to determine PTGR-3 enzyme activity as described previously (2). Briefly, PTGR-3 recombinant protein (OriGene, Rockville, MD) was incubated with 0.5 mM NADPH and 0.6 mM prostaglandins (13,14-dihydro-15-keto-PGE<sub>2</sub>, 15-keto-PGE<sub>2</sub>, 15-keto-PGF<sub>2 $\alpha$</sub> , 15-keto-PGF<sub>1 $\alpha$</sub> , or 15-keto-PGE<sub>1</sub>) in 0.1 M Tris-HCl (pH 7.4) at 37°C for 30 min. After enzymatic reaction, color reagent (790  $\mu$ M indonitrotetrazolium chloride, 60  $\mu$ M phenazene methosulfate, and 1% Tween 20) was added and incubated at 37°C in the dark for 10 min. To stop the reaction, phthalate buffer (pH 3.0) was added. PTGR-3 activity was determined by measuring the absorbance of formazans, an indicator of remaining NADPH, at 490 nm with a spectrophotometer. One unit of the enzyme was defined as the amount of enzyme catalyzing the production of 1  $\mu$ mol NADP<sup>+</sup>/min. The apparent  $K_m$  and  $V_{max}$  values were determined using the Michaelis-Menten equation and calculated by nonlinear regression.

### LC-MS/MS analysis of prostaglandins

Details for the prostaglandin extraction and derivation were described by Chou et al. (2). Deuterated 13,14-dihydro-15-keto-PGE<sub>2</sub> standard (Cayman Chemical, Ann Arbor, MI) was added to homogenized samples as an internal control. Chromatographic analyses were performed using Accela UHPLC (Thermo Scientific, Hemel Hempstead, UK) and Acquity UPLC systems (Waters, Hertsfordshire, UK). The samples were separated on a C18 reversed-phase LC column (Phenomenex Luna, 150 mm × 2 mm × 3  $\mu$ m) using a linear mobile phase gradient (A) water and (B) 5% methanol/95% acetonitrile. Mass spectrometry analyses were performed on LTQ Velos (Thermo Scientific) linear ion trap (LIT)-orbitrap and QTRAP 4000 (AB Sciex, Concord, ON, Canada) quadrupole-linear ion trap (QqLIT) mass spectrometers.

### Transient transfection and reporter assay

For 3T3-L1 preadipocyte transfection, confluent cells were cotransfected with the PPRE×3-TK-LUC plasmid and pHRG-TK plasmid (an internal control) by lipofection (Lipofectamine 2000, Invitrogen, Carlsbad, CA). 3T3-L1 preadipocytes, which were transfected with the TK-LUC reporter plasmid (without the PPRE×3 promoter), were the control group. One day after transfection, media were changed to adipogenic medium without or with chemicals (1  $\mu$ M troglitazone, 10  $\mu$ M 15-keto-PGE<sub>2</sub>, 10  $\mu$ M 13,14-dihydro-15-keto-PGE<sub>2</sub>, and 1  $\mu$ M GW9662). Twenty-four hours after treatment, cells were harvested and luciferase was assayed (Dual-Glo luciferase assay system; Promega, Madison, WI). For 293T cell transfection, confluent cells were incubated with plasmids (GAL4-PPAR $\gamma$ , UAS<sub>C</sub>×4-TK-LUC, and Flag-PTGR-3) and lipofectamine. After 6 h of transient transfection, media were changed to growth media without or with chemicals (1  $\mu$ M troglitazone, 10  $\mu$ M 15-keto-PGE<sub>2</sub>, 10  $\mu$ M 13,14-dihydro-15-keto-PGE<sub>2</sub>, and 1  $\mu$ M GW9662). Twenty-four hours after treatment, cells were harvested for determination of luciferase activity.

### Western blot

Total protein from tissue or cells was extracted by radioimmunoprecipitation assay buffer with protease inhibitors. The sample was centrifuged at 12,000 rpm for 10 min, and the supernatant

was subjected to Western blot. For Western blot, 20  $\mu$ g of protein lysate was separated by SDS-PAGE and then transblotted onto a polyvinylidene fluoride membrane (Perkin Elmer, Norwalk, CT). The PTGR-3 and PTGR-2 primary antibodies were purchased from Abcam (Cambridge, MA). Adiponectin antibody, aP2 antibody, and PPAR $\gamma$  were purchased from Cell Signaling (Boston, MA).  $\alpha$ -Tubulin and GAPDH antibodies (Cell Signaling, Boston, MA) were used for the loading control in the lysates of total protein. The secondary antibody coupled to horseradish peroxidase was used in the chemiluminescence procedure (Immobilon Western; Millipore, Billerica, MA). The Western blotting procedure was performed according to the manufacturer's instruction.

### Approvals for usage of animals

All animal experiments were performed in accordance with protocols approved by the National Taiwan University Institutional Animal Care and Use Committee (IACUC Approval No. 20100329).

### Statistical analysis

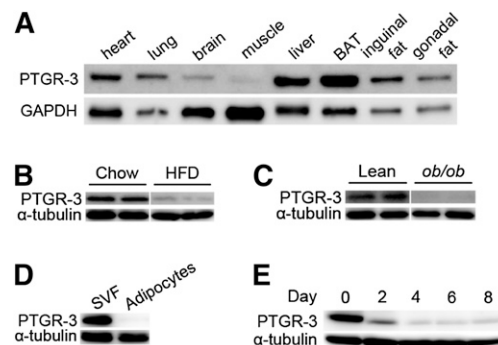
Results are expressed as means  $\pm$  SE. Comparison between two groups were performed using unpaired *t*-test.  $P < 0.05$  was considered statistically significant. A two-way ANOVA (ANOVA) procedure was used to determine the main effects of PPAR ligands, PTGR-3, and their interaction on intracellular triacylglycerol content and luciferase activity. Multiple comparisons between groups were performed using post-hoc Tukey test (SAS Institute Inc., Cary, NC). A significant difference indicated  $P \leq 0.05$ .

## RESULTS

### PTGR-3 is a negative regulator of adipocyte differentiation

To explore the physiological function of PTGR-3, we first assayed the tissue expression pattern of PTGR-3. PTGR-3 protein and mRNA are ubiquitously expressed in several tissues, including heart, brown adipose tissue, and white adipose tissue (Fig. 1A and supplementary Fig. I-A). We also examined whether PTGR-3 levels were altered in murine models of obesity. We found that PTGR-3 levels were decreased in white adipose tissue of high-fat-diet (HFD) fed mice (Fig. 1B) and *ob/ob* mice (Fig. 1C and supplementary Fig. I-B). Furthermore, PTGR-3 is specifically expressed in stromal vascular fractions (SVF) rather than mature adipocytes (Fig. 1D), and PTGR-3 levels remarkably decreased during induced adipogenesis of 3T3-L1 cells (Fig. 1E). These results suggest that PTGR-3 may be involved in adipocyte differentiation.

To investigate whether PTGR-3 affects adipocyte differentiation, 3T3-L1 preadipocytes were infected with lentivirus carrying PTGR-3 shRNA and were then induced for adipocyte differentiation. shRNAs targeting PTGR-3 efficiently attenuated PTGR-3 protein levels but not PTGR-2 levels (Fig. 2A). After 8 days of adipogenic induction, adipocyte differentiation was accelerated in PTGR-3-knockdown cells compared with shRNA control cells (shLuc) (Fig. 2B), accompanied by increased intracellular triglyceride levels (Fig. 2C). Consistently, adiponectin and aP2, two markers for adipocyte differentiation, were increased in PTGR-3-knockdown cells compared with shRNA control cells after hormonal induction (Fig. 2D). Quantitative

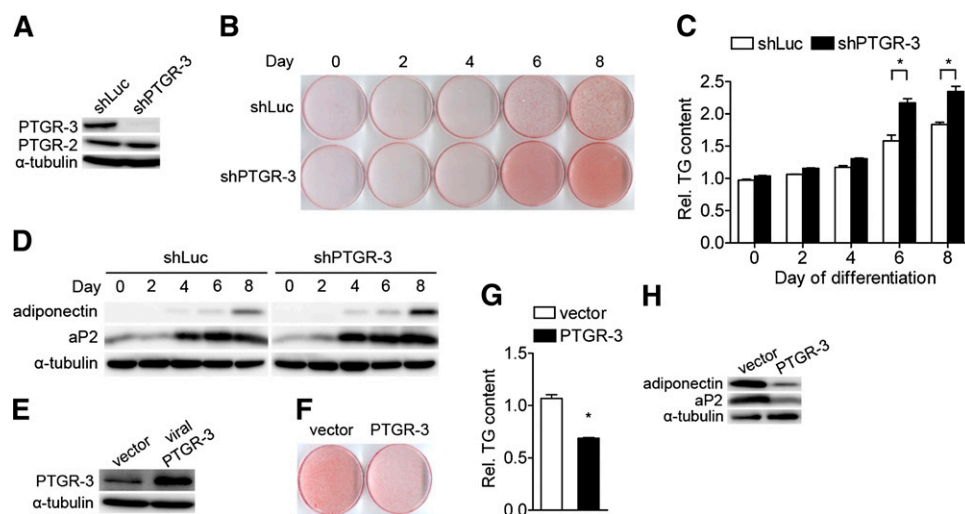


**Fig. 1.** PTGR-3 expression pattern in adipose tissue and during adipocyte differentiation. (A) Tissue distribution of PTGR-3 protein from 12-week-old C57BL/6 mice. (B) Expression of PTGR-3 protein from white adipose tissue of 12-week-old chow/HFD-fed mice. (C) Expression of PTGR-3 protein from white adipose tissue of 12-week-old lean and *ob/ob* mice. (D) Expression of PTGR-3 protein from mouse SVF and adipocytes. (E) Expression of PTGR-3 protein during induced 3T3-L1 adipocyte differentiation.

PCR with reverse transcription showed increased expression of PPAR $\gamma$ -targeted genes, including *Lpl* and *Cd36*, in PTGR-3-knockdown 3T3-L1 cells after induction (supplementary Fig. II-A). By contrast, adipocyte differentiation was remarkably decreased when we ectopically expressed PTGR-3 protein in 3T3-L1 preadipocytes (Fig. 2E, F), which was accompanied by a decrease in intracellular triglyceride content (Fig. 2G) and in expression of adipocyte differentiation markers (Fig. 2H and supplementary Fig. II-B). Taken together, these results demonstrate PTGR-3 negatively regulates adipocyte differentiation.

### PTGR-3 is a 15-oxoprostaglandin- $\Delta^{13}$ -reductase

It has been demonstrated that 15-keto-PGE<sub>2</sub> can be metabolized to 13,14-dihydro-15-keto-PGE<sub>2</sub> by PTGR-2 (2). Therefore, we asked whether PTGR-3 is also an enzyme that catalyzes the same reaction. We first tested various concentrations of PTGR-3 recombinant protein on 15-keto-PGE<sub>2</sub> catabolism in vitro. The results showed that PTGR-3 metabolizes 15-keto-PGE<sub>2</sub> in a NADPH-dependent reaction and that this reaction was linearly accelerated with increased concentration of PTGR-3 recombinant protein (Fig. 3A), prolonged reaction time, and increased 15-keto-PGE<sub>2</sub> concentration (Fig. 3B, C). In addition, we found that PTGR-3 is able to catalyze the metabolism of other prostaglandins, including 15-keto-PGF<sub>2 $\alpha$</sub> , 15-keto-PGF<sub>1 $\alpha$</sub> , and 15-keto-PGE<sub>1</sub> (Table 1). Enzyme kinetic studies revealed that PTGR-3 had the highest efficiency for 15-keto-PGF<sub>2 $\alpha$</sub>  catabolic reaction ( $K_{cat}/K_m = 271.76 \pm 8.82 \text{ mM}^{-1} \text{ min}^{-1}$  compared with 15-keto-PGF<sub>1 $\alpha$</sub>  ( $214.41 \pm 12.92 \text{ mM}^{-1} \text{ min}^{-1}$ ), 15-keto-PGE<sub>2</sub> ( $157.24 \pm 14.00 \text{ mM}^{-1} \text{ min}^{-1}$ ) and 15-keto-PGE<sub>1</sub> ( $58.45 \pm 4.59 \text{ mM}^{-1} \text{ min}^{-1}$ ) (Table 2). We next asked whether PTGR-3 is a 15-oxoprostaglandin- $\Delta^{13}$ -reductase converting 15-keto-PGE<sub>2</sub> to 13,14-dihydro-15-keto-PGE<sub>2</sub>. We analyzed the mass spectrum of reaction product obtained from the incubation of 15-keto-PGE<sub>2</sub> with PTGR-3 recombinant protein by LC-MS/MS. A specific peak (Fig. 3D) was detected with mass spectrum identical to standard 13,14-dihydro-15-keto-PGE<sub>2</sub> (supplementary Fig. III-A) and



**Fig. 2.** PTGR-3 is a negative regulator of adipocyte differentiation. (A) Efficiency of shRNA control (shLuc) and PTGR-3 shRNA targeted on mouse PTGR-3 protein levels of 3T3-L1 cells. (B) Oil Red O staining of shRNA control and PTGR-3-knockdown cells during adipocyte differentiation. (C) Quantification of Oil Red O in shRNA control and PTGR-3-knockdown cells during adipocyte differentiation. \* $P < 0.05$  versus shLuc. (D) Expression of adipogenic genes (adiponectin and aP2) in shRNA control and PTGR-3-knockdown cells during adipocyte differentiation. (E) Efficiency of ectopic expression of mouse PTGR-3 protein in 3T3-L1 cells. (F) Oil Red O staining of vector only and PTGR-3-overexpressing cells after 8-day hormonal induction. (G) Quantification of Oil Red O in vector only and PTGR-3-overexpressing cells after 8-day hormonal induction. \* $P < 0.05$  versus vector. (H) Protein level of adipogenic marker genes (adiponectin and aP2) in vector only and PTGR-3-overexpressing cells after 8-day hormonal induction. The bars indicate the means  $\pm$  SE for cells from three independent replicates ( $n = 3$ ).

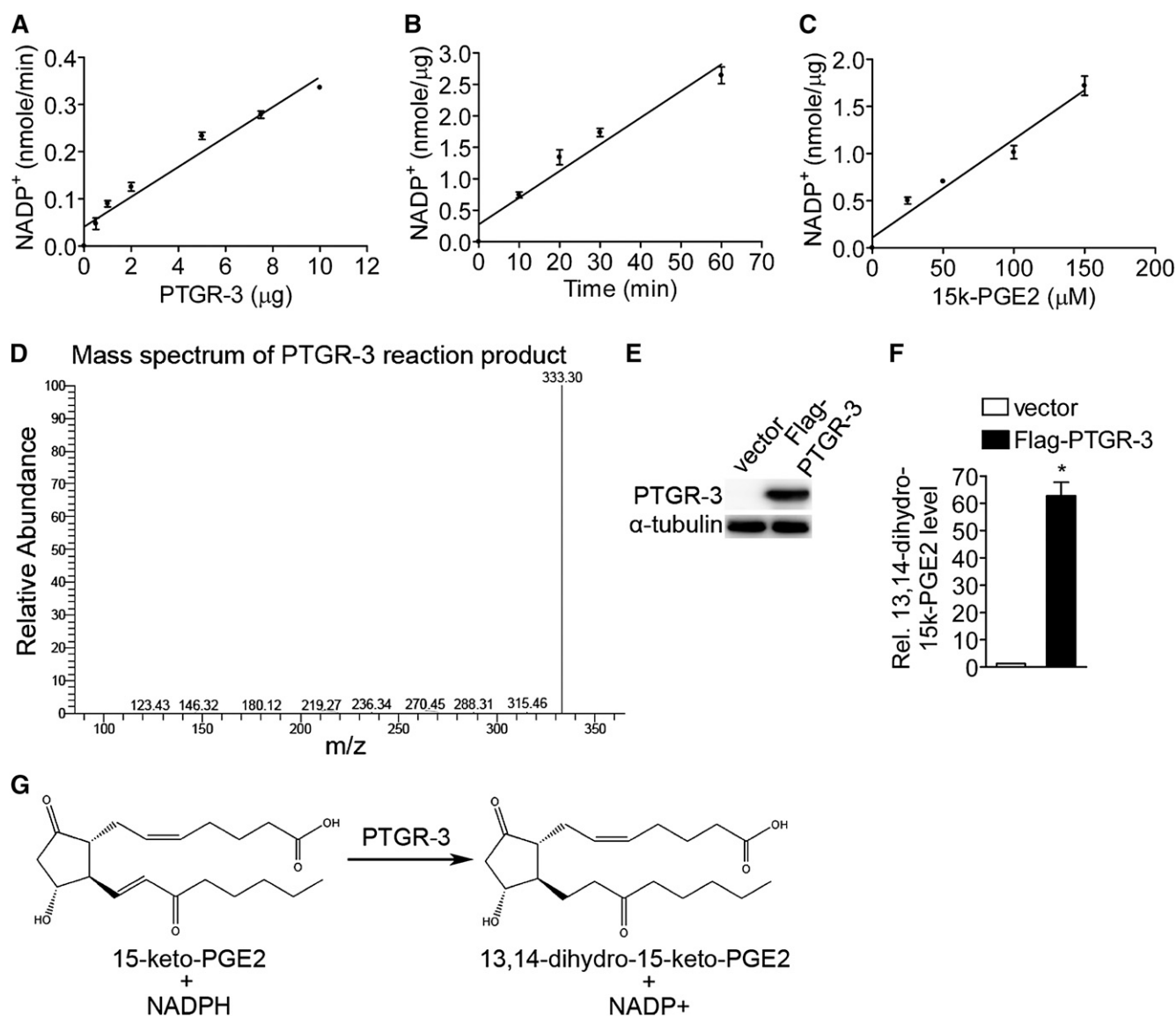
product obtained by reaction of 15-keto-PGE<sub>2</sub> and PTGR-2 recombinant protein (supplementary Fig. III-B), demonstrating 13,14-dihydro-15-keto-PGE<sub>2</sub> is a 15-keto-PGE<sub>2</sub> metabolite in PTGR-3-mediated reaction. To examine whether PTGR-3 is involved in conversion of 15-keto-PGE<sub>2</sub> to 13,14-dihydro-15-keto-PGE<sub>2</sub> in a cell-based reaction, we ectopically expressed Flag-PTGR-3 protein in 293 cells (Fig. 3E) and treated these cells with 15-keto-PGE<sub>2</sub>. Intracellular prostaglandins were extracted and analyzed by LC-MS/MS. After 15-keto-PGE<sub>2</sub> treatment, a mass spectrum of 13,14-dihydro-15-keto-PGE<sub>2</sub> was clearly detected in PTGR-3-overexpressing cells compared with vector-only cells (Fig. 3F, G). Taken together, these results demonstrate that PTGR-3 is a 15-oxoprostaglandin- $\Delta^13$ -reductase converting 15-keto-PGE<sub>2</sub> to 13,14-dihydro-15-keto-PGE<sub>2</sub> in vitro and in cell-based reactions.

#### 15-keto-PGE<sub>2</sub>, a PTGR-3 substrate, promotes adipogenesis through activation of PPAR $\gamma$

To determine whether 15-keto-PGE<sub>2</sub>, a substrate of PTGR-3, has a significant effect on adipogenesis, we measured the intracellular 15-keto-PGE<sub>2</sub> levels during adipocyte differentiation. Consistent with the reduced expression of PTGR-3 expression in initiation of differentiation (Fig. 1E and Fig. 4A), 15-keto-PGE<sub>2</sub> level was remarkably increased to a peak at 48 h after hormonal induction and then declined (Fig. 4B). To examine whether 15-keto-PGE<sub>2</sub> has a direct effect on adipocyte differentiation, we treated 3T3-L1 preadipocytes with 15-keto-PGE<sub>2</sub> during adipocyte differentiation. After induction of adipocyte differentiation, a remarkable increase in the lipid droplet deposition (Fig. 4C), intracellular triglyceride content (Fig. 4D), and

adipogenic marker genes expression (Fig. 4E) was observed in 3T3-L1 preadipocytes treated with 15-keto-PGE<sub>2</sub>. However, the product of PTGR-3, 13,14-dihydro-15-keto-PGE<sub>2</sub>, did not cause a significant effect on adipocyte differentiation. The effect of 15-keto-PGE<sub>2</sub> on adipocyte differentiation was similar to PPAR $\gamma$  agonist troglitazone. Furthermore, adipocyte differentiation was not further accelerated when 3T3-L1 preadipocytes were simultaneously treated with 15-keto-PGE<sub>2</sub> and troglitazone, suggesting that 15-keto-PGE<sub>2</sub> and troglitazone positively regulated adipogenesis through similar mechanism.

It has been demonstrated that troglitazone enhances adipogenesis by direct activation of PPAR $\gamma$  via binding to the ligand-binding domain. We tested whether 15-keto-PGE<sub>2</sub> promotes adipocyte differentiation by enhancing PPAR $\gamma$  transcriptional activity. We transfected a PPRE-driven luciferase plasmid into 3T3-L1 preadipocytes and then treated these cells with 15-keto-PGE<sub>2</sub>. The results showed that 15-keto-PGE<sub>2</sub> significantly activated the PPRE-driven luciferase activity (Fig. 4F). However, PGE<sub>2</sub>, the precursor of 15-keto-PGE<sub>2</sub>, and 13,14-dihydro-15-keto-PGE<sub>2</sub>, the metabolite of 15-keto-PGE<sub>2</sub>, do not have any effect on PPRE-driven luciferase activity (supplementary Fig. IV). 15-keto-PGE<sub>2</sub> also does not affect the activity of PPAR $\gamma$ 's obligate heterodimer partner, retinoid X receptor (RXR) (supplementary Fig. V). We further asked whether 15-keto-PGE<sub>2</sub> directly activates PPAR $\gamma$  via its ligand-binding domain. As expected, 15-keto-PGE<sub>2</sub> increased PPAR $\gamma$  activity by direct interaction with activation of PPAR $\gamma$  ligand-binding domain (Fig. 4G). Taken together, these results demonstrate that 15-keto-PGE<sub>2</sub>, a PTGR-3 substrate, enhances adipogenesis through a PPAR $\gamma$ -dependent pathway.



**Fig. 3.** PTGR-3 is a 15-oxoprostaglandin- $\Delta^{13}$ -reductase. (A) Effect of various dose of PTGR-3 recombinant protein on the PTGR-3 activity in the enzymatic reaction. Each data point represents means  $\pm$  SE for three independent replicates ( $n = 3$ ). (B) Time-dependent formation of NADP<sup>+</sup> in the PTGR-3 enzymatic reaction. Each data point represents means  $\pm$  SE for three independent replicates ( $n = 3$ ). (C) Effect of various dose of 15-keto-PGE<sub>2</sub> on PTGR-3 activity in the enzymatic reaction. Each data point represents means  $\pm$  SE for three independent replicates ( $n = 3$ ). (D) LC-MS/MS spectrum of PTGR-3 enzymatic reaction product. Mass spectrum of 13,14-dihydro-15-keto-PGE<sub>2</sub> from enzymatic reaction product was identified by LC-MS/MS. (E) Efficiency of ectopic expression of Flag-PTGR-3 protein in 293 cells. (F) Verification of intracellular 13,14-dihydro-15-keto-PGE<sub>2</sub> level in 15-keto-PGE<sub>2</sub>-treated Flag-PTGR-3-overexpressing 293 cells. Intracellular 13,14-dihydro-15-keto-PGE<sub>2</sub> from 15-keto-PGE<sub>2</sub>-treated vector only and Flag-PTGR-3-overexpressing 293 cells were extracted and analyzed by LC-MS/MS ( $n = 3$ ). The bars indicate the means  $\pm$  SE for three independent replicates. \* $P < 0.05$  versus vector. (G) The scheme of PTGR-3 enzyme reaction mechanism.

### PTGR-3 decreases proadipogenic effect of 15-keto-PGE<sub>2</sub> through regulation of PPAR $\gamma$ activity

To assess whether PTGR-3 has a direct effect on 15-keto-PGE<sub>2</sub>-mediated adipogenesis, we treated vector-only and PTGR-3-overexpressing 3T3-L1 cells with or without 15-keto-PGE<sub>2</sub>. Similar to previous results, 15-keto-PGE<sub>2</sub> significantly promoted adipocyte differentiation (Fig. 5A, two-way ANOVA,  $P < 0.05$ ). Conversely, overexpression of PTGR-3 suppressed adipocyte differentiation in 3T3-L1 cells (Fig. 5A, two-way ANOVA,  $P < 0.05$ ). However, the proadipogenic effect of 15-keto-PGE<sub>2</sub> was remarkably abolished when PTGR-3 protein was overexpressed in 3T3-L1 preadipocytes

(Fig. 5A,  $P < 0.05$  for interaction). Similar results were found for intracellular triglyceride content, expression of adipogenic marker genes, and PPRE-driven luciferase activity (Fig. 5B–D).

We further tested whether GW9662, an irreversible competitive PPAR $\gamma$  antagonist that covalently binds to a cysteine residue in the PPAR $\gamma$  ligand-binding domain, can erase the enhanced adipocyte differentiation in PTGR-3-knockdown 3T3-L1 preadipocytes. After GW9662 treatment, adipocyte differentiation, triglyceride content, adipogenic marker genes, and PPRE-driven luciferase activity were significantly decreased in PTGR-3-knockdown cells compared

TABLE 1. Specific activities of PTGR-3 on various compounds

Substrate	Specific Activity(nmol/min-mg protein)
15-Keto-PGE <sub>2</sub>	215.04 ± 1.78
15-Keto-PGE <sub>1</sub>	109.61 ± 4.40
15-Keto-PGF <sub>2α</sub>	303.34 ± 2.39
15-Keto-PGF <sub>1α</sub>	286.20 ± 3.46
13,14-Dihydro-15-keto-PGD <sub>2</sub>	ND
13,14-Dihydro-15-keto-PGE <sub>2</sub>	ND
13,14-Dihydro-15-keto-PGF <sub>2α</sub>	ND
Leukotriene B <sub>4</sub>	ND

ND, nondetectable.

with absence of GW9662 treatment (Fig. 5E–H, two-way ANOVA,  $P < 0.05$  for interaction).

To confirm our hypothesis that PTGR-3 directly suppresses 15-keto-PGE<sub>2</sub>-mediated transactivation of PPAR $\gamma$ , we cotransfected 293 cells with Flag-PTGR-3, CMX-GAL4-PPAR $\gamma$ , and UAS $\times$ 4-TK-LUC plasmids (Fig. 5I), and then treated these cells with or without 15-keto-PGE<sub>2</sub>. Similar to previous results, 15-keto-PGE<sub>2</sub> significantly activated luciferase activity, but this effect was remarkably reduced by PTGR-3 overexpression in a dose-dependent manner (Fig. 5J, one-way ANOVA,  $P < 0.05$ ). Taken together, these results clearly demonstrate that PTGR-3 is involved in 15-keto-PGE<sub>2</sub>-mediated adipogenesis and plays a negative regulator role in this process.

## DISCUSSION

The major finding of this study is PTGR-3, a novel 15-oxoprostaglandin- $\Delta^{13}$ -reductase that catalyzes the reaction in converting 15-keto-PGE<sub>2</sub> to 13,14-dihydro-15-keto-PGE<sub>2</sub>. We found that PTGR-3 expression in white adipose tissue was reduced in genetic *ob/ob* and HFD-induced obese mice models. PTGR-3 expression decreased drastically during adipocyte differentiation. Ectopic expression of PTGR-3 in 3T3-L1 preadipocytes decreased PPAR $\gamma$ -dependent transcriptional activity, thereby attenuating adipogenesis. In contrast, suppression of PTGR-3 expression in cells accelerated adipogenesis by increasing PPAR $\gamma$  transcriptional activity. These results reveal that PTGR-3 negatively modulates adipocyte differentiation by regulation of PPAR $\gamma$  transcriptional activity.

It has been demonstrated that several prostaglandins act as bioactive modulators for regulating adipocyte differentiation through either direct binding on the PPAR $\gamma$  ligand-binding domain or indirect mechanisms mediated

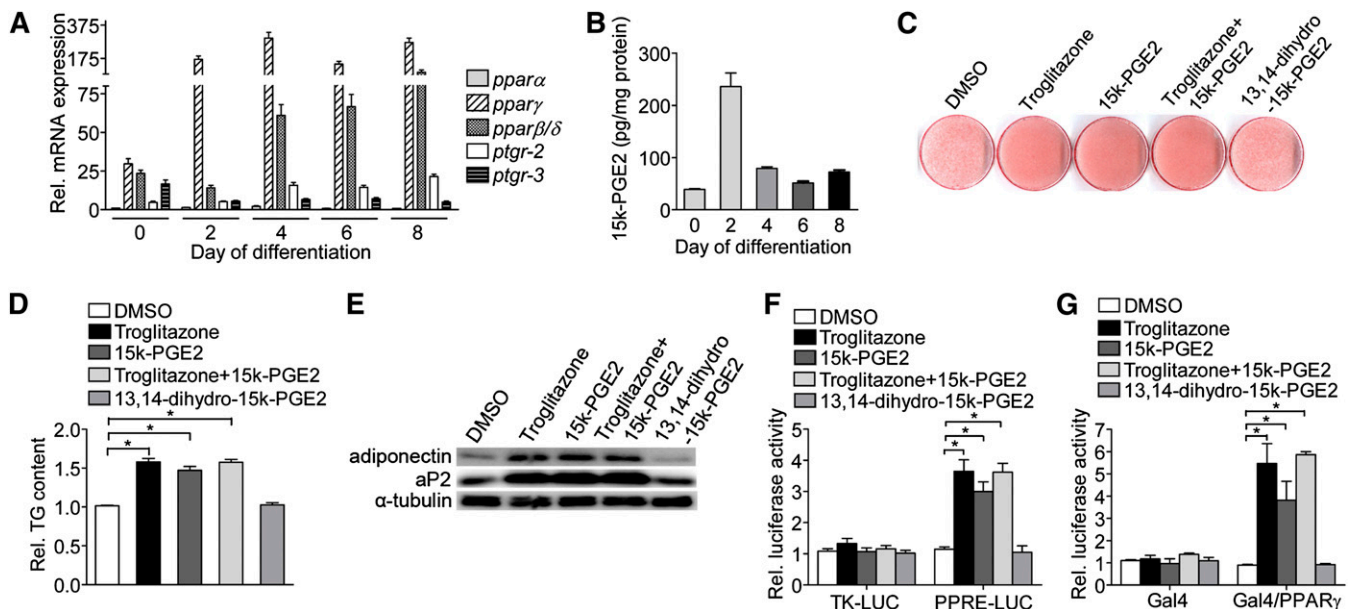
by signaling transduction. For the past decade, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>) has been considered a potent natural ligand for PPAR $\gamma$  (1). 15d-PGJ<sub>2</sub> is derived from prostaglandin H<sub>2</sub> (PGH<sub>2</sub>), which is converted by PGD synthase to prostaglandin D<sub>2</sub> and further chemically dehydrated to form 15d-PGJ<sub>2</sub>. Several lines of evidence have demonstrated that inhibition of the COX pathway or dysregulation of genes involved in this series of reactions in 3T3-L1 preadipocytes affects adipocyte differentiation (6–9). The highest level of 15d-PGJ<sub>2</sub> was detected in 3T3-L1 cells during the maturation phase of adipocytes (10). In contrast, other groups have demonstrated that no differences in formation of 15d-PGJ<sub>2</sub> are observed during adipocyte differentiation (11). Thus, it remains uncertain whether 15d-PGJ<sub>2</sub> is truly an endogenous ligand for PPAR $\gamma$  that modulates adipocyte differentiation. Prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) is generated from PGH<sub>2</sub> by PGF<sub>2α</sub> synthase (PGFS), and PGF<sub>2α</sub> is reported to be an inhibitory modulator of adipocyte differentiation (12). The level of PGF<sub>2α</sub> rapidly increased to a peak 3 h after initiation of differentiation and then declined, indicating that PGF<sub>2α</sub> suppresses in the early phase of adipogenesis (13). Knockdown of aldo-keto reductase 1B3 (AKR1B3), a PGFS, decreased de novo PGF<sub>2α</sub> biosynthesis in 3T3-L1 cells and then further promoted lipid accumulation (13). It has been shown that PGF<sub>2α</sub> suppressed adipogenesis by enhancing phosphorylation of PPAR $\gamma$  via prostaglandin F receptor-activated MEK/ERK cascade (14). On the other hand, PGE<sub>2</sub>, a precursor of 15-keto-PGE<sub>2</sub>, is the most abundant prostaglandin produced in 3T3-L1 preadipocytes, and the highest level of PGE<sub>2</sub> was detected during the early phase of adipogenesis (15, 16). The activity of 15-hydroxy prostaglandin dehydrogenase, an enzyme catalyzing the conversion of PGE<sub>2</sub> to 15-keto-PGE<sub>2</sub>, was also detected in adipose tissue (16), implying that PGE<sub>2</sub> metabolites or its catabolizing enzymes may play a potential role in modulating adipocyte differentiation. However, the role of PGE<sub>2</sub> metabolites in lipid metabolism has not been widely studied.

In our previous study, we found that 15-keto-PGE<sub>2</sub> is a PPAR $\gamma$  ligand and that 15-keto-PGE<sub>2</sub> is reduced to 13,14-dihydro-15-keto-PGE<sub>2</sub> by PTGR-2 (2). Here, we identified that PTGR-3 is a new member of prostaglandin reductase family. Similar to PTGR-2, PTGR-3 converts 15-keto-PGE<sub>2</sub> to 13,14-dihydro-15-keto-PGE<sub>2</sub> by a similar reductive mechanism, thereby influencing intracellular contents of bioactive 15-keto-PGE<sub>2</sub> in cells, which may modulate the process of adipogenesis in 3T3-L1 preadipocytes. Enzyme kinetics studies showed relatively higher efficiency of PTGR-2 in converting 15-keto-PGE<sub>2</sub> to 13,14-dihydro-15-keto-PGE<sub>2</sub>

TABLE 2. Enzymatic substrates and kinetic parameters for PTGR-3

Substrate	$K_m$	$V_{max}$	$K_{cat}$	$K_{cat}/K_m$
	$\mu M$	milliunits/mg <sup>a</sup>	min <sup>-1</sup>	mM <sup>-1</sup> min <sup>-1</sup>
15-Keto-PGE <sub>2</sub>	55.03 ± 5.45	215.04 ± 1.78	8.60 ± 0.07	157.24 ± 14.00
15-Keto-PGE <sub>1</sub>	75.11 ± 7.78	109.61 ± 4.40	4.37 ± 0.15	58.45 ± 4.59
15-Keto-PGF <sub>2α</sub>	42.49 ± 1.00	303.34 ± 2.39	11.55 ± 0.57	271.76 ± 8.82
15-Keto-PGF <sub>1α</sub>	53.54 ± 3.70	286.20 ± 3.46	11.45 ± 0.14	214.41 ± 12.92

<sup>a</sup>1 unit = 1  $\mu$ mol NADP<sup>+</sup>/min.

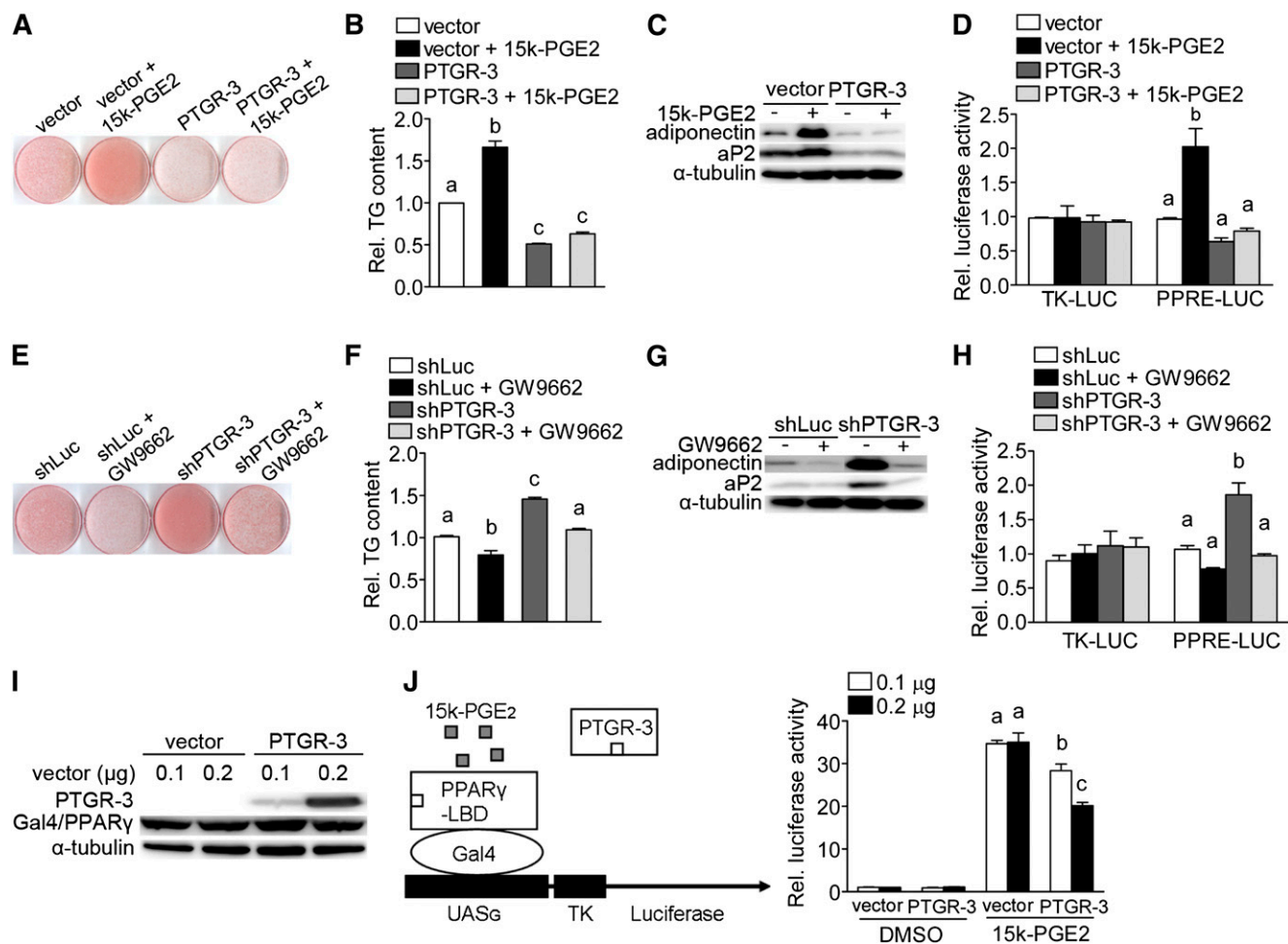


**Fig. 4.** 15-keto-PGE<sub>2</sub>, a PTGR-3 substrate, promotes adipogenesis through activation of PPAR $\gamma$ . (A) Expression of PTGR and PPAR mRNA during adipocyte differentiation. (B) Measurement of 15-keto-PGE<sub>2</sub> level during adipocyte differentiation. Intracellular 15-keto-PGE<sub>2</sub> level isolated from 3T3-L1 cells were extracted and analyzed by LC-MS/MS ( $n = 3$ ). (C) 3T3-L1 preadipocytes were maintained in induction medium with or without treatments (1  $\mu$ M troglitazone, 10  $\mu$ M 15-keto-PGE<sub>2</sub>, and 10  $\mu$ M 13,14-dihydro-15-keto-PGE<sub>2</sub>) for 2 days. After 8 days of adipogenic stimulation, cells on the plates were stained with Oil Red O and quantified (D), and adipogenic gene (adiponectin and aP2) expression (E) was determined ( $n = 3$ ). \* $P < 0.05$  versus DMSO. (F) 15-keto-PGE<sub>2</sub> triggers PPRE-driven luciferase activity in differentiating 3T3-L1 cells. Differentiating 3T3-L1 cells were transfected with reporter vectors (TK-LUC and PPRE-LUC) for 24 h and then cells were incubated in induction medium with or without treatments (1  $\mu$ M troglitazone, 10  $\mu$ M 15-keto-PGE<sub>2</sub>, and 10  $\mu$ M 13,14-dihydro-15-keto-PGE<sub>2</sub>) for 2 days. The activity of firefly luciferase was determined and normalized to the activity of renilla luciferase. The bars indicate the means  $\pm$  SE for three independent replicates ( $n = 3$ ). \* $P < 0.05$  versus DMSO. (G) Transactivation of 15-keto-PGE<sub>2</sub> on PPAR $\gamma$  via ligand-binding domain of PPAR $\gamma$ . Differentiating 3T3-L1 cells were transfected with expression vectors (CMX-GAL4 and CMX-GAL4-PPAR $\gamma$ ) and UAS<sub>C</sub>4-TK-LUC reporter plasmid for 24 h, and then cells were incubated in induction medium with or without treatments (1  $\mu$ M troglitazone, 10  $\mu$ M 15-keto-PGE<sub>2</sub>, and 10  $\mu$ M 13,14-dihydro-15-keto-PGE<sub>2</sub>) for 2 days. The activity of firefly luciferase was determined and normalized to the activity of renilla luciferase. The bars indicate the means  $\pm$  SE for three independent replicates ( $n = 3$ ). \* $P < 0.05$  versus DMSO.

compared with PTGR-3. Both PTGR-2 and PTGR-3 were expressed abundantly in white adipose tissue (supplementary Fig. I-A) and were downregulated in white adipose tissue of *ob/ob* mice (supplementary Fig. I-B). Interestingly, expression of PTGR-3 in 3T3-L1 preadipocytes decreased rapidly within 48 h during the course of induced adipocyte differentiation, accompanied by a rise of 15-keto-PGE<sub>2</sub> level and PPAR $\gamma$  expression, implying that PTGR-3 may modulate PPAR $\gamma$  activity by regulation of 15-keto-PGE<sub>2</sub> production in an early stage of adipocyte differentiation. After 2 days of induction, PTGR-2 expression started to increase and endogenous 15-keto-PGE<sub>2</sub> level gradually decreased, indicating PTGR-2 may play a more dominant role in the late stage of adipocyte differentiation than PTGR-3. Cumulatively, these findings demonstrate that these enzymes display different kinetic parameters and different expression patterns during adipogenesis. Whether and how PTGR-2 and PTGR-3 coordinately regulate 15-keto-PGE<sub>2</sub> homeostasis and regulate adipogenesis remains to be investigated.

Conversion of preadipocytes to adipocytes is a complicated process, which is regulated by several factors, including hormonal and nutrient stimulation. Preadipocytes have to integrate these signals from the environment for initiation of adipocyte differentiation. Several factors secreted

from preadipocytes, such as DLK1/PREF1 and Wnt proteins, play an important role in maintaining the undifferentiated state in preadipocytes by extracellular signaling transduction. It has been demonstrated that dysregulation of these genes promotes adipocyte differentiation by influencing C/EBP $\alpha$  and PPAR $\gamma$  expression in vivo or in vitro (17, 18). These results suggest that maintenance of preadipocytes in an undifferentiated state requires the activity of extracellular signaling molecules. However, little is known about whether intracellular proteins, such as enzymes involved in proadipogenic molecule metabolism, also modulate maintenance of the preadipose state. Here, we demonstrate that expression of PTGR-3, similar to DLK1/PREF1 and Wnt proteins, is predominantly expressed in preadipocytes and decreases remarkably during adipocyte differentiation. In addition, PTGR-3 protein is abundantly expressed in adipose tissue of lean mice, which contains a relatively high percentage of undifferentiated preadipocytes compared with adipose tissue of obese mice, implying that PTGR-3 may play a role in maintaining the undifferentiated state in preadipocytes. Forced PTGR-3 expression attenuates adipocyte differentiation, whereas knocking down PTGR-3 in 3T3-L1 preadipocytes accelerates adipocyte differentiation. Overall, these observations suggest that PTGR-3 protein may facilitate maintaining the undifferentiated state



**Fig. 5.** PTGR-3 decreases proadipogenic effect of 15-keto-PGE<sub>2</sub> on PPAR $\gamma$  activity. Vector-only and PTGR-3 overexpressing preadipocytes were maintained in induction medium with or without 10  $\mu$ M 15-keto-PGE<sub>2</sub> treatment for 2 days. After 8 days of adipogenic stimulation, cells on the plates were stained with Oil Red O (A) and quantified (B) and adipogenic gene (adiponectin and aP2) expression was determined (C). The bars indicate the means  $\pm$  SE for three independent replicates ( $n = 3$ ). Different superscripts indicate significant difference between groups (two-way ANOVA,  $P \leq 0.05$  with post-hoc Tukey test). The expression of adipogenic markers (adiponectin and aP2) was determined and normalized to  $\alpha$ -tubulin. (D) PPRE-driven luciferase activity of vector-only and PTGR-3-overexpressing cells after 15-keto-PGE<sub>2</sub> treatment. Vector-only and PTGR-3-overexpressing preadipocytes were transiently transfected with reporter vectors (TK-LUC and PPRE-LUC) for 24 h, and then cells were incubated in induction medium with or without 10  $\mu$ M 15-keto-PGE<sub>2</sub> treatment. The activity of firefly luciferase was determined and normalized to the activity of renilla luciferase. The bars indicate the means  $\pm$  SE for three independent replicates ( $n = 3$ ). Different superscripts indicate significant difference between groups (two-way ANOVA,  $P \leq 0.05$  with post-hoc Tukey test). shRNA control and PTGR-3-knockdown cells were maintained in induction medium with or without 1  $\mu$ M GW9662 treatment for 2 days. After 8 days of adipogenic stimulation, cells on the plates were stained with Oil Red O (E) and quantified (F) and adipogenic gene (adiponectin and aP2) expression was determined (G). The bars indicate the means  $\pm$  SE for three independent replicates ( $n = 3$ ). Different superscripts indicate significant difference between groups (two-way ANOVA,  $P \leq 0.05$  with post-hoc Tukey test). Expression of adipogenic genes (adiponectin and aP2) in GW9662-treated control and PTGR-3-knockdown cells. The expression of adipogenic markers (adiponectin and aP2) was determined and normalized to  $\alpha$ -tubulin. (H) PPRE-driven luciferase activity of shRNA control and PTGR-3-knockdown cells after GW9662 treatment. shRNA control and PTGR-3-knockdown cells were transiently transfected with reporter vectors (TK-LUC and PPRE-LUC) for 24 h and then cells were incubated in induction medium with or without 1  $\mu$ M GW9662 treatment. The activity of firefly luciferase was determined and normalized to the activity of renilla luciferase. The bars indicate the means  $\pm$  SE for three independent replicates ( $n = 3$ ). Different superscripts indicate significant difference between groups (two-way ANOVA,  $P \leq 0.05$  with post-hoc Tukey test). (I) Efficiency of ectopic expression of UAS $\times$ 4-TK-LUC, CMX-GAL4-PPAR $\gamma$ , and Flag-PTGR-3 protein in 293 cells. The expression of PTGR-3 and Gal/PPAR $\gamma$  was determined and normalized to  $\alpha$ -tubulin. (J) Transactivation of 15-keto-PGE<sub>2</sub> on PPAR $\gamma$  ligand-binding domain in Flag-PTGR-3-overexpressing 293 cells. 293 cells were transiently transfected with expression vectors (CMX-GAL4, CMX-GAL4-PPAR $\gamma$ , and Flag-PTGR-3) and UAS $\times$ 4-TK-LUC reporter plasmid for 24 h, and then cells were incubated in growth medium with or without 10  $\mu$ M 15-keto-PGE<sub>2</sub> for 2 days. The activity of firefly luciferase was determined and normalized to the activity of renilla luciferase. The bars indicate the means  $\pm$  SE for three independent replicates ( $n = 3$ ). Different superscripts indicate significant difference between groups (one-way ANOVA,  $P \leq 0.05$  with post-hoc Tukey test).

in preadipocytes by inhibiting endogenous 15-keto-PGE<sub>2</sub> production and that suppression of PTGR-3 expression is required for normal adipocyte differentiation by regulating PPAR $\gamma$  transcriptional activity.

In conclusion, we provide evidence that PTGR-3 is a novel 15-oxoprostaglandin- $\Delta$ <sup>13</sup>-reductase that affects adipocyte differentiation by regulation of PPAR $\gamma$  activity. PTGR-3 expression is reduced in adipose tissue from murine models

of obesity. Modulation of PTGR-3 expression or activity might provide a novel avenue in treating obesity and related metabolic disorders.

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