15-Hydroxyprostaglandin Dehydrogenase-derived 15-Keto-prostaglandin E₂ Inhibits Cholangiocarcinoma Cell Growth through Interaction with Peroxisome Proliferator-activated Receptor-γ, SMAD2/3, and TAP63 Proteins*\(^\text{f}\)

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Background: 15-PGDH catalyzes PGE2 oxidation to form 15-keto-PGE2.

Results: 15-PGDH-derived 15-keto-PGE₂ is a PPAR-γ-ligand that causes Smad2/3 association with TGFBR1/SARA and induces formation of pSmad2/3-TAP63-p53 ternary complex.

Conclusion: 15-PGDH-mediated 15-keto-PGE₂ signaling cascade interacts with PPAR-γ, Smad2/3, and TAP63.

Significance: Induction of 15-PGDH expression or administration of 15-keto-PGE₂ may present a promising anti-cancer therapeutic strategy.

Prostaglandin E₂ (PGE₂) is a potent lipid mediator that plays a key role in inflammation and carcinogenesis. NAD⁺-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) catalyzes the oxidation of the 15(S)-hydroxyl group of PGE₂, which is subsequently converted to other prostaglandins via cyclooxygenase (COX) enzymes catalyzing the rate-limiting step in prostaglandin synthesis, converting arachidonic acid into prostaglandin H₂, which is subsequently converted to other prostaglandins via prostaglandin synthases. Numerous studies have documented the pro-inflammatory and pro-tumorigenic actions of COX-2 and PGE₂ in various human and animal cancers (1–3). Overproduction of PGE₂ enhances cell proliferation, invasion, metastasis, angiogenesis, and inhibits apoptosis. Cyclooxygenase (COX) inhibitors have been found to reduce cancer and precancerous growths, although the risk of cardiovascular side effects has limited its use (4, 5). As suppression of COX/PGE₂ has been one of the most successful approaches for cancer prevention, there is great interest in identifying alternative novel targets for inhibition of the prostaglandin pathway for effective anticancer therapy with fewer side effects.

The amount of biologically active PGE₂ in the inflammatory and tumor microenvironment is regulated by the balance of PGE₂ synthesis and degradation. The NAD⁺-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) catalyzes the oxidation of the 15(S)-hydroxyl group of PGE₂, converting PGE₂ into 15-keto-PGE₂ (6). Consistent with the documented catabolism of PGE₂ by 15-PGDH, accumulating evidence suggests an important role of 15-PGDH in cancer development and progression (7–15). For example, 15-PGDH is down-regulated in several malignancies, such as colorectal, lung, gastric, bladder, and pancreatic cancers (7, 8, 10, 16–20). Reduction of 15-PGDH is an independent predictor of poor survival associated with enhancement of cell proliferation in gastric adenocarcinoma (18). A haplotype in the 15-PGDH gene is positively

pneumocystis jiroveci pneumonia; PPAR-γ, peroxisome proliferator activated receptor-γ; PPRE, peroxisome proliferator response element; SARA, Smad anchor for receptor activation; SCID, severe combined immunodeficiency; TGFBRI, TGF-β receptor I; TRITC, tetramethylrhodamine isothiocyanate; Q-PCR, quantitative PCR; IP, immunoprecipitation; ETE, eicosatetraenoic acid; LTB, leukotriene B; LX, lipoxin.

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associated with colorectal cancer risk (12). In mouse models of carcinogenesis, overexpression of 15-PGDH decreases cancer cell growth or delays tumor formation, whereas deletion of 15-PGDH increases the susceptibility to chemically or genetically induced colon tumors (8). Targeted adenovirus-mediated delivery of 15-PGDH gene inhibited colon cancer growth in a mouse xenograft model (21). The hepatocyte growth factor and its receptor c-Met signaling promotes PGE2 biogenesis in colorectal cancer cells via up-regulation of COX-2 and down-regulation of 15-PGDH (22). ω3-Polynsaturated fatty acids reduce the level of PGE2 in cholangiocarcinoma cells through down-regulation of COX-2 and induction of 15-PGDH (23). Indeed, reciprocal regulation between COX-2 and 15-PGDH expression has been documented in several cancers (24). Anti-cancer therapeutics, such as transforming growth factor (TGF)-β1, glucocorticoids, and histone deacetylase inhibitors, have been shown to exert their anti-carcinogenic activity in part through induction of 15-PGDH expression (17, 25). All of these findings point toward an important tumor suppressive function of 15-PGDH.

To date, the action of 15-PGDH is largely attributable to its conversion of biologically active PGE2, with 15-keto-PGE2 being considered as largely inactive. This study provides novel evidence for an active role of 15-keto-PGE2 in 15-PGDH-mediated anti-tumor effect. Our data reveal a novel 15-PGDH-keto-PGE2-mediated signaling cascade that interacts with oxisome proliferator-activated receptor-γ (PPAR-γ) and TAP63 in human cholangiocarcinoma cells. We have shown that 15-keto-PGE2 is a natural ligand that binds to PPAR-γ and causes its dissociation from Smad2/3, which allows subsequent Smad2/3 phosphorylation and activation of tumor-suppressive 15-PGDH (22). Moreover, induced 15-keto-PGE2 levels in CCLP1 cells. For prostaglandin extraction, CCLP1 cells in 50:50 hexane/ethyl acetate were vortex-mixed, and the samples (4 ml) were dried under nitrogen and reconstituted in 180 μl of 50:50 methanol, 10 mM ammonium acetate. 20 μl of PGE2-d4 was added to each sample and vortexed for ~15 s. 3 μl of each sample was injected for LC/MS/MS analysis using the AB Sciex API 4000 mass spectrometer system. For HPLC, prostaglandins were chromatographically resolved using a 2.6-μm Phenomenex Kinetex Phenyl Hexyl, 100 × 2.1-mm analytical column, and a linear gradient with mobile phase A (10 mM ammonium acetate in water) and mobile phase B (methanol). Individual analytes were detected using electrospray negative ionization and monitoring the transitions m/z 339 → 315 for 15-keto-PGE2 and m/z 355 → 275 for PGE2-d4. The identification of the compounds was confirmed by comparison with reference standards. A calibration curve for 15-keto-PGE2 was constructed and used to determine the concentration of 15-keto-PGE2 in each sample. Standards of PGE2 and PGD2 were analyzed during method development to ensure chromatographic separation because they were structural isomers of 15-keto-PGE2 as well as having similar product ion spectra.

Cell Transfection and Stable Cell Lines—CCLP1 cells were transfected with pCMV6-AC-GFP, pCMV6-AC-GFP-15PGDH (NM_000860.3), pGFP-V-RS, and pGFP-V-RS-15PGDH using transfection reagent Lipofectamine® 2000 (Invitrogen) according to the manufacturer’s instructions. For screening CCLP1 stable cell lines with 15-PGDH overexpression or knockdown, 48 h after transfection, the cells were cultured with the selective media containing 1–2 mg/ml G418 (Calbiochem) for 15-PGDH overexpression or 1–2 μg/ml puromycin (Invitrogen) for 15-PGDH knockdown. For the next 4 weeks or so, the selective media were replaced every 3 days. Transfection efficiency was measured by immunofluorescence staining with anti-GFP antibody and Western blot with anti-15-PGDH antibody.

Stable Cell Lines with Double Transfections—The CCLP1 cells were first transfected with pCMV6-AV-GFP-15PGDH vector using Lipofectamine® 2000 (Invitrogen), and the stably transfected cells were selected by using 1 mg/ml G418 (Invitrogen). pGFP-V-TAP63 was then co-transfected into the above stable cell line, and the transfected cells were grown in presence of 1–2 μg/ml puromycin (Invitrogen) for selection. Alternatively, the above cells were transfected with pCMV6-15PGDH (NM_000860.3), pCMV6-TAP63, and pcDNA3-TAP63 was then co-transfected into the above stable cell line, and the transfected cells were grown in presence of 1–2 μg/ml puromycin according to the manufacturer’s instructions. The antibody against TGF-β1 was purchased from eBioscience. The cytotoxicity of CCLP1 cells was determined by comparison with reference standards. A calibration curve for 15-keto-PGE2 was constructed and used to determine the concentration of 15-keto-PGE2 in each sample. Standards of PGE2 and PGD2 were analyzed during method development to ensure chromatographic separation because they were structural isomers of 15-keto-PGE2 as well as having similar product ion spectra.
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**Western Blotting**—The logarithmically growing cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in a lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, pH 8.0) with protease inhibitor mixtures and phosphatase inhibitor from Roche Applied Science. Cells lysates were centrifuged at 12,000 × g for 20 min at 4 °C after sonication on ice, and supernatants were collected. Protein concentration was measured using a protein technology by rotation for 1 h at room temperature, and the samples were subjected to an additional round of IP as described above.

**EMSAs**—Cells were washed and scraped in ice-cold PBS to prepare nuclei for electrophoretic gel mobility shift assay with the use of the gel shift assay system (Promega) modified according to the manufacturer’s instructions. In brief, consensus oligonucleotides for TAP63-binding site were biotin-labeled (hot probe) as follows: 5'-biotin forward, GATGGATTG-GACAGTAAAG-3', and reverse CTTTACCTGTCATTCCCTC-3'; no-biotin-labeled (cold probe) forward, 5'-GATGGA-TTGACAGTAAAG-3'; and reverse, 5'-CTTTACCTGTC-AATCCCTC-3' (cold probe). Each binding reaction was carried out with 1 μg of related dsDNA probe and 200 μg of purified nuclear extract of binding buffer containing 0.5 mg/ml poly(dI-dC) (25 mM HEPES, pH 8.0, with 50 mM KCl, 0.13 M NaCl, 0.5 mM EDTA, 10 mM DTT, and 5% glycerol). Samples containing cells or tissue lysate proteins were separated on any kDa mini-protein TGX precast gels (Bio-Rad), transferred onto a nitrocellulose membrane (Bio-Rad), and then blocked in 10% dry milk/PBST (PBS with 0.1% Tween 20) for 1 h at RT. The blots were incubated with 0.2 μg/ml antibody overnight at 4 °C. Following three washes, membranes were then incubated with secondary antibody (horseradish peroxidase-conjugated immunoglobulin G or IRDye 680LT/IRDye 800CW secondary antibodies from LI-COR Biosciences) for 60 min at RT or 4 °C overnight in PBST. Signals were enhanced chemiluminescence plus kit (GE Healthcare) or IRDye 680LT/IRDye 800CW secondary antibodies from LI-COR Biosciences. Detection was carried out with 1 ml of binding buffer containing 0.5 mg/ml poly(dI-dC) (25 mM HEPES, pH 8.0, with 50 mM KCl, 0.13 M NaCl, 0.5 mM EDTA, 10 mM DTT, and 5% glycerol). The blots were incubated with 30 μg/ml anti-PCNA antibody (or appropriate control, duplicate sections were immunostained without primary mouse anti-human Ki67, PCNA, and TAP63 antibodies (Santa Cruz Biotechnology). The primary antibodies were appropriately diluted. As the secondary antibody, anti-mouse IgG (horseradish peroxidase linked whole antibody from sheep, GE Healthcare) was used at 200× dilution. In brief, deparaffinized sections were treated with 0.3% H2O2 in methanol for 30 min to abolish endogenous peroxidase activity and then microwaved in antigen unmasking solution for antigen retrieval. Sections were stained with 10% goat serum in PBS for 1 h at 37 °C and then incubated with 2 μg/ml anti-PCNA antibody (or appropriate diluted other antibodies) at 4 °C overnight. Then the sections were incubated with anti-mouse IgG (horseradish peroxidase-linked whole antibody) at 37 °C for 1 h. Staining was performed using 3,3-diaminobenzidine substrate kit for peroxidase according to the manufacturer’s instructions (Vector Laboratories) and counterstained with hematoxylin. As a negative control, duplicate sections were immunostained without exposure to the primary antibodies. The frequency of positive cells was determined by counting the total number of cells and positive stained cells in randomly selected ×200 magnification fields, including at least 1000 cells. Average numbers from the field sets were then determined and reported as the percentage of positively stained cells to the total number of cells.

**EMSA**

**Co-immunoprecipitation (IP) and Repeat IP**—For co-immunoprecipitation, cells were transfected using Lipofectamine® 2000 (Invitrogen) in a 100-mm diameter dish. At the end of each treatment, the cells were lysed in 1 ml of the whole-cell extract buffer A (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 0.5–1% Nonidet P-40, 0.1 mM EDTA, and 1.0 mM DTT) with protease inhibitor mixtures. In brief, 500-μl cell lysates were pre-cleared with 30 μl of protein G/plus agarose beads (Santa Cruz Biotechnology) by rotation for 1 h at room temperature, and the supernatant was obtained after centrifugation (1000 × g) for 3 min at 4 °C. Precleared supernatants were incubated with 2 μg of antibody by rotation for 4 h at 4 °C. The immunoprecipitates were incubated with 30 μl of protein A/G-plus agarose beads by rotation overnight at 4 °C and then centrifuged at 5000 rpm for 5 min at 4 °C. The precipitates were washed five times for 10 min with beads wash solution (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA) and then resuspended in 40 μl of 2× SDS-PAGE sample loading buffer to incubate for 5 min at 100 °C. Then Western blot was performed with another related antibody indicated in Western blotting. Repeat IP was conducted by cleansing the first precipitates with elution buffer (0.1% Triton X-100, 0.1% SDS, 0.5% BSA in PBS). Specifically, a 40-μl aliquot of immunoprecipitates was eluted with 750 μl of elution buffer and incubated for 50 min at room temperature, and the samples were subjected to an additional round of IP as described above.

**RT PCR**

**Immunohistochemistry**—Tissues were fixed with 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned at 4 μm. Sections were immunohistochemically stained using primary mouse anti-human Ki67, PCNA, and TAP63 antibodies (Santa Cruz Biotechnology). The primary antibodies were appropriately diluted. As the secondary antibody, anti-mouse IgG (horseradish peroxidase linked whole antibody from sheep, GE Healthcare) was used at 200× dilution. In brief, deparaffinized sections were treated with 0.3% H2O2 in methanol for 30 min to abolish endogenous peroxidase activity and then microwaved in antigen unmasking solution for antigen retrieval. Sections were stained with 10% goat serum in PBS for 1 h at 37 °C and then incubated with 2 μg/ml anti-PCNA antibody (or appropriate diluted other antibodies) at 4 °C overnight. Then the sections were incubated with anti-mouse IgG (horseradish peroxidase-linked whole antibody) at 37 °C for 1 h. Staining was performed using 3,3-diaminobenzidine substrate kit for peroxidase according to the manufacturer’s instructions (Vector Laboratories) and counterstained with hematoxylin. As a negative control, duplicate sections were immunostained without exposure to the primary antibodies. The frequency of positive cells was determined by counting the total number of cells and positive stained cells in randomly selected ×200 magnification fields, including at least 1000 cells. Average numbers from the field sets were then determined and reported as the percentage of positively stained cells to the total number of cells.
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Immunofluorescence—Following treatment, cells on a cover-slip were washed twice with cold PBS and were then fixed in 4% paraformaldehyde for 10 min. The fixed cells were treated with 0.3% H2O2 in methanol for 30 min to abolish endogenous per-oxidase activity and then treated with 0.1% Triton and 5% DMSO. After washing three times with PBS, the samples were blocked with 5% BSA at 37 °C for 1 h and then were incubated with 2 μg/ml primary antibody at RT for 1 h or at 4 °C overnight. Following three washings with PBS, the sections were incubated with TRITC-linked IgG or FITC-linked IgG. The samples were counterstained with DAPI (diluted 1:1000 in double distilled H2O) for 15 min. Coverslips were applied using Mounting Medium for fluorescence, and images were captured using Olympus FV1000 II laser scanning confocal microscope (Olympus) by FV10-ASW1.7 software or fluorescence microscope (Olympus).

BrdU Staining—80% confluent cells were cultured for 24 h before treatment with 10 μl of BrdU (Roche Applied Science) for 4 h. Immunofluorescent staining with an anti-BrdU antibody was performed according to the manufacturer’s instructions (BD Biosciences). BrdU-positive cells from 10 random chosen fields of at least three independent samples were counted.

Wound Healing Assay—Cells were cultured to >90% confluence in 10-cm dishes. The cells were rinsed with PBS and starved in low serum media (1.5 ml; 0.5–0.1% serum in DMEM) overnight. A sterile 200-μl pipette tip was used to scratch wounds through the cells. The cells were cultured with PBS. Photographs were taken taken at 10× at 4, 8, and 24 h (the mean) measurement.

Culture Plate Colonization Assay—103 cells were plated in a 10-cm dish and allowed to grow for 14 days. The colonies were stained with 0.5 ml of 2% crystal violet in methanol and 10% glacial acetic acid. The colonies were counted, and the data were obtained from three independent experiments.

Soft Agar Colony Formation Assay—102 cells were plated on a 6-well plate containing 0.5% (lower) and 0.35% (upper) double layer soft agar. To form lower layer soft agar, 1% agar was melted in a microwave and cooled to 40 °C in a water bath, and 2× DMEM containing 20% FCS was warmed to 40 °C. After allowing for the temperature (40 °C) to equilibrate at least 30 min, the two solutions were mixed in equal volumes to give 0.5% lower layer soft agar (0.5% agar + 1× DMEM + 10% FCS). Then 1 ml of 0.5% lower layer soft agar was rapidly added into each well of a 6-well plate and set aside for 5 min or more until solidification at RT. To form the upper layer soft agar, 0.7% agar was melted in a microwave and cooled to 40 °C in a water bath, and 2× DMEM containing 20% FCS was warmed to 40 °C. After allowing for the temperature (40 °C) to equilibrate at least 30 min, 3 ml of the each solution (0.7% agarose, 2× DMEM containing 20% FCS) was added into a 10-ml tube containing 0.1 ml of 12,000 cells/ml of cell suspension. After mixing gently by swirling, the 1-ml mixture was added into each well of the three replicate 6-well plates. Then the 6-well plates were incubated at 37 °C in humidified incubator for 21 days. The cells were fed 1–2 times per week with cell culture media (DMEM). Soft agar colonies on the 6-well plates were stained with 0.5 ml of 0.005% crystal violet for more than 1 h, and the colonies were counted.

Dual-Luciferase Reporter Assay—Cells (1 × 105/well of a 6-well plate) were transiently transfected with 1 μg of luciferase construct (pGL3/PPRE-luc, p3TP-luc, pGL3/TAP63 promoter-luc, and pGL3-Luc) and 0.1 μg of pRL-Tk (Promega) together with the indicated plasmids using Lipofectamine/plus reagent (Invitrogen). After transfection for 36 h, the cells were harvested with the lysis buffer, and luciferase activities of cell extracts were measured by DLReady™ Centro XS™ LB960 with the use of the Dual-luciferase assay system (Promega) according to the manufacturer’s instructions. Luciferase activity was normalized for transfection efficiency with Renilla luciferase activity.

Cell Proliferation WST-1 Assay—To describe growth curves, cells were synchronized in G0 phase by serum deprivation and then released from growth arrest by re-exposure to complete medium with serum. Cell proliferation was detected by reagent WST-1 kit (Roche Applied Science) according to the manufacturer’s instructions. WST-1 absorbance curve was based on the normalized absorbance. Each point represents the mean of three replicates.

Chromatin Immunoprecipitation—Formaldehyde cross-linking and chromatin immunoprecipitation assays were performed as described by Shang et al. (52) or according to the protocol provided by Upstate Biotechnology with modifications. In brief, cells with 90% confluence in a 150-mm dish were cross-linked by adding formaldehyde to a final concentration of 1% formaldehyde (0.68 ml of 37% formaldehyde in 25 ml of media) and rocked for 10 min at room temperature. The DNA was purified using a QIAquick spin column and eluted in 50 μl of column of 10 μl Tris, pH 8.0. PCR conditions were 60 s at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by 10 min at 72 °C. The Tap63 promoter PCR primer sequences are as follows: P1, 5'-ACTTATTGAGATGGAAGGAGG-3', P2, 5'-GATAACAACTCAACTCC-3'.

Real Time Quantitative PCR—Q-PCR was used to analyze ChIP DNA and INPUT DNA in triplicate using Fast SYBR Green PCR Master Mix (Qiagen) according to the manufacturer-
Results

15-PGDH Inhibits CCLP Cell Proliferation and Migration—CCLP1 cells (human intrahepatic cholangiocarcinoma cell line) (23, 26) were transfected with the GFP control vector (pCMV6-AC-GFP), the 15-PGDH expression vector, the RNAi control vector (pGFP-V-RS), and the 15-PGDH RNAi vector (pGFP-V-RS-15PGDH), respectively. Successful alteration of 15-PGDH in the stably transfected cell lines were confirmed by immunofluorescence and Western blotting (Fig. 1A). Immunofluorescence staining showed that the level of 15-PGDH was increased in cells transfected with the 15-PGDH expression vector compared with transfection with the corresponding GFP control vector; the 15-PGDH staining intensity in 15-PGDH knockdown cells was significantly lower than the corresponding RNAi control vector cells. Western blotting analysis revealed that the expression of 15-PGDH increased in 15-PGDH-overexpressed cells (56 kDa, GFP-15PGDH fusion protein) and decreased in 15-PGDH knockdown cells (29 or 58 kDa). The 15-PGDH-overexpressed cells had reduced PGE2, and high levels of 15-keto-PGE2, whereas the 15-PGDH knockdown cells show accumulation of PGE2 and reduction of 15-keto-PGE2 (Fig. 1B). Because 15-keto-PGE2 is known to activate PPAR-γ (27), we examined the PPAR response element (PPRE) reporter activity in CCLP1 cells with altered 15-PGDH expression. As shown in Fig. 1B, 15-PGDH overexpression significantly increased the PPRE reporter activity, whereas 15-PGDH knockdown reduced it. The effect of 15-PGDH on PPRE reporter activity was abolished when the cells were co-transfected with a vector expressing 15-oxo-prostaglandin-D13 (PGR-2) (PGR-2 overexpression reduces the level of 15-keto-PGE2, as the enzyme catalyzes the reaction converting 15-keto-PGE2 to 13,14-dihydro-15-keto-PGE2 (27)). Furthermore, treatment of wild type CCLP1 cells with the 15-PGDH metabolite, 15-keto-PGE2, increased the PPRE-luciferase activity. This effect was abolished in the PLM1 vehicle control experiment and the EMSA analysis showed that the specificity of PPAR-γ binding to PPRE, was abolished when the enzyme catalyzes the reaction converting 15-keto-PGE2 to 13,14-dihydro-15-keto-PGE2 (27)). The overexpression of 15-PGDH increased the PPRE reporter activity (see supporting information for Fig. 1, part I). Treatment of wild type CCLP1 cells with 15-keto-PGE2 metabolism, 15-PGDH knockdown reduced it (see supporting information for Fig. 1, part II). Because PGE2 synthesis serves as the precursor for 15-keto-PGE2, we further evaluated whether knockdown of mPGES-1, a rate-limiting enzyme in the PGE2 synthetic pathway, would prevent 15-PGDH effects. DNA pulldown, EMSA and luciferase reporter activity assays showed that RNAi knockdown of mPGES-1 attenuated 15-PGDH-induced PPAR-γ activation (see supporting information for Fig. 1, part III). However, mPGES-1 knockdown did not alter the effect of exogenous 15-keto-PGE2. These observations support that it is the endogenous PGE2, derivative that activates PPAR-γ. Collectively, the above findings indicate activation of PPAR-γ by 15-PGDH-derived 15-keto-PGE2 in CCLP1 cells.

We next examined the growth curves of CCLP1 cells with overexpression or knockdown of 15-PGDH. As shown in Fig. 1C, overexpression of 15-PGDH significantly inhibited tumor cell growth, whereas 15-PGDH knockdown accelerated tumor cell growth. Accordingly, 15-PGDH overexpression decreased clonogenic capacity, and 15-PGDH knockdown increased it (Fig. 1D). In comparison with the two mock CCLP1 cells that resulted in 22.13 ± 8.07 and 27.67 ± 5.12% colony formation rates, respectively, the 15-PGDH-overexpressed CCLP1 cells had a colony formation rate of 6.02 ± 2.21% (p < 0.01), and the 15-PGDH-depleted cells had a colony formation rate of 89.67 ± 18.47% (p < 0.01) (Fig. 1D). The percentages of the BrdU-pos-
Live cells were lower in 15-PGDH-overexpressed cells (6.12 ± 1.16% versus 25.78 ± 5.23%, p < 0.01) and higher in 15-PGDH knockdown cells (75.23 ± 10.64% versus 27.74 ± 6.21%, p < 0.01) (see supporting information for Fig. 1, part IV). Wound healing assays showed that 15-PGDH overexpression inhibited cell migration, and 15-PGDH depletion enhanced cell migration. Taken together, these data indicate that 15-PGDH decreases tumor cell malignancy potential, in vitro.

15-PGDH Inhibits Cholangiocarcinoma Growth in Vivo—To further examine the effect of 15-PGDH on tumor growth in vivo, CCLP1 cells with 15-PGDH overexpression or knockdown were inoculated subcutaneously into SCID mice, and the animals were closely monitored for tumor development. As shown in Fig. 2A, 15-PGDH overexpression inhibited tumor growth, and 15-PGDH depletion accelerated growth. The weight of the 15-PGDH-overexpressed tumors (0.11 ± 0.02 g)
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![Image of a graph and table]

**FIGURE 2.** 15-PGDH inhibits CCLP1 cell growth in vitro and in xenograft tumors.

Panel A, photomicrographs of CCLP1 cell colonies cultured in semisolid agar medium. The colony numbers were counted. The data represent mean ± S.E. from three independent experiments. Panel B, 15-PGDH expression was determined by semiquantitative RT-PCR analysis. The data represent mean ± S.E. from three independent experiments. Panel C, 15-PGDH protein expression was determined by Western blotting analysis. The data represent mean ± S.E. from six independent experiments. Panel D, proliferation marker, Ki67, is also higher in 15-PGDH knockdown group (90.28% versus 9.37%, p < 0.01); the weight of the 15-PGDH-depleted tumors was approximately one-fifth of the control weight (0.52 ± 0.09g, p < 0.01); the weight of the 15-PGDH-depleted tumors (1.45 ± 0.18g) was about 2.5 times of the control tumor weight (0.59 ± 0.08g, p < 0.01) and 12 times of the 15-PGDH overexpressed tumor weight. The time of the tumor appearance in the 15-PGDH overexpressed group was significantly longer compared with the control group (18.65 ± 3.12 days versus 9.12 ± 2.14 days, p < 0.01). Conversely, the tumor appearance time in the 15-PGDH knockdown group was significantly shortened compared with the control group (6.01 ± 1.27 days versus 9.37 ± 2.12 days, p < 0.01). As shown in Fig. 2B, the percentage of cells staining for the PCNA was significantly higher in 15-PGDH knockdown tumor cells (90.28 ± 12.66% versus 45.25 ± 5.87%, p < 0.01) but lower in 15-PGDH-overexpressed tumor cells (20.15 ± 2.85% versus 51.21 ± 5.98%, p < 0.01). Similarly, the percentage of cells staining for another cell proliferation marker, Ki67, was also higher in 15-PGDH knockdown group (82.16% ± 0.18g, p < 0.01) but lower in 15-PGDH overexpressed group 18.24 ± 2.36% versus 48.41 ± 6.97%, p < 0.01).

We next employed an additional tumor xenograft model in which an adenoviral vector expressing 15-PGDH (pAd-15-PGDH) was directly injected into the tumors grown in SCID mice (at 3-day intervals, starting 11 days after inoculation until the end of the experiment). As shown in Fig. 2C, pAd-15-PGDH injection significantly inhibited the growth of the xenograft
tumors. The tumor size in the pAd-15-PGDH-injected group was significantly smaller compared with the pAd control group. The average tumor weight in the pAd-15-PGDH-treated group was also significantly lower compared with the adenoviral control group (1.79 ± 0.55 versus 0.43 ± 0.15, p < 0.01). The percentages of the PCNA and Ki67-positive cells in pAd-15-PGDH-treated group were lower than in pAd control group. These findings further demonstrate a tumor-suppressive role of 15-PGDH in vivo.

15-PGDH Metabolite 15-Keto-PGE2 Is a PPAR-γ Ligand That Disrupts PPAR-γ-Smad2/3 Complex Leading to Smad2/3 Activation—Consistent with the documented inhibition of Smad2/3 by PPAR-γ (28, 29), our data showed that PPAR-γ overexpression inhibited the phosphorylation of Smad2/3 in CCLP1 cells (successful overexpression of PPAR-γ was confirmed by Western blotting analysis) (Fig. 3). The PPAR-γ-mediated inhibition of Smad2/3 phosphorylation persisted in the presence of PPAR-γ agonists (cigitazone, rosiglitazone, and 15-d-PGJ2) or PPAR-γ antagonist (GW9662) as well as arachidonic acid and PGE2. Surprisingly, the phosphorylation of Smad2/3 was substantially enhanced in the presence of 15-keto-PGE2, suggesting that 15-keto-PGE2 differs from other PPAR-γ agonists in regulation of Smad2/3 phosphorylation (Fig. 3B). The protein levels of total Smad2/3, TGF-β receptors (TGFβRI and TGFβRII), and Smad anchor for receptor activation (SARA) were not altered by 15-keto-PGE2 treatment. Luciferase reporter activity assays showed that PPAR-γ overexpression inhibited Smad2/3 transcription activity, and this effect persisted in the presence of other PPAR-γ agonists but not 15-keto-PGE2 (Figs. 3A and 4D). PPAR-γ-overexpressed cells treated with 15-keto-PGE2 showed significantly higher Smad2/3 transcription activity compared with PPAR-γ overexpression alone or 15-keto-PGE2 treatment alone. Accordingly, overexpression of both PPAR-γ and 15-PGDH led to higher Smad2/3 transcription activity compared with either PPAR-γ or 15-PGDH overexpression alone. These observations suggest that 15-PGDH-derived 15-keto-PGE2 induces Smad2/3 phosphorylation and transcription activation, in contrast to other PPAR-γ ligands. Immunoprecipitation and Western blotting analysis showed that PPAR-γ is associated with Smad2; this association is disrupted by 15-keto-PGE2 but not by other PPAR-γ ligands (Fig. 3C). Furthermore, PPAR-γ overexpres-
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We carried out further experiments to examine the direct effect of 15-keto-PGE₂ on Smad2/3 phosphorylation and activation. As shown in Fig. 4D, 15-keto-PGE₂ treatment enhanced Smad2/3 phosphorylation and transcription activity; these effects were inhibited when the cells were treated with the anti-TGF-β antibody. The 15-keto-PGE₂-induced Smad2/3 phosphorylation and transcription activity were inhibited by GW9662, a PPAR-γ antagonist. Although PPAR-γ overexpression alone inhibited Smad2/3 phosphorylation and transcription activity, the presence of 15-keto-PGE₂ reversed the inhibitory effect of PPAR-γ and enhanced Smad2/3 phosphorylation and transcription activity. Whereas PPAR-γ associates with Smad2/3 and inhibits the interaction between Smad2 and SARA, treatment with 15-keto-PGE₂ causes Smad2/3 dissociation from PPAR-γ and subsequent association with SARA.

Given that 15-PGDH is known to convert several substrates, including prostaglandins and lipoxins, to produce various keto- and oxo-metabolites and that some of these lipid mediators are known to influence tumor cell proliferation (32–34), we performed further experiments to evaluate the potential effect of other 15-PGDH-derived metabolites in our system. We observed that PPAR-γ and Smad2 induced by 15-keto-PGE₂, but not by 15-keto-ETE, 15-oxo-LTB₄, or 12-oxo-LTB₄, two other potential substrates of 15-PGDH (33, 34), did not affect 15-PGDH-induced dissociation of Smad2 from PPAR-γ and association of Smad2 with SARA and TGFBR1. These results suggest that the 15-PGDH metabolite, 15-keto-PGE₂, is a PPAR-γ ligand that disrupts PPAR-γ-Smad2/3 complex leading to Smad2/3 activation.

15-PGDH-derived 15-Keto-PGE₂ Induces the Expression of TAP63—Given the emerging connection between TGF-β/Smad and TAP63 (35, 36), we performed further experiments to determine whether 15-PGDH and 15-keto-PGE₂ might regulate TAP63. Immunofluorescence and Western blotting analysis showed that 15-PGDH overexpression increased TAP63 expression, and 15-PGDH knockdown decreased it (Fig. 5). Consistent with the role of 15-keto-PGE₂ in 15-PGDH actions, overexpression of PGR-2 inhibited 15-PGDH-induced Smad-association with SARA; this effect was reversed by treatment with 15-keto-PGE₂ but not by other PPAR-γ ligands. We further observed that 15-keto-PGE₂ is the only PPAR-γ agonist that increases Smad2 association with TGFBR1. Taken together, these findings indicate that the 15-PGDH product 15-keto-PGE₂ disrupts the PPAR-γ-Smad2/3 binding complex, leading to increased Smad2/3 association with SARA and TGFBR1 and subsequent Smad2/3 phosphorylation and activation. This effect is unique for 15-keto-PGE₂, in contrast to the other PPAR-γ ligands.

Furthermore, we observed that TGF-β treatment increased the expression of 15-PGDH in three human cholangiocarcinoma cell lines (CCLP1, SG231, and HuCCT1) (see supporting information for Fig. 3). Our results suggest a positive feedback loop between TGF-β and 15-PGDH/15-keto-PGE₂ signaling pathways in cholangiocarcinoma cells. This finding is consistent with the previous reports that TGF-β induces 15-PGDH in human gastrointestinal and lung cancers (30, 31).

To further examine the effect of 15-PGDH on Smad2/3 activation, we performed immunofluorescence staining and Western blotting analysis to detect the phosphorylation of Smad2/3 in CCLP1 stable cell lines. As shown in Fig. 4A, Western blotting and immunofluorescence staining showed that the level of pSmad2/3 increased when 15-PGDH was overexpressed but decreased when 15-PGDH was knocked down. The effect of 15-PGDH on Smad2/3 phosphorylation was not through TGF-β production, as 15-PGDH overexpression or knockdown decreased when 15-PGDH was knocked down. The effect of 15-PGDH on Smad2/3 phosphorylation was not through TGF-β production, as 15-PGDH overexpression or knockdown decreased when 15-PGDH was knocked down. The effect of 15-PGDH on Smad2/3 phosphorylation was not through TGF-β production, as 15-PGDH overexpression or knockdown decreased when 15-PGDH was knocked down. The effect of 15-PGDH on Smad2/3 phosphorylation was not through TGF-β production, as 15-PGDH overexpression or knockdown decreased when 15-PGDH was knocked down. The effect of 15-PGDH on Smad2/3 phosphorylation was not through TGF-β production, as 15-PGDH overexpression or knockdown decreased when 15-PGDH was knocked down. The effect of 15-PGDH on Smad2/3 phosphorylation was not through TGF-β production, as 15-PGDH overexpression or knockdown decreased when 15-PGDH was knocked down. The effect of 15-PGDH on Smad2/3 phosphorylation was not through TGF-β production, as 15-PGDH overexpression or knockdown decreased when 15-PGDH was knocked down. The effect of 15-PGDH on Smad2/3 phosphorylation was not through TGF-β production, as 15-PGDH overexpression or knockdown decreased when 15-PGDH was knocked down. The effect of 15-PGDH on Smad2/3 phosphorylation was not through TGF-β production, as 15-PGDH overexpression or knockdown decreased when 15-PGDH was knocked down. The effect of 15-PGDH on Smad2/3 phosphorylation was not through TGF-β production, as 15-PGDH overexpression or knockdown decreased when 15-PGDH was knocked down. The effect of 15-PGDH on Smad2/3 phosphorylation was not through TGF-β production, as 15-PGDH overexpression or knockdown decreased when 15-PGDH was knocked down. The effect of 15-PGDH on Smad2/3 phosphorylation was not through TGF-β production, as 15-PGDH overexpression or knockdown decreased when 15-PGDH was knocked down.
TAP63 expression. The direct effect of 15-keto-PGE2 was confirmed by the observation that 15-keto-PGE2 treatment increased TAP63 expression. We observed that the expression of p53 was also regulated by 15-PGDH and 15-keto-PGE2 in a similar pattern. Maximal induction of TAP63 and p53 was observed when both 15-PGDH and PPAR-γ were overexpressed. Knockdown of either 15-PGDH or PPAR-γ led to reduced expression of TAP63 and p53. These findings provide novel evidence for induction of TAP63 and p53 by 15-PGDH-derived 15-keto-PGE2 through a PPAR-γ-dependent mechanism.

Given that pharmacological PPAR-γ ligands are known to induce the expression of p53 and its downstream genes p21WAF1/CIP1 and GADD45 in cholangiocarcinoma (37), we performed further experiments to determine whether 15-PGDH and 15-keto-PGE2 might influence p21WAF1/CIP1 and GADD45 expression. As shown in Fig. 5, the levels of p21WAF1/CIP1 and GADD45 were increased in cells with 15-PGDH overexpression but decreased in cells with 15-PGDH depletion. Accordingly, 15-keto-PGE2 treatment also induced the expression of p21WAF1/CIP1 and GADD45; this effect was abolished by the PPAR-γ antagonist GW9662. These findings suggest that 15-PGDH and 15-keto-PGE2 can activate p53 downstream genes in cholangiocarcinoma cells.

We observed that the reduction of pSmad2/3, TAP63, and p53 by 15-PGDH RNAi was partially reversed by 15-keto-PGE2 but not by other potential 15-PGDH metabolites, including 15-keto-PGF2α, 5-oxo-ETE, 12-oxo-ETE, 15-oxo-ETE, or 12-oxo-LTB4 (see supporting information for Fig. 5). LXA4 and LXB4 did not alter 15-PGDH-induced increase of pSmad2/3, TAP63, and p53. These results further support the role of 15-keto-PGE2 in mediating the effect of 15-PGDH in cholangiocarcinoma cells.

15-PGDH-derived 15-Keto-PGE2 Facilitates the Formation of TAP63, p53, and pSmad2/3 Ternary Complex That Binds to the TAP63 Promoter Consensus Sequence—As the expression of TAP63 is autoregulated in which TAP63 binds to its own promoter consensus sequence along with Smad2/3 and p53 (38, 39), we performed further experiments to address whether 15-PGDH and 15-keto-PGE2 might influence the binding of TAP63, p53, and pSmad2/3 to the TAP63 promoter consensus sequence. As shown in Fig. 6, the formation of the TAP63-pSmad2/3 ternary complex was increased in 15-PGDH-overexpressed cells but reduced in 15-PGDH knockdown cells. EMSA indicated that 15-PGDH overexpression increased the binding of TAP63, p53, and pSmad2/3 to the TAP63 promoter consensus sequence, whereas 15-PGDH knockdown decreased their interaction (Fig. 6, B–D). DNA pulldown analysis revealed...
that 15-PGDH overexpression increased the amount of TAP63, p53, and pSmad2/3 bound to the TAP63 promoter consensus sequence, whereas 15-PGDH knockdown decreased their association (Fig. 6, A and B). No TAP63 binding to its promoter was detected when both 15-PGDH and Smad2/3 were knocked down. However, overexpression of Smad2/3 enhanced 15-PGDH-induced TAP63 binding to its promoter. The 15-PGDH-induced binding of TAP63, p53, and pSmad2 to the TAP63 promoter consensus sequence was inhibited by pretreatment with the anti-TGF-β antibody (Fig. 7C), by overexpression of PGR-2 (Fig. 7D), or by pretreatment with the PPAR-γ antagonist GW9662 (Fig. 7E). Consistent with these observations, 15-keto-PGE₂ treatment also increased the binding of TAP63, p53, and pSmad2 to the TAP63 promoter consensus sequence, and this effect was inhibited by pretreatment with the anti-TGF-β antibody (Fig. 7F) or by the PPAR-γ antagonist
These observations demonstrate the involvement of PPAR-γ in 15-PGDH/15-keto-PGE₂-induced TAP63 autotranscription. We further performed chromatin immunoprecipitation assays to determine the binding of TAP63, p53, and pSmad2/3 to the TAP63 promoter consensus site. As shown in Fig. 8A, the GW9662 (Fig. 7G). These observations demonstrate the involvement of PPAR-γ in 15-PGDH/15-keto-PGE₂-induced TAP63 autotranscription.
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binding of TAP63, p53, and pSmad2/3 to the TAP63 promoter consensus site was increased in 15-PGDH-overexpressed cells but decreased in 15-PGDH knockdown cells. Knockdown of PPAR-γ or treatment with the PPAR-γ antagonist GW9662 inhibited 15-PGDH- and 15-keto-PGE2-induced TAP63 binding to its promoter (Fig. B8). The 15-PGDH RNAi-induced reduction of TAP63 binding to the TAP63 promoter was partially reversed by 15-keto-PGE2 but not by 15-keto-PGF2α, 5-oxo-ETE, 12-oxo-ETE, 15-oxo-ETE, or 12-oxo-LTB4 (see supporting information for Fig. B8). Moreover, the 15-PGDH-induced TAP63 association with the TAP63 promoter was not altered by LXA4 and LXB4 treatment. These results further support the role of 15-keto-PGE2 in mediating 15-PGDH-induced TAP63 binding to the TAP63 promoter in cholangiocarcinoma cells.

We observed that PPAR-γ knockdown prevented 15-PGDH-induced pSmad association with the TAP63 promoter (Fig. B8). Accordingly, knockdown of Smad2/3 prevented 15-PGDH-induced TAP63 binding to its promoter consensus site (Fig. 8C). It is of note that TAP63 DNA binding was completely eliminated when both 15-PGDH and Smad2/3 were knocked down. 15-PGDH Enhances TAP63 Promoter Activity—Luciferase reporter activity assays showed that the TAP63 promoter reporter activity was higher in cells with 15-PGDH overexpression but decreased in cells with 15-PGDH depletion (Fig. 8B). The effect of 15-PGDH on TAP63 promoter reporter activity was no longer observed when PGR-2 was knocked down. Knockdown of Smad2/3 or p53 or treatment with the PPAR-γ antagonist GW9662 abolished 15-PGDH overexpression plus TAP63 promoter activity. These results support an involvement of PPAR-γ in TAP63 promoter induction. However, 15-keto-PGE2-mediated 15-PGDH-mediated TAP63 expression was not altered in cells with 15-PGDH knockdown plus PPAR-γ knockdown. TAP63 knockdown prevented 15-PGDH overexpression plus TAP63 promoter luciferase activity, whereas TAP63 knockdown prevented 15-PGDH overexpression plus inhibition of tumor cell proliferation, colony formation, and migration (Fig. 9A). However, TAP63 overexpression prevented the increase of cell proliferation, colony formation, and migration induced by 15-PGDH depletion (Fig. 9B). These results demonstrate that 15-PGDH signaling inhibits tumor growth at least in part through TAP63 (illustrated in Fig. 10).

DISCUSSION

To date, the action of 15-PGDH is largely attributable to its conversion of biologically active PGE2, with its enzymatic products being considered as largely inactive. This study provides novel evidence for an active role of 15-keto-PGE2 in 15-PGDH-mediated anti-tumor effect. We have shown that the 15-PGDH-derived 15-keto-PGE2 is an endogenous PPAR-γ ligand that causes PPAR-γ dissociation from Smad2/3, allowing Smad2/3 binding to TGFBR1 and SARA and subsequent Smad2/3 phosphorylation and activation. By using chromatin immunoprecipitation assay as well as immunoprecipitation and Western blotting analysis, we were able to detect the PPAR-γ and Smad2/3 binding complex in cell lysates and nuclear extracts of cells with 15-keto-PGE2. Smad2/3 is associated with TGFBR1 and SARA in cells, so PPAR-γ leads to dissociation of Smad2/3 association with TGFBR1 and SARA. Knocking Smad2/3 association with TGFBR1 and SARA results in subsequent Smad2/3 phosphorylation and transcriptional activity.

Our data show that 15-PGDH/15-keto-PGE2-induced Smad2/3 activation is not mediated through TGF-β production, given that the level of TGF-β was not altered in cells with 15-PGDH overexpression or knockdown or with 15-keto-PGE2 treatment. However, we observed that 15-PGDH-mediated Smad2/3 phosphorylation and transcriptional activity was influenced by TAP63 (illustrated in Fig. 10).
enced by TGF-β signaling status. Although TGF-β treatment enhanced 15-PGDH-mediated increase in Smad2/3 phosphorylation and reporter activity, inhibition of TGF-β prevented 15-PGDH-induced Smad2/3 phosphorylation and activation. These findings suggest a novel interplay between 15-PGDH-derived 15-keto-PGE₂ and TGF-β for regulation of Smad2/3 activation.

We observed that the 15-PGDH/15-keto-PGE₂-induced Smad2/3 phosphorylation facilitated the formation of the pSmad2/3, TAP63, and p53 ternary complex, which binds to the TAP63 promoter and induces the transcription of the TAP63 gene. These findings are consistent with the observations that TAP63 is able to bind to its own promoter (along with Smad2/3 and p53) to induce its autotranscription (35, 38, 42–50).

The observations that knockdown of PPAR-γ, Smad2/3, and p53 prevented 15-PGDH/15-keto-PGE₂-induced TAP63 tran-

FIGURE 9. Effect of TAP63 on 15-PGDH-regulated tumor cell growth, colony formation, and cell migration. A, knockdown of TAP63 prevents 15-PGDH-induced inhibition of cell growth, colony formation, and cell migration. Panels a, Western blotting for 15-PGDH and TAP63 in CCLP1 stable cell lines. Ctrl, control. Panel b, WST cell proliferation assay. Each sample was assayed in triplicates for 6 consecutive days, and the data represent means ± S.E. from three independent experiments (***, p < 0.01; *, p < 0.05). Panel c, soft agar colony formation assay. The cells were incubated at 37 °C for 21 days (the culture medium was changed 1–2 times per week). The culture plates were stained with 0.5 ml of 0.005% Crystal Violet (Sigma) for at least 1 h, and the numbers of colonies were counted. The data are expressed as means ± S.E. from three independent experiments (**, p < 0.01). Panel d, wound healing assay at 0 and 24 h in indicated CCLP1 stable cell lines. The data are expressed as means ± S.E. from three independent experiments (**, p < 0.01). B, overexpression of TAP63 prevents 15-PGDH knockdown effects. Panels a, Western blotting for 15-PGDH and TAP63 in CCLP1 cells stably transfected with 15-PGDH RNAi vector and/or TAP63 expression vector. Panel b, WST cell proliferation assay. Each sample was assayed in triplicates for 6 consecutive days, and the data represent means ± S.E. from three independent experiments (**, p < 0.01; *, p < 0.05). Panel c, soft agar colony formation assay. The cells were incubated at 37 °C for 21 days (the culture medium was changed 1–2 times per week). The culture plates were stained with 0.5 ml of 0.005% crystal violet (Sigma) for at least 1 h, and the numbers of colonies were counted. The data are expressed as means ± S.E. from three independent experiment (**, p < 0.01). Panel d, wound healing assay at 0 and 24 h in indicated CCLP1 stable cell lines. The data are expressed as means ± S.E. from three independent experiment (**, p < 0.01).
15-keto-PGE₂ in 15-PGDH-mediated inhibition of tumor cell growth. This assertion is based on several observations. 1) 15-Keto-PGE₂ treatment and 15-PGDH overexpression were found to have comparable effects on PPAR-γ/H₉₂₅₃ activation and Smad2/3 phosphorylation/activation. 2) 15-Keto-PGE₂ treatment and 15-PGDH overexpression had comparable effects on Smad2/3 association with TAP63 and p53, their DNA binding ability, and induction of TAP63 expression. 3) 15-PGDH-induced Smad2/3 association with TAP63 and p53, their DNA binding ability, and induction TAP63 expression were all inhibited by PGR-2. 4) The effects induced by 15-PGDH depletion (e.g. binding of PPAR-γ to Smad2, dissociation of Smad2 from SARA and TGFBRI, inhibition of Smad2/3 activity, and inhibition of TAP63 expression) were partially reversed by 15-keto-PGE₂ but not by the other 15-PGDH metabolites. 6) Two other 15-PGDH substrates (LXA₄ and LXB₄) did not significantly alter Smad2/3 activation and TAP63 expression in 15-PGDH-overexpressed cells. All of these observations support an active role of 15-keto-PGE₂ in 15-PGDH-mediated inhibition of cholangiocarcinoma cell growth. In our system, we observed that 15-PGDH overexpression led to a 2-fold reduction of PGE₂ and a 5-fold increase of 15-keto-PGE₂. The difference in the fold changes may relate to the low basal level of 15-keto-PGE₂ in the cells, although it is possible that other factors that regulate PGE₂ and 15-keto-PGE₂ biosynthesis and degradation may also be implicated. Given the relatively high concentrations of 15-PGDH metabolites utilized in the experiments in vitro (at micromolar concentrations), the physiological implication of these findings in vivo remains to be further defined.

In this study, the effect of 15-keto-PGE₂ was compared with several other PPAR-γ agonists, including ciglitazone, rosiglitazone, and 15-d-PGJ₂. We found that only 15-keto-PGE₂ was able to cause PPAR-γ dissociation from Smad2/3 (thus removing PPAR-γ inhibition on Smad2/3), whereas ciglitazone, rosiglitazone, and 15-d-PGJ₂ (15-deoxy-Δ₁₂,₁₄-prostaglandin J₂) were unable to dissociate PPAR-γ from the Smad2/3 binding complex, despite their ability to enhance PPAR-γ transcription activity in the PPRE reporter activity assays. These findings suggest differential regulation of Smad2/3 by PPAR-γ via specific ligands. Although 15-keto-PGE₂ enhances PPAR-γ-mediated PPRE reporter activity (as an agonist), our data showed that 15-keto-PGE₂ counteracted PPAR-γ action in the regulation of Smad2/3 activity (as an antagonist). Thus, 15-keto-PGE₂ differentially regulates PPAR-γ activity depending on specific downstream targets. To our knowledge, this is the first study describing activation of Smad2/3 by a PPAR-γ ligand. The importance of 15-keto-PGE₂ in regulation of Smad2/3 is underscored by the diverse functions of TGFB-β and Smad signaling and by the fact that 15-keto-PGE₂ is an endogenous PPAR-γ ligand produced by 15-PGDH, an enzyme that is abundantly expressed in a vari-
15-keto-PGE2 and other PPAR-γ ligands, showed no significant effect on Smad2/3 activation. The exact mechanism for the different actions between 15-keto-PGE2 and other PPAR-γ ligands is not clear and remains speculative. It is possible that this process might involve conformational change of the Smad2/3-binding site of the PPAR-γ triggered by 15-keto-PGE2, but not thiazolidinediones. However, it is also possible that some other yet to be identified factors might be involved.

In summary, this study depicts a novel 15-PGDH-mediated 15-keto-PGE2 signaling cascade that interacts with PPAR-γ, Smad2/3, and TAP63 to inhibit cholangiocarcinoma cell growth. 15-Keto-PGE2 is identified as a natural ligand that binds to PPAR-γ and causes its dissociation from Smad2/3, which allows subsequent Smad2/3 phosphorylation and activation of TAP63. Given that 15-PGDH converts the pro-inflammatory and pro-tumorigenic PGE2 to the anti-inflammatory and tumor-suppressive 15-keto-PGE2, induction of 15-PGDH expression or administration of 15-keto-PGE2 may represent a promising anti-cancer therapeutic strategy that warrants further investigation.

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