miR-21 Targets 15-PGDH and Promotes Cholangiocarcinoma Growth

Lu Lu, Kathleen Byrnes, Chang Han, Ying Wang, and Tong Wu

Abstract

miRNAs are a group of small, noncoding RNAs that modulate the translation of genes by binding to specific target sites in the target mRNA. This study investigated the biologic function and molecular mechanism of miR-21 in human cholangiocarcinoma. *In situ* hybridization analysis of human cholangiocarcinoma specimens showed increased miR-21 in cholangiocarcinoma tissue compared with the noncancerous biliary epithelium. Lentiviral transduction of miR-21 enhanced human cholangiocarcinoma cell growth and clonogenic efficiency *in vitro*, whereas inhibition of miR-21 decreased these parameters. Overexpression of miR-21 also promoted cholangiocarcinoma growth using an *in vivo* xenograft model system. The NAD⁺-linked 15-hydroxyprostaglandin dehydrogenase (15-PGDH/HPGD), a key enzyme that converts the protumorigenic prostaglandin E2 (PGE₂) to its biologically inactive metabolite, was identified as a direct target of miR-21 in cholangiocarcinoma cells. In parallel, cyclooxygenase-2 (COX2) overexpression and PGE₂ treatment increased miR-21 levels and enhanced miR-21 promoter activity in human cholangiocarcinoma cells.

Implications: Cholangiocarcinogenesis and tumor progression are regulated by a novel interplay between COX-2/PGE₂ and miR-21 signaling, which converges at 15-PGDH. *Mol Cancer Res;* 12(6); 1–11. ©2014 AACR

Introduction

Cyclooxygenase (COX)-2–derived prostaglandin E₂ (PGE₂) is the most abundant prostaglandin in inflammation and various human malignancies (1, 2). The tumorigenic actions of PGE₂ are attributable to its modulation of cell proliferation, survival, migration, and invasion. The level of PGE₂ in the inflammatory and tumor microenvironments is controlled by the status of PGE₂ synthesis and degradation. Whereas the cyclooxygenases are rate-limiting key enzymes that control PGE₂ biosynthesis, the NAD⁺-linked 15-hydroxyprostaglandin dehydrogenase (15-PGDH) is a key enzyme that converts PGE₂ to its biologically inactive metabolite, 13,14-dihydro-15-keto-PGE₂, thus leading to PGE₂ inactivation (1, 3). Consistent with the anti-inflammatory and antitumorigenic effect of 15-PGDH, downregulation of 15-PGDH expression has been observed in several human cancers, including colon, gastric, and breast cancer (4–12). It is conceivable that reduced 15-PGDH may lead to PGE₂ accumulation, which sustains carcinogenesis and tumor progression. However, the exact mechanisms for 15-PGDH downregulation in carcinogenesis have not been fully elucidated.

Cholangiocarcinoma is a highly malignant cancer of the biliary tract. It often arises from background conditions that cause long-standing inflammation, injury, and reparative biliary epithelial cell proliferation (13, 14). Cholangiocarcinogenesis involves a series of sequential events including chronic inflammation, cholangiocyte proliferation, dysplasia, and ultimately malignant transformation (15). Studies have documented upregulation and activation of COX-2/PGE₂ signaling in human cholangiocarcinoma and demonstrated an important role of this cascade in regulating cholangiocarcinoma cell growth and apoptosis (16–20).

miRNAs are a group of small, noncoding RNAs that target specific sites in mRNAs, leading to translational inhibition or cleavage of target mRNAs. Recent studies have pointed toward a potentially important role of miRNAs in hepatobiliary carcinogenesis (21), although the functions and mechanisms of miRNAs in hepatobiliary cancers remain to be further defined. In the current study, we have identified 15-PGDH as a novel target of miR-21 in cholangiocarcinoma and our results disclose a novel feed-forward loop between the COX-2/PGE₂ and miR-21 signaling pathways, which is crucial in cholangiocarcinogenesis and tumor progression.

Materials and Methods

Materials

Dulbecco’s modified minimum essential medium (DMEM) and fetal bovine serum (FBS) were purchased...
from Sigma. Opti-MEM reduced serum medium and Lipofectamine 2000 reagent were purchased from Invitrogen. miR-21 expression and scramble control lentivirus particles with enhanced green fluorescent protein (eGFP) were purchased from GeneCopoeia. Rabbit polyclonal Anti-15-PGDH antibody was purchased from Cayman Chemical. Mouse monoclonal anti-β-actin antibody was from Sigma; anti-COX-2 antibody was from Cayman Chemical. Synthetic miR-21 mimic and inhibitor RNAs were purchased from Qiagen. 15-PGDH siRNA pool was purchased from Dharmacon; COX-2 siRNAs were from OriGene. PGE2 and the COX-2 inhibitor NS398 were purchased from Calbiochem.

**In situ hybridization**

The miRCURY LNA microRNA ISH Optimization Kit (Exiqon) was used for *in situ* hybridization of mature miR-21 in the formalin-fixed and paraffin-embedded tissue specimens surgically resected from patients with cholangiocarcinoma according to the approval of the institutional review board. Briefly, the tissue sections were deparaffinized and treated with proteinase K for 10 minutes at 37°C. After dehydration, slides were incubated with miR-21 locked nucleic acid probe for 60 minutes at 50°C, followed by stringent washes with 5×, 1×, and 0.2× saline sodium citrate buffers at 50°C; DIG blocking reagent (Roche) in maleic acid buffer containing 2% sheep anti-digoxigenin for 60 minutes at room temperature. The slides were developed by incubating with 4-nitro-blue tetrazolium and 5-brom-4-chloro-3-indolylphosphate substrate (Roche) for 2 hours at room temperature. The slides were developed by incubating with 4-nitro-blue tetrazolium and 5-brom-4-chloro-3-indolylphosphate substrate (Roche) for 2 hours at 30°C, followed by nuclear fast red counterstain (Vector Laboratories) for 1 minute at room temperature. Scrambled probe and U6 small nuclear RNA-specific probe were used as system control. The same procedure was used to analyze the cholangiocarcinoma tissue arrays (obtained from BioCat GmbH; 44 cases of cholangiocarcinoma and 4 cases of nonneoplastic tissues). The miR-21 staining was evaluated using a 0 to 4+ semiquantitative scale: 0, completely negative; 1+, weak cytoplasmic staining; 2+, moderate cytoplasmic staining of >50% of the cells; 3+, strong cytoplasmic staining of >50% of the cells; and 4+, extremely strong cytoplasmic staining of >50% of the cells.

**Cell culture**

Four human cholangiocarcinoma cell lines, including CCLP1, SG231, HuCCT1, TFK1, and one immortalized nontumorigenic human cholangiocyte cell line (H69) were used in this study (HuCCT1 and TFK1 cells were obtained from the Japanese Cancer Research Resources Bank, Osaka, Japan; H69 cells were kindly provided by Dr. Gregory J. Gores at the Mayo Clinic College of Medicine, Rochester, MN). The CCLP1, SG231, and HuCCT1 cells were transduced with miR-21–expressing lentiviral particles or scramble control overnight. After 72 hours, the medium was replaced with fresh medium containing puromycin for selection and the subsequent cultures were continued in the presence of 1 μg/mL puromycin. After the cells reached confluence, total RNA was extracted and quantitative reverse transcription PCR (qRT-PCR) was performed to verify the levels of miR-21.

**Stable transfection**

The CCLP1, SG231, and TFK1 cells were transduced with miR-21–expressing lentiviral particles or scramble control overnight. After 72 hours, the medium was replaced with fresh medium containing puromycin for selection and the subsequent cultures were continued in the presence of 1 μg/mL puromycin. After the cells reached confluence, total RNA was extracted and quantitative reverse transcription PCR (qRT-PCR) was performed to verify the levels of miR-21.

**Cell proliferation assay**

The growth of human cholangiocarcinoma cells was measured by using the WST-1 reagent from Roche. Briefly, the cells were plated in 96-well plates and cultured for 24 hours to allow attachment. Then the medium was replaced with fresh medium containing 1% FBS or specific reagents as indicated and the cultures were continued for 1 to 5 days. For cell growth measurement, WST-1 reagent was added to each well and the cells were incubated at 37°C for 1 hour; absorbance at 450 nm was measured using an ELISA plate reader.

**Colony-formation assay**

For soft agar colony-formation assay, the cell culture medium and the 5% agar solution were warmed to 40°C in a water bath. Then, the cell culture medium and the agar solution were mixed in the proportion of 9:1 and added to 6-well plates at 1 mL of mixture per well. The plates were placed under room temperature until solidification. For preparation of top agar, the cell suspensions and the 3.5% agar solution were warmed to 40°C and then mixed in the proportion of 9:1. One milliliter of mixture containing 500 cells was added on the top of base agar in each well of 6-well plates. The cells were cultured for 15 days and the colonies were counted under a microscope. For plate colony-formation assay, 1 × 105 cells were cultured in 10-cm dishes for 10 days to allow colony formation. Colonies were fixed in 100% methanol and stained with 0.1% crystal violet solution (Amresco) and counted.

**Cell invasion assay**

The cell invasion assay was performed in Matrigel-coated Transwell chambers (BD Biosciences Discovery Labware). Five hundred microliters of cell suspension (5 × 104 cells/mL) was added to each of the upper chambers. Cell culture medium containing 5% FBS was added to each of the lower chambers as chemoattractant. After incubating cells at 37°C for 24 hours, the cells on the upper surface of the membrane were removed with a cotton swab. The invading cells on the lower surface of the membrane were fixed in 100% methanol and stained with 0.05% crystal violet solution. Then, the invading
cells were counted under a microscope and for each chamber eight fields were randomly selected for counting.

**Transient transfection**

The cells were transfected with the 15-PGDH expression plasmid (OriGene; pCMV6-AC-GFP as control plasmid) or the COX-2 expression plasmid (pDNA3 as control plasmid) using Lipofectamine2000 reagent according to the manufacturer’s instructions. After transfection at indicated time periods, the transfection reagents were removed, and the cells were used for further experiments. Transfection of miR-21 inhibitor, 15-PGDH and COX-2 siRNAs was performed by using Lipofectamine 2000 reagent. The levels of 15-PGDH and COX-2 proteins in the transfected cells were verified by Western blotting.

**qRT-PCR**

Cellular total RNA was isolated using the TRIzol reagent (Invitrogen). For quantification of mature miR-21 levels, reverse transcription was performed by using the miScript Reverse Transcription Kit (Qiagen). The mature form of miR-21 was amplified by using the miScript SYBR Green PCR Kit (Qiagen) on Bio-Rad C1000 Thermal Cycler. U6 small nuclear 2 (U6B) was used as the internal control. The U6B (Hs_RNU6B_2) and miR-21 (Hs_miR-21_2) primers were purchased from Qiagen. For quantitation of miR-21 levels, reverse transcription was performed by using the miScript SYBR Green PCR Kit (Invitrogen). For quantitation of mature miR-21 levels, miR-21 was amplified by using the miScript SYBR Green PCR Kit (Qiagen) by using the Dual-Luciferase Reporter Assay System (Promega). The Renilla luciferase activity was used as internal control and the ratio of Firefly to Renilla activity was calculated for normalization. For miR-21 promoter luciferase reporter activity measurement, the cells were transfected with 1.6 µg pEZX-PG04-miR-21 promoter plasmid (Genecopoeia). After 24 hours, the cells were treated with specific reagents as indicated in the figure legends. The cell culture medium was collected after the treatment and luciferase activity was measured in a FLUOstar Omega microplate reader (BMG LABTECH) by using the Secrete-Pair Dual Luminescence Assay Kit (Genecopoeia). The secreted alkaline phosphatase (SEAP) luminescence was used as internal control and the ratio of *Gaussia* luciferase (Gluc) activity to SEAP was calculated for normalization.

**PGE₃ enzyme immunoassay**

The cells were incubated overnight at 37°C in serum-free medium. The cell culture medium was then collected and centrifuged for 10 minutes at 12,000 × g to remove the floating cells and cell debris. The concentration of PGE₃ in the culture medium was measured by using a specific PGE₃ EIA kit (Cayman Chemical).

**Tumor xenograft studies**

Four-week-old male nonobese diabetic (NOD) CB17-prkdc/scid (severely combined immunodeficient) mice were purchased from The Jackson Laboratory. For each mouse, 3 × 10⁶ miR-21 overexpressed or control CCLP1 cells in 100 µL PBS were subcutaneously inoculated in the left and right flank areas (N = 6). The mice were observed over 16 days for tumor formation. Upon sacrifice, the tumors were recovered and the wet weight of each tumor was measured. Portion of each tumor was used for Hema-toxilin and eosin (H&E) staining, Western blotting, and qRT-PCR analysis.

**Statistical analysis**

The *in vitro* data were presented as mean ± standard deviation (SD) or standard error (SE) from a minimum of three replicates. Differences between groups were evaluated...
by SPSS 13.0 statistical software with the Student $t$ test, one-
way analysis of variance (ANOVA), repeated measures ANOVA or Wilcoxon signed ranks test. A $P$ value less than 0.05 was considered as statistically significant.

**Results**

**The expression of miR-21 is increased in human cholangiocarcinoma tissues**

We performed *in situ* hybridization to determine the expression of miR-21 in human cholangiocarcinoma tissues. The tissue specimens analyzed include archived formalin-fixed paraffin-embedded human cholangiocarcinoma tissues from 9 patients who underwent surgical resections and 44 cases of cholangiocarcinoma tissue arrays. In the archived cholangiocarcinoma tissues, miR-21 was expressed in 8 of 9 cases (88.9%). In nonneoplastic peribiliary glands, miR-21 was expressed in only 1 of 9 cases (11.1%). The staining intensity of miR-21 was significantly higher in cholangiocarcinoma cells compared with the matched nonneoplastic bile duct epithelial cells ($P < 0.01$; Fig. 1A and B). In the tissue array analysis, miR-21 was expressed in 24 of 44 cholangiocarcinoma cases at different levels (47.7%, 1+; 4.5%, 2+; 2.3%, 4), but not in the 4 nonneoplastic cases (Fig. 1C). These findings confirm that the expression of miR-21 is upregulated in human cholangiocarcinoma cells.

**miR-21 enhances cholangiocarcinoma cell growth and invasion *in vitro***

To further determine the biologic role and molecular mechanism of miR-21 in cholangiocarcinoma, we generated cholangiocarcinoma cell lines (CCLP1, SG231, and TFK1) with stable overexpression of miR-21. These cells were produced by infecting the parental cell lines with lentivirus particles carrying the miR-21 and eGFP genes under the control of the same promoter; the stably transduced cells were selected in the presence of 1 μg/mL puromycin. Satisfactory infection efficiency was confirmed by high eGFP expression under fluorescence microscopy and by qRT-PCR for miR-21. As shown in Fig. 2A, the selected miR-21 lentivirus–infected cell lines showed increased miR-21 expression and increased cell growth compared with the corresponding lentivirus control cells. In addition, miR-21 overexpression increased colonogenic efficiency in soft agar (Supplementary Fig. S1) and enhanced tumor cell invasion (Supplementary Fig. S2). We next used a parallel approach in which cholangiocarcinoma cells were transfected with the miR-21–specific inhibitor or the scramble control. HuCCT1 cell line was used for this purpose, as this cell line...
Figure 2. miR-21 regulates cholangiocarcinoma cell growth in vitro. A, top, cell growth curves of human cholangiocarcinoma cells (CCLP1, SG231, and TFK1) stably transduced with miR-21 lentivirus (indicated as L/miR-21) or scramble control (indicated as L/control; N = 4; P < 0.001, repeated measures ANOVA). Bottom, miR-21 levels were measured by qRT-PCR. The data are presented as mean ± SD (N = 3; *** P < 0.001; ** P < 0.01; * P < 0.05; Student t test). B, the levels of miR-21 in different human cholangiocarcinoma cell lines (CCLP1, SG231, TFK1, and HuCCT1). C, the effect of miR-21 inhibition on cell growth in HuCCT1 cells. The cells were transfected with miR-21 inhibitor (Anti/miR-21) or scramble control (Anti/Control) and WST-1 assay was performed to determine cell growth (the data are presented as mean ± SE; N = 6; P < 0.01, repeated measures ANOVA). D, the effect of miR-21 inhibition on colony formation in HuCCT1 cells transfected with miR-21 inhibitor (Anti/miR-21) or scramble control (Anti/Control). The number of colonies was counted after 10 days. Left, representative images showing colonies formed in cell culture dishes. Right, average colony forming efficiency (the data is presented as mean ± SD; N = 3; ** P < 0.01).
Figure 3. 15-PGDH is a direct target of miR-21 in cholangiocarcinoma cells. A, putative miR-21 binding site in the 3'-UTR of 15-PGDH mRNA. A mutant 15-PGDH 3'-UTR with one-nucleotide deletion was constructed as indicated. B, Western blotting for 15-PGDH in an immortalized human cholangiocyte cell line (H69) and four human cholangiocarcinoma cell lines (CCLP1, SG231, HuCCT1, and TFK1). C, qRT-PCR analysis for 15-PGDH mRNA in miR-21-overexpressed and control cells. The data are presented as mean ± SD (N=3; **, P<0.01; *** P<0.001; Student t test). D, 15-PGDH protein levels in miR-21-overexpressed and control cells as determined by Western blotting. E, CCLP1 and SG231 cells were transfected with miR-21 mimic or scramble control together with a wild-type 15-PGDH 3'-UTR reporter plasmid, or a mutant 15-PGDH 3'-UTR reporter plasmid. The luciferase activity was analyzed 48 hours after transfection. The data are presented as mean ± SE (**, P<0.001, Student t test). F, equal number of miR-21-overexpressed and control cells were plated in 6-well plates and cultured in serum-free medium overnight. The cell culture medium was collected to measure PGE2 concentration. The data are presented as mean ± SD (*, P<0.05; ***, P<0.001, Student t test).
line expresses relatively high basal level of miR-21 among the four cholangiocarcinoma cell lines used in this study (Fig. 2B). We observed that inhibition of miR-21 significantly decreased cell growth ($P < 0.01$) and reduced clonogenic efficiency ($P < 0.01$; Fig. 2C and D). These results demonstrate that miR-21 promotes anchorage-dependent cell growth and anchorage-independent colony formation and enhances cell invasion, indicating protumorigenic effect of miR-21 in cholangiocarcinoma.

**miR-21 targets the tumor suppressor 15-PGDH**

Next, we sought to identify the direct target of miR-21 in human cholangiocarcinoma cells. By using the microRNA.org resource, we found that the 15-PGDH mRNA harbors the miR-21 binding site in the 3'-UTR (illustrated in Fig. 3A). 15-PGDH is a key enzyme that degrades the proinflammatory and protumorigenic PGE2 and has been shown to inhibit the growth of several human cancers (5, 6), although the role of 15-PGDH in human cholangiocarcinogenesis has not been previously reported. In this study, we observed decreased 15-PGDH protein expression in human cholangiocarcinoma cells (CCLP1, SG231, HuCCT1, and TFK1) compared with the immortalized nontumorigenic human cholangiocyte cell line, H69 (Fig. 3B). qRT-PCR and Western blotting results showed that both 15-PGDH mRNA and protein levels were decreased in cells with stable overexpression of miR-21 (Fig. 3C and D). Transient transfection of CCLP1 and SG231 cells with miR-21 mimic also decreased 15-PGDH protein level (data not shown). On the other hand, transfection of SG231 cells with anti-miR-21 increased 15-PGDH mRNA and protein levels (Supplementary Fig. S3). To document the role of the putative miR-21 binding site for regulation of 15-PGDH in cholangiocarcinoma cells, we cotransfected miR-21 mimic with 15-PGDH wild-type or mutant 3'-UTR reporter plasmid. As shown in Fig. 3E, miR-21 mimic decreased 15-PGDH 3'-UTR luciferase reporter activity and this effect was abolished when the one nucleotide in the miR-21 seed binding site was deleted. Given that 15-PGDH is a key enzyme involved in PGE2 inactivation, we further measured the level of PGE2 and observed that miR-21 overexpression increased the accumulation of PGE2 in CCLP1 and SG231 cells (Fig. 3F). As COX-2 is a rate-limiting key enzyme that mediates PGE2 synthesis, we also examined the level of COX-2.
protein and found that miR-21 did not alter the expression of COX-2 (data not shown). Our results suggest that miR-21–induced PGE2 increase in cholangiocarcinoma cells is mediated predominantly through inhibition of 15-PGDH and blockade of PGE2 degradation. The role of 15-PGDH for inhibition of cholangiocarcinoma growth is supported by the observation that 15-PGDH overexpression inhibited CCLP1 cell growth (Fig. 4A), whereas 15-PGDH siRNAs accelerated tumor cell growth (Fig. 4B).

miR-21 promotes cholangiocarcinoma growth in tumor xenograft model

To investigate whether miR-21 promotes cholangiocarcinoma growth in vivo, miR-21–overexpressed and control CCLP1 cells were subcutaneously inoculated into the flank areas of SCID mice. As shown in Fig. 5A and B, miR-21–overexpressed tumors were larger in size and had higher tumor weight compared with control (0.72 ± 0.10 g vs. 0.37 ± 0.18 g; *P < 0.05). qRT-PCR result confirmed elevated miR-21 levels in miR-21–overexpressed tumors (Fig. 5C). Consistent with the in vitro results, the levels of 15-PGDH mRNA and protein were also decreased in miR-21–overexpressed tumors (Fig. 5D and E). Thus, miR-21 reduces 15-PGDH level and enhances cholangiocarcinoma growth, both in vitro and in vivo.

PGE2 upregulates miR-21 level and increases miR-21 transcription

We performed further experiments to determine whether miR-21 might be regulated by the COX-2/PGE2 signaling cascade. As shown in Fig. 6A, CCLP1 and SG231 cells transfected with the COX-2 expression plasmid showed increased PGE2 production and increased miR-21 level. COX-2 overexpression also increased miR-21 level.

![Figure 5. miR-21 promotes cholangiocarcinoma growth in vivo. A and B, the gross image and weight of xenograft tumors recovered from the 6 SCID mice. *P < 0.05 (paired t test). C and D, miR-21 and 15-PGDH mRNA levels in xenograft tumor tissues were determined by qRT-PCR. The data are presented as mean ± SD (**, *P < 0.01; ***P < 0.001, paired t test). E, 15-PGDH protein levels in xenograft tumor tissues were determined by Western blotting.](image-url)
in H69 cells (Supplementary Fig. S4). Accordingly, treatment of CCLP1 and SG231 cells with PGE2 also increased miR-21 expression (Fig. 6B). Furthermore, PGE2 treatment also increased miR-21 promoter luciferase reporter activity (Supplementary Fig. S5). Given that the HuCCT1 cell line expresses highest basal level of COX-2 protein among

Figure 6. COX-2 and PGE2 signaling enhances miR-21 expression in cholangiocarcinoma cells. A, the effect of COX-2 overexpression on miR-21 level. Top, CCLP1 and SG231 cells were transfected with COX-2 expression plasmid or control PCDNA3 vector. 48 hours after transfection, total cellular RNA was isolated and miR-21 level was determined by qRT-PCR. The expression of COX-2 was confirmed by Western blotting (Middle). The level of PGE2 released from the transfected cells is shown at the bottom. The results were obtained from three experiments (\(P < 0.05; \quad \star; \quad P < 0.01; \quad \star\star; \quad P < 0.001\), Student t test). B, the effect of PGE2 treatment on miR-21 level. CCLP1 and SG231 cells were treated with PGE2 at indicated concentrations for 24 hours. Total cellular RNA was isolated and miR-21 level was determined by qRT-PCR. The results were obtained from three individual experiments (left, \(P < 0.001\); right, \(P < 0.05\), one-way ANOVA). C, COX-2 siRNAs decreased miR-21 expression in HuCCT1 cells. The cells were transfected with two individual COX-2 siRNAs. 48 hours after transfection, total cellular RNA was isolated and miR-21 level was determined by qRT-PCR (\(N = 3; \quad P < 0.001\), one-way ANOVA). Decreased COX-2 protein levels in the cells transfected with COX-2 siRNAs were confirmed by Western blotting. D, COX-2 siRNAs decreased miR-21 promoter luciferase reporter activity in HuCCT1 cells. The cells were transfected with miR-21 promoter reporter plasmid. 24 hours after transfection, the cells were transfected with two individual COX-2 siRNAs. 72 hours after the siRNA transfection, the cell culture medium was obtained for analysis of the luciferase reporter activity (\(N = 3; \quad P < 0.01\), one-way ANOVA).
the four cholangiocarcinoma cell lines used in this study, the HuCCT1 cells were selected for COX-2 inhibition studies. As shown in Fig. 6C and D, COX-2 siRNAs significantly decreased miR-21 expression as well as miR-21 promoter luciferase reporter activity in HuCCT1 cells. Moreover, inhibition of COX-2 by its pharmacologic inhibitor, NS-398, also significantly decreased miR-21 level in HuCCT1 cells (Supplementary Fig. S6). These data suggest that the COX-2-derived PGE2 upregulates miR-21 expression in cholangiocarcinoma cells and the effect is mediated at least in part through induction of miR-21 gene transcription.

Discussion

The current study identifies 15-PGDH as a novel target of miR-21 in cholangiocarcinoma. Our results disclose a novel feed-forward loop between the COX-2/PGE2 and miR-21 signaling pathways via 15-PGDH, which is crucial in cholangiocarcinogenesis and tumor progression (illustrated in Fig. 7). These findings are noteworthy as 15-PGDH is an important tumor suppressor that antagonizes the proinflammatory and tumorigenic effect of PGE2. Moreover, by using in situ hybridization technique, we observed that miR-21 is exclusively expressed in cholangiocarcinoma cells but not in other cell types in the tumor microenvironment (such as myofibroblasts, endothelial cells, and inflammatory cells); this observation is consistent with the reported increase of miR-21 in cholangiocarcinoma cells by qRT-PCR analysis (22, 23). In the present study, the tumorigenic effect of miR-21 in cholangiocarcinoma is documented by complementary in vitro and in vivo studies.

In normal tissues, 15-PGDH acts as a physiologic antagonist of the COX2/PGE2 signaling. Thus, loss of 15-PGDH expression in tumor tissues may be one of the key mechanisms for enhanced PGE2 signaling in human cancers. However, to date, the exact mechanism for loss of 15-PGDH expression in cancer has not been completely defined. The current study shows that the expression of 15-PGDH is reduced in human cholangiocarcinoma cell lines (compared with the nontumorous biliary epithelial cell line, H69). Our results in this study provide the first evidence that 15-PGDH is posttranscriptionally downregulated by miR-21. This conclusion is based on the following findings: (i) miR-21 binding site was identified in the 3′-UTR of 15-PGDH mRNA by sequence alignment analysis; (ii) miR-21 overexpression decreased 15-PGDH mRNA and protein levels; and (iii) transfection of miR-21 mimic decreased 15-PGDH 3′-UTR luciferase reporter activity and the effect was abolished by miR-21 seed binding site mutation. The observations that 15-PGDH overexpression inhibited tumor cell growth and that 15-PGDH inhibition accelerated tumor cell growth support a tumor-suppressive role of 15-PGDH in human cholangiocarcinoma. Our findings described in this study, along with the documented role of 15-PGDH in tumorigenesis, support a key role of miR-21–mediated inhibition of 15-PGDH in cholangiocarcinogenesis and tumor progression.

Although much attention has been paid to miRNA target studies to understand the role of miRNAs in cancer biology, there are fewer studies on how miRNAs are regulated in cancer cells. Whereas some of miRNAs that are contained in introns might be generated as a by-product of pre-miRNA splicing, most miRNAs likely come from intergenic regions and are transcribed from their own promoters (24). A previous study described a miR-21 regulatory region mapping −3,565 to −2,415 upstream of the primary miR-21 (pri-miR-21), which is inducible by interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3; ref. 25). In addition to transcriptional regulation, miR-21 can be also posttranscriptionally regulated by the transforming growth factor beta (TGF-β) and bone morphogenetic protein (BMP) signaling (26). In the current study, we show that miR-21 is induced by PGE2 at least in part through transcriptional regulation. The latter assertion is based on the findings that COX-2 overexpression and PGE2 treatment increased miR-21 level and miR-21 promoter luciferase reporter activity and that inhibition of COX-2 decreased miR-21 level and miR-21 promoter luciferase reporter activity.

In summary, this study shows that miR-21 enhances cholangiocarcinoma growth by inhibiting 15-PGDH, which leads to PGE2 accumulation and that enhanced PGE2 signaling further stimulates miR-21 transcription and increases the level of cellular miR-21. Such a feed-forward regulatory loop between PGE2 and miR-21 likely plays an important role in cholangiocarcinogenesis and tumor progression. Thus, blocking the COX2/PGE2 signaling in
Combination with targeting miR-21 may represent a promising therapeutic strategy for the treatment of human cholangiocarcinoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: L. Lu, C. Han, T. Wu
Development of methodology: L. Lu, K. Byrnes, Y. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Lu, K. Byrnes

References

Writing, review, and/or revision of the manuscript: L. Lu, C. Han, T. Wu
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