Supplementary Materials and Methods

miR-26a1 expressed and scramble control lentiviral particles with enhanced green fluorescent protein were obtained from GeneCopoeia (Rockville, MD), 15-PGDH expressed adenovirus and control viral vector were kindly provided by Dr Hsin-Hsiung Tai at the University of Kentucky, Lexington, KY. Rabbit polyclonal antibodies against GSK-3β, β-catenin, peroxisome proliferator-activated receptor β/δ, TCF-4, LEF-1, and Axin were purchased from Santa Cruz Biotechnology; against 15-PGDH were purchased from Cayman Chemical (Ann Arbor, MI); and against p-β-catenin (S33/37/T41), CyclinD1, and epidermal growth factor receptor were purchased from Cell Signaling (Danvers, MA). Mouse monoclonal antibodies against GSK-3β, β-catenin, Axin, c-Myc, TCF-1, CD44, CyclinE1, and Ki67 were obtained from Santa Cruz Biotechnology, against cyclooxygenase-2 from Cayman Chemical, and against β-actin from Sigma. The anti-rabbit IgG (horseradish peroxidase–linked F(ab′)2 fragment from donkey) and the anti-mouse IgG (horseradish peroxidase–linked whole antibody from sheep) were purchased from GE Healthcare Limited (Piscataway, NJ). All other chemical reagents were analytical grades (purchased from Sigma).

In Situ Hybridization for miRNA

In situ hybridization for miR-26a was performed in the formalin-fixed and paraffin-embedded tissue specimens surgically resected from patients diagnosed with cholangiocarcinoma by using the MiRCURY LNA microRNA ISH Optimization Kit (Exiqon, Vedbaek, Denmark) with the approval of the Institutional Review Board. Briefly, 6-μm-thick paraffin sections were deparaffinized and treated with proteinase-K (15 μg/mL) at 37°C for 10 min. After dehydration, slides were incubated with 40 nM miR-26a locked nucleic acid probe (5′-DIG-AGCCTATCCTGGATTACTTGAA-3′-DIG) at 50°C for 60 min, followed by stringent washes with 5× standard saline citrate, 1× saline sodium citrate, and 0.2× saline sodium citrate buffers at 50°C; DIG blocking reagent (Roche, Mannheim, Germany) in maleic acid buffer containing 2% sheep serum at room temperature for 15 min; and alkaline phosphatase–conjugated anti-digoxigenin (diluted 1:500 in blocking reagent; Roche) at room temperature for 60 min. Enzymatic development was performed by incubating the slides with 4-nitro-blue tetrazolium and 5-brom-4-chloro-3′-Indolylphosphate substrate (Roche) at 30°C for 2 h to allow formation of dark-blue 4-nitro-blue tetrazolium formazan precipitate, followed by nuclear fast red counterstain (Vector Laboratories, Burlingame, CA) at room temperature for 1 min. Slides were then dismantled in water, dehydrated in alcohol solutions, and mounted with mounting medium (Vector Laboratories). Scrambled probe and U6 small nuclear RNA–specific probe were used as system control. A standard 4-point scale method was used to evaluate the staining intensity under microscope and results were scored as 0 (negative), 1 (+), 2 (++), or 3 (+++) according to established criteria.[1]

Cell Culture

Four human cholangiocarcinoma cell lines, including CCLP1,[2] SG231,[3] HuCCT1,[4] TFK1,[5] and 1 noncancerous cholangiocyte cell line (H69) were utilized in this study (HuCCT1 and TFK1 cells were obtained from the Japanese Cancer Research Resources Bank; 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 cultured according to our methods as described previously[6-9]; TFK1 cells were cultured in RPMI-1640 containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, as described previously.[5] The H69 cells were cultured in Bronchial Epithelial Cell Basal Medium (Lonza) supplemented with growth factors in BEGM SingleQuot Kit and 10% heat-inactivated fetal bovine serum. All cells were incubated in a humidified atmosphere of 5% CO_2 incubator at 37°C. The miR-26a1–overexpressed and scramble control stable cell lines were established by transduction with miR-26a1 lentiviral vector or miRNA-scramble control lentiviral vector, followed by selection with media containing puromycin.

Anti-miR/siRNA Transfection

siRNA targeting COX-2, β-catenin and GSK-3β (Ambion, Grand Island, NY), or miR-26a–specific anti-miR (Qiagen, Valencia, CA) were transfected into cells by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as per manufacturer's instruction. After transfection at indicated time periods, cells were analyzed for proliferation and the cell lysates were obtained for Western blotting.

3′-UTR Luciferase Reporter Plasmid Construction

The 500-bp 3′-UTR of human GSK-3β complementary DNA was amplified by polymerase chain reaction with the following primers: forward primer 5′-AAGCTTTACCAAATGGGCGAGACACACCT-3′ and reverse primer 5′-CTCGAGTGTCGTGGGAGAGAGATT-GTATGTTCT-3′. Endonuclease restriction sites were incorporated in primers to facilitate ligation into the luciferase reporter plasmid pMIR-REP-dCMV. Correct sequence of the insert was verified by sequencing. Creation of the 3 nucleotides mutation was achieved by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA) followed by sequence verification.

Cells Proliferation WST-1 Assay

Cells were cultured in serum-free medium for 24 h to synchronize cell cycle. The 2×10^3 cells were seeded onto each well of 96-well plates in 200 μL culture medium containing 1% fetal bovine serum. Ninety microli-

ters serum-free medium mixed with 10 μL WST-1 was added to each well and the cells were incubated for 4 h at 37°C and 5% CO_2. After incubating, A450 nm was measured by an enzyme-linked immunosorbent assay plate reader.

Culture Plate Colony-Formation Assay

The 1×10^3 cells were plated into 10-cm dish and cultured for 14 days to allow colony formation. Colonies were stained with 0.1% crystal violet (Amersco, Solon, OH) in 50% methanol and 10% glacial acetic acid for counting.

Wound Healing Assay

Cells cultured in 10-cm dishes (in approximately 90% confluence) were washed with phosphate-buffered saline (PBS) and cultured overnight in serum-free medium. A line was drawn using a sterile 20-μL pipette tip, and the cell debris was gently rinsed with PBS. At the time points of 0, 8, and 24 h, 100× magnification pictures were taken at the same view field (the culture media were changed after each measurement).

Western Blotting

The logarithmically growing cells were washed twice with ice-cold PBS and lysed in a lysis buffer containing 50 mM HEPES, 1 mM EDTA (pH 8.0); 1 tablet of Roche protease inhibitors cocktail was added per 10 mL buffer. After sonication on ice, the cell lysates were centrifuged at 12,000*g* for 20 min at 4°C and the supernatants were collected for Western blotting. The protein concentration was measured with the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA). After boiling for 5 min in the protein loading buffer with 2-mercaptoethanol, the samples were separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred onto the nitrocellulose membrane (BioRad). The membranes were blocked in PBS with 0.1% Tween 20 (PBS-T) containing 5% nonfat milk for 1 h. Blots were incubated with different primary antibodies (at appropriate dilutions) in PBS-T containing 5% nonfat milk at 4°C overnight. After 3 washes with PBS-T, the membranes were incubated with secondary antibody (horseradish peroxidase–conjugated IgG) in PBS-T containing 5% nonfat milk for 1 hour. After 3 washes with PBS-T, signals were visualized with Enhanced Chemiluminescence Plus Kit (GE Healthcare).

Co-immunoprecipitation

Cells in 100-mm dishes were lysed in 1 mL whole-cell extract buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1 mM EDTA, 1% NP-40, 1.0 mM dithiothreitol, and protease inhibitors. Five hundred microliters cell lysates were used for immunoprecipitation with specific antibodies. In brief, cell lysate was precleared with 30 μL protein A/G-plus agarose beads (Santa Cruz) by rotation at 4°C for 1 h. The supernatant was obtained after centrifugation 5000 rpm at 4°C for 5 min. The precleared supernatants were incubated with 2 μg antibodies by rotation at 4°C for 4 h, and then incubated with 30 μL protein A/G-plus agarose beads by rotation at 4°C overnight. The samples were collected by centrifugation at 5000 rpm at 4°C for 5 min, followed by 5 times wash with beads wash solution (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1 mM EDTA, and 0.1% NP-40), and then suspended in 2× SDS-PAGE sample loading buffer. After boiling for 5 min, the samples were subjected to SDS-PAGE and Western blotting with specific antibodies.

DNA Pull-Down Assay

Cells were lysed by sonication in HKMG buffer containing 10 mM HEPES (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, and 0.5% NP-40 with protease inhibitors for the preparation of nuclear exact. The obtained nuclear extracts were precleared with streptavidin-agarose resin (Thermo) for 1 h; the precleared samples were then incubated with 1 μg biotinylated double-stranded oligonucleotide TCF/LEF-site (5′-Biotin-CCCTTTGATCT-3′ and 5′-Biotin-AGATCAAAGGG-3′; synthesized by Integrated DNA Technologies) and 10 μg poly(dI-dC) at 4°C for 24 h. DNA-protein complex was collected by incubation with streptavidin-agarose resin on a shaker to prevent precipitation at 4°C for 1 h. The resin-bound complex was washed at least 5 times with 1.0 mL PBS, followed by centrifugation at 2000*g* for 1 to 2 min to discard the supernatant. After the resin-bound complex was boiled in SDS-PAGE sample loading buffer, the released protein was loaded onto the 4% to 20% SDS-PAGE gel and identified by Western blotting with specific antibodies. Ten times more unlabeled TCF/LEF DNA oligonucleotide was used as the cold competing probe. β-actin was used as the loading control.

Enzyme Immunoassay for PGE_2

The 1×10^6 cells were incubated for 24 h in 1 mL serum-free medium, and the supernatants were collected and centrifuged for 10 min at 12,000*g* to remove the floating cells and cellular debris. The amount of PGE_2 in the spent media was measured by enzyme immunoassay using a specific EIA kit (GE Healthcare).

qRT-PCR

Total RNA was prepared using Trizol reagent (Invitrogen). Reverse transcription was performed by using Qiagen miScript Reverse Transcription Kit (Valencia, CA). Qiagen miScript SYBR Green PCR Kit and miR-26a–specific miScript Primer Assay were used to amplify the mature form of miR-26a on Bio-Rad C1000 Thermal Cycler. U6 small nuclear 2 (U6b) was used as the internal control. The primers for GSK-3β were: 5′-GCTTTGAAAGTAATCCCTGGGGTTTGG-3′ (forward) and

5′-TGCAGAGGTGCAAAACGGAGCA-3' (reverse). The primers for the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: 5′-GGCCCACATGGCCTCCAAGG-3′ (forward) and 5′-GGCAGGGACTCCCCAGCAGT-3′ (reverse).

SCID Mice Tumor Xenograft Studies

Four-week-old male athymic nude NOD CB17-prkdc/SCID mice were purchased from Jackson Laboratory (Bar Harbor, ME). Subcutaneous xenografts were established by inoculating 1.5×10^6 miR-26a–overexpressed or control CCLP1 cells in the flanks of mice (6 mice per group). The mice were observed over 4 weeks for tumor formation. The tumor volume was measured twice weekly with a caliper and calculated by using the formula: larger diameter $\times$ (smaller diameter)^2/2. Upon sacrifice, the tumors were recovered and the wet weights of each tumor were determined. Portion of each tumor was selected for H&E staining, immunohistochemical staining for Ki67, Western blotting for GSK-3β, and qRT-PCR analysis for miR-26a and GSK-3β mRNA. Immunohistochemical staining for Ki67 was performed according to standard methodology, and the percentages of Ki67-positive cells were determined by counting the number of positively stained cells vs total cells in more than 3 randomly selected 200$\times$ magnification fields (at least 1000 cells).

Immunofluorescence Localization

Cells with 70% confluence grown on coverslips were washed twice with cold PBS and fixed in 2% paraformaldehyde for 10 min. Cells were penetrated with 0.1% Triton X-100 in PBS and blocked with 5% bovine serum albumin at room temperature for 1 h. Subsequently, cells were incubated with primary antibodies at 4°C overnight, followed by 3 washings with PBS and incubation with anti-mouse Rhodamine-linked IgG (Santa Cruz). After counterstaining with 4′,6-diamidino-2-phenylindole (0.1 μg/mL) for 1 min, the coverslips were applied with mounting medium and images were captured using fluorescence microscope.

Luciferase Activity Assay

Cells were collected 24 h after transfection and analyzed by using the Dual-Luciferase reporter assay system (Promega, Madison, WI). Luciferase activity was measured by centro xs^3 lb 960 microplate fluoroscence reader (Mandel, Ontario, Canada). The pRL-TK plasmid with constitutive expression of Renilla luciferase was cotransfected with different firefly luciferase-based reporter as internal control.

Statistical Analysis

Results are presented as mean $\pm$ standard deviation from a minimum of 3 replicates. Difference between groups was evaluated by SPSS 13.0 statistical software with one-way analysis of variance, repeated measure analysis of generalized linear model or Wilcoxon signed ranks test methods. $P < .05$ was considered as statistically significant.

Results and Discussion

We did not observe tumor metastasis in SCID mice with miR-26a overexpression; however, the possibility of miR-26a on tumor metastasis cannot be entirely excluded, given that the tumor growth time period in our model is within 1 month and this duration may be too short for manifestation of metastasis.

Our results show that miR-26a overexpression increases β-catenin, whereas miR-26a knockdown reduces β-catenin in cholangiocarcinoma cells. However, when different cholangiocarcinoma cell lines are compared, the expression levels of miR-26a and β-catenin are not positively correlated. The latter phenomenon may be explained by the fact that the levels of β-catenin in different cell lines may be influenced by other factors, including the characteristics of individual cell lines and the activation status of other β-catenin regulatory signaling molecules.

References

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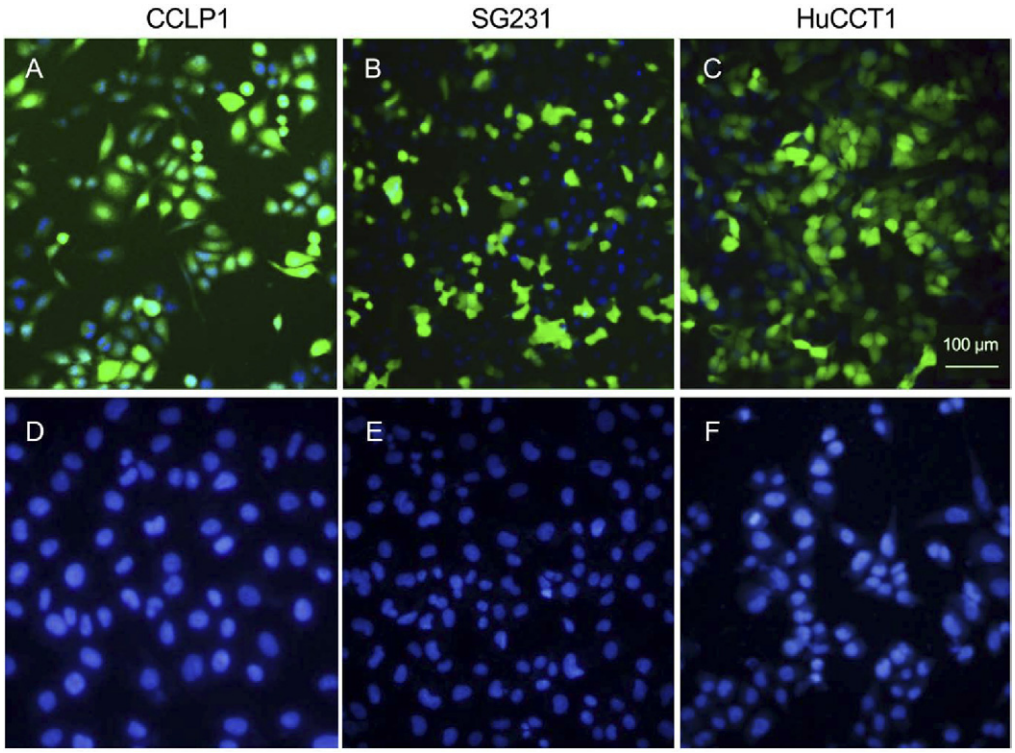
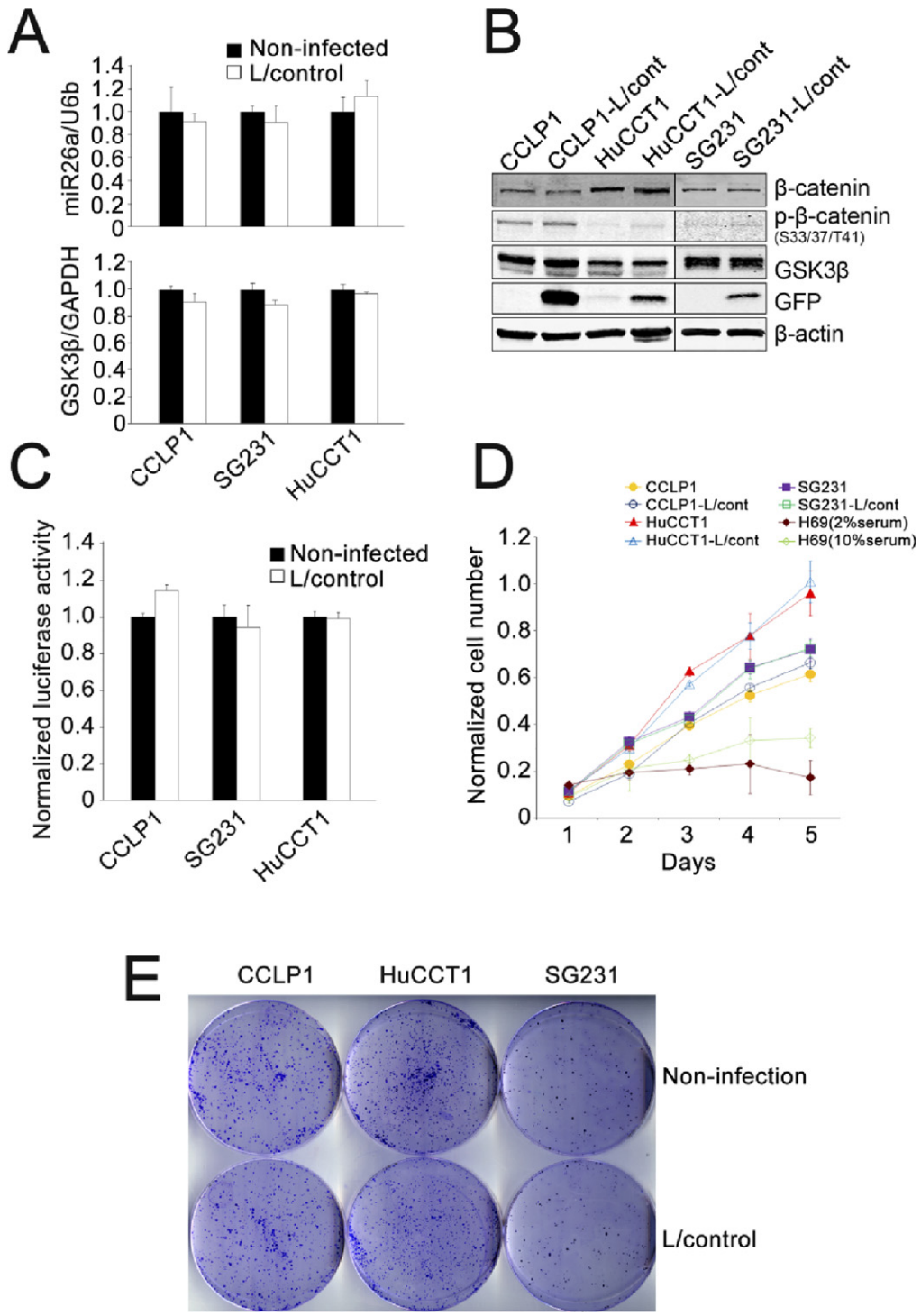
Supplementary Table 1. miR-26a Level in Human Cholangiocarcinoma Tissues

Staining intensity	Cholangiocarcinoma	Benign bile duct epithelium
0 (−)	2/21 (9.5%)	14/21 (66.7%)
1 (+)	9/21 (42.9%)	6/21 (28.6%)
2 (++)	6/21 (28.6%)	1/21 (4.8%)
3 (+++)	4/21 (19.0%)	0/21 (0%)

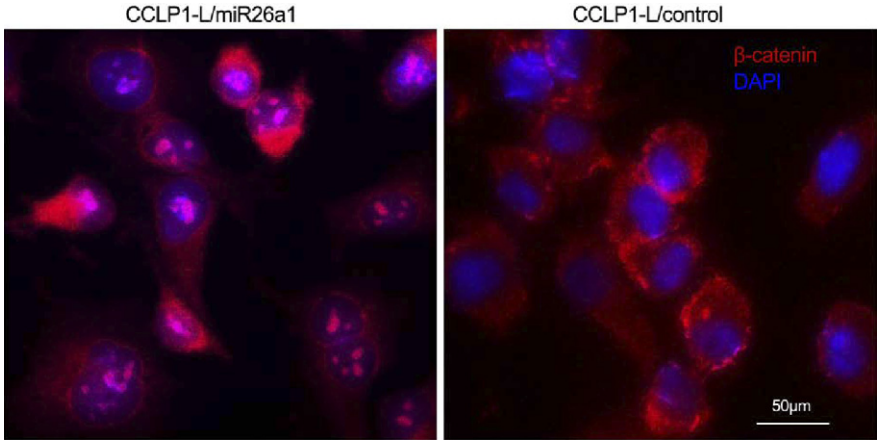
NOTE. $P < .001$, Wilcoxon signed-ranks test (2-tailed).



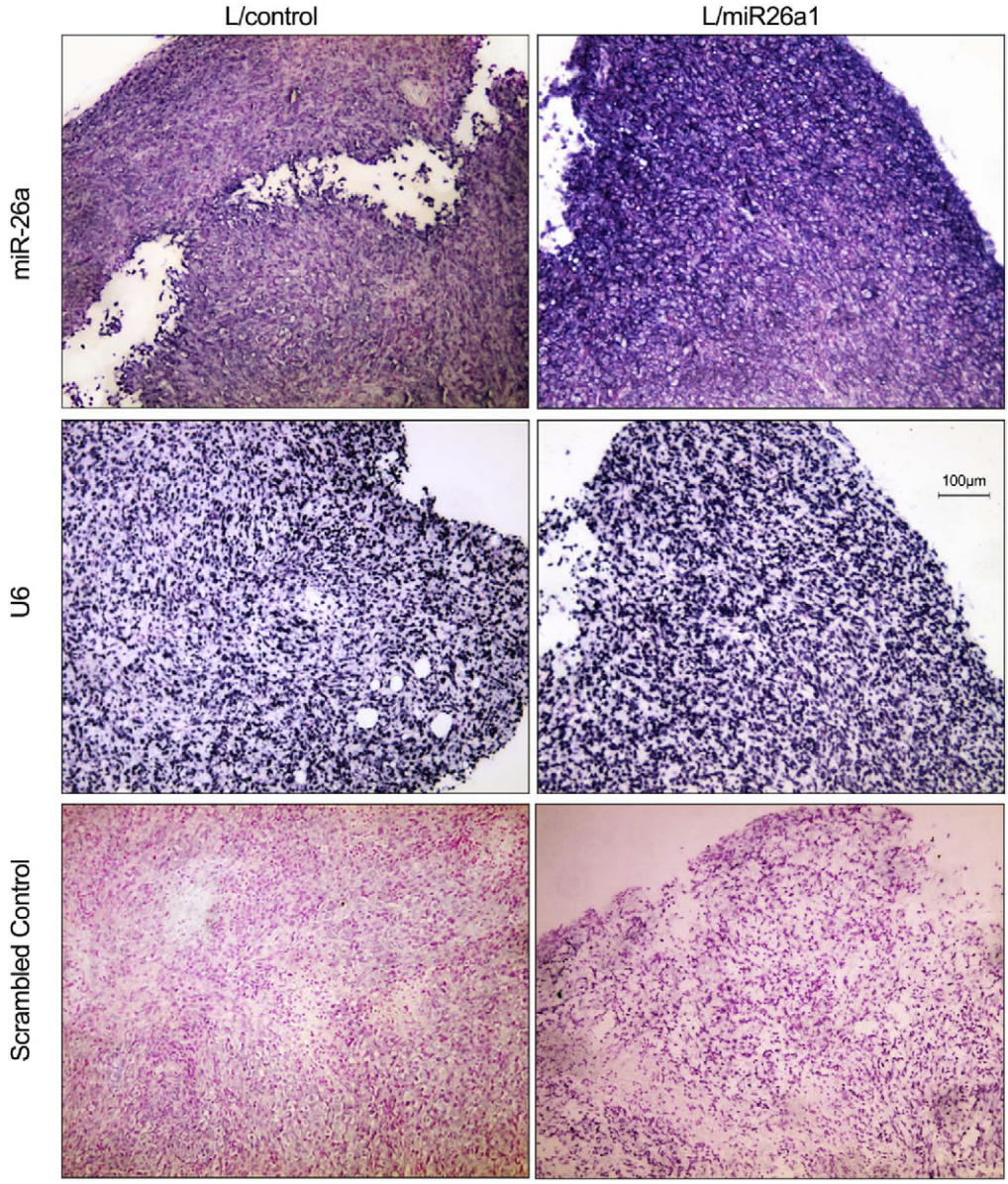
Supplementary Figure 1. Expression of enhanced green fluorescent protein (eGFP) in stably transduced cell lines. Immunofluorescent microscopy for eGFP was performed in 3 human cholangiocarcinoma cell lines (ie, CCLP1, SG231, and HuCCT) stably transduced with the miR-26a1 lentivirus (L/miR-26a1, in which the eGFP and has-miR-26a1 genes were fused and controlled by CMV promoter). (*A, B, C*) L/miR-26a1–transduced cells; (*D, E, F*) noninfected control cells.



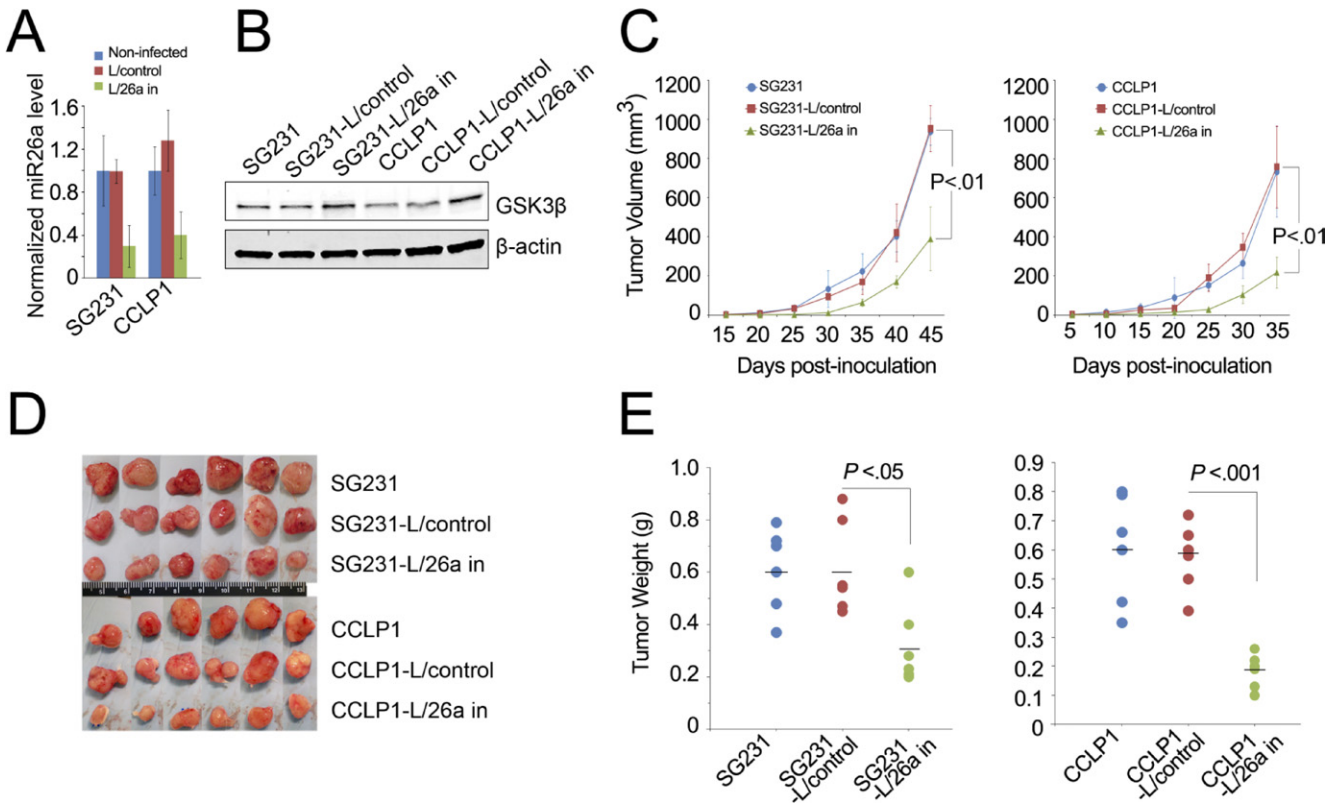
Supplementary Figure 2. Cholangiocarcinoma cells with or without stable transduction of control lentivirus. (*A*) qRT-PCR analysis for miR-26a and GSK3β mRNAs in cholangiocarcinoma cells with or without scramble control lentivirus transduction. Data are shown as mean ± standard error of mean (SEM) from 3 experiments. (*B*) Representative Western blots showing the protein levels of GSK-3β, β-catenin, and phosphorylated β-catenin in cholangiocarcinoma cells with or without scramble control lentivirus transduction. (*C*) TCF/LEF luciferase activity assay. Cholangiocarcinoma cells with or without scramble control lentivirus infection were transfected with the TCF/LEF luciferase reporter plasmid. The cell lysates were obtained 24 h after transfection to measure the luciferase activity by using a luminometer. Renilla luciferase expression plasmid pRL-TK was used as internal control. Data are presented as mean ± SEM from 3 independent experiments. (*D*) Cell proliferation assay. Cholangiocarcinoma cells with or without scramble control lentivirus infection were analyzed by the WST-1 assay. All cells were cultured in OPTI-MEM medium (Invitrogen) with 2% fetal bovine serum unless otherwise indicated. Three hours after WST-1 reagent incubation, the absorption was measured by an enzyme-linked immunosorbent assay reader under wavelength of 450 nm. Data are presented as mean ± SEM from 3 independent experiments. (*E*) Colonogenic assay in cholangiocarcinoma cells with or without scramble control lentivirus infection. One thousands cells were plated in 10-cm dishes and cultured for 10 to 14 days. The colonies were stained with 0.1% crystal violet.



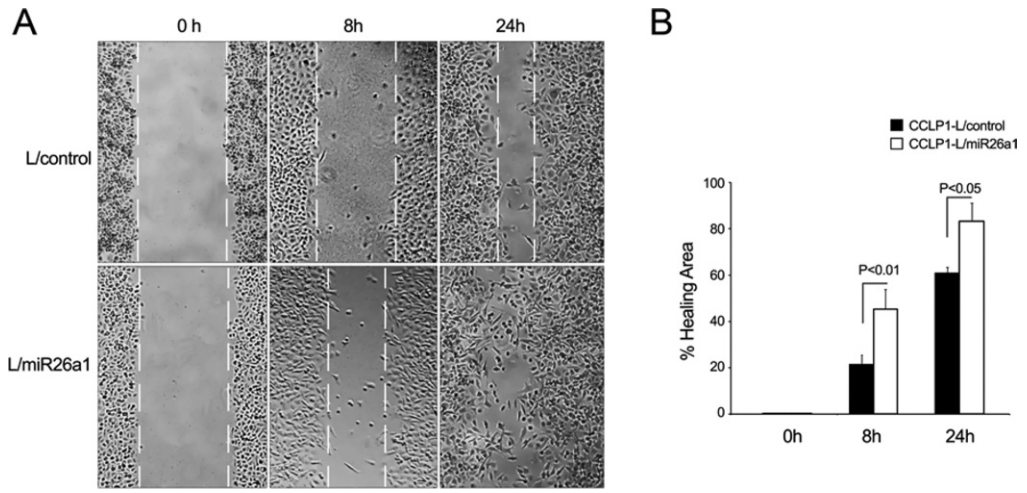
Supplementary Figure 3. Representative immunofluorescence staining for β-catenin in CCLP1 cells transduced with miR-26a1 or control lentivirus. miR-26a–overexpressed cells show more β-catenin accumulation in the nuclei compared to the miRNA-scramble control cells.



Supplementary Figure 4. miR-26a expression in xenograft tumor tissues. In situ hybridizations for miR-26a and U6 small nuclear RNA were performed by using locked nucleic acid probe as described in the Materials and Methods section. Locked nucleic acid miRNA-scramble probe was used as the negative control.



Supplementary Figure 5. The effect of miR-26a inhibition. SG231 and CCLP1 cells were stably transduced with the lentivirus expressing miR-26a–specific inhibitor (L/26a in) or the scramble control lentivirus vector (L/control). Cells without viral vector transduction were used as an additional control. (*A*) The level of miR-26a in cultured cells as determined by qRT-PCR analysis. The level of miR-26a in noninfected cells was set as 1.0. Data are presented as mean ± standard error of mean (SEM) from 3 experiments. (*B*) Representative Western blots for GSK-3β in cultured cells (with β-actin as the loading control). (*C–E*) The effect of miR-26a inhibitor on cholangiocarcinoma growth in SCID mice. miR-26a inhibitor expressed, vector control, or noninfected CCLP1 and SG231 cells (5×10^6 for CCLP1, 2×10^6 for SG231) were inoculated subcutaneously into *SCID* mice (n = 6), and the mice were closely monitored for tumor growth. The mice were sacrificed 35 (for CCLP1) or 45 days (for SG231) post inoculation to recover the tumors. (*C*) The growth curves of xenograft tumors (data represent mean ± standard deviation [SD] from 6 SCID mice). (*D*) Photography of xenograft tumors recovered from SCID mice. (*E*) The weight of xenograft tumors (data represent mean ± SD from 6 SCID mice).



Supplementary Figure 6. Wound-healing assay. (*A*) Representative photographs from CCLP1 cells stably transduced with the miR-26a1 lentivirus (L/miR-26a1) and the control lentivirus (L/control) taken at 0, 8, and 24 h after scratch. (*B*) The mean wound healing rate for miR-26a–overexpressed and control cells at 0, 8, and 24-h time points.