1 # SUPPLEMENT

2 ## SUPPLEMENTAL MATERIALS AND METHODS

3 **Materials.** rhTRAIL, rhPDGF-BB, anti-human PDGF-BB antiserum AB-220-NA,
4 rhSHH (R&D Systems, Minneapolis, MN), PKA inhibitor H-89 (Cayman Chemical, Ann
5 Arbor, MI), non-specific cAMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine
6 (IBMX), topoisomerase II inhibitor etoposide (Sigma, St. Louis, MO), and cyclopamine
7 (LC Laboratories, Woburn, MA) were prepared according to the suppliers protocols.
8 Imatinib mesylate/**STI**-571, an inhibitor of the kinase activity of PDGFR(-β), was a
9 generous gift from E. B. Leof (Div. of Pulmonary and Critical Care Medicine, Mayo
10 Clinic, Rochester, MN). Imatinib was dissolved in sterile water (10 mmol/l stock
11 solution) and subsequently diluted in cell culture medium.

12 **Generation of a stable transfectant expressing SMO short hairpin RNA.**
13 Short hairpin RNA (shRNA) lentiviral plasmid for SMO was from Thermo Fisher
14 Scientific (Hunsville, AL; Oligo ID: V2LHS_56569; GenBank accession no.:
15 NM_005631). KMCH-1 cells were transfected using OptiMEM I (Gibco-Invitrogen,
16 Carlsbad, CA) containing 6 μL/mL Lipofectamine (Invitrogen),1 μg/mL plasmid DNA,
17 and 6 μL/mL Plus reagent (Invitrogen). Forty-eight hours after transfection, fresh DMEM
18 containing 0.5 μg/mL puromycin was added. Surviving clones were separated using
19 cloning rings and individually cultured. A clone with a scrambled shRNA was employed
20 as a control (stable scrambled KMCH-1 cells).The expression/knockdown of SMO in the
21 clones was assessed by immunoblot analysis.

22 **Quantitation of PDGF-BB and cAMP.** Levels of secreted human PDGF-BB and
23 intracellular cAMP in CCA and MFB cell experiments were determined by enzyme-

1 linked immunosorbent assays using commercially available kits (PDGF-BB: RayBiotech,
2 Norcross, GA; cAMP: Cell Biolabs, San Diego, CA) according to the suppliers protocols.
3 **Co-culture experiments.** Cell co-culture experiments were performed using a
4 transwell insert co-culture system (24 wells) equipped with 0.4 µm pore size polyester
5 (PET) inserts (Corning Coster, Acton, MA) for 6 days according to the manufacturer's
6 recommendations. Briefly, KMCH-1 or KMBC cells were plated alone or together with
7 myofibroblastic human primary HSCs or LX-2 cells in the transwell insert co-culture
8 system (KMCH-1 or KMBC cells in the bottom and human primary HSCs or LX-2 cells
9 in the top wells; 1:1 ratio). First, all cells were plated alone at a density of
10 2×10^3 cells/well overnight. The co-culture insert chambers with the human primary
11 HSCs or LX-2 cells then were transferred the next day. Cells were treated as indicated
12 whereas rhTRAIL was added at the end of the experiment (day 6) for 6 hrs and the anti-
13 human PDGF-BB antiserum was added on day 5 for 24 hrs (anti-human PDGF-BB
14 antiserum was added not longer than 24 hrs to minimize confounding effects on apoptosis
15 measurement due to decreased cell proliferation). After rhTRAIL treatment, the KMCH-1
16 or KMBC cells in the bottom wells were analyzed for apoptosis by DAPI-staining and
17 TUNEL assay as described in the "Quantitation of apoptosis" section (for the TUNEL
18 assay, cells were plated on sterilized trimmed coverslips that were placed in the bottom
19 wells prior to cell seeding).
20 **Quantitation of apoptosis.** Apoptosis in CCA cells was quantified by assessing
21 the characteristic nuclear changes of apoptosis after staining with
22 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma, St. Louis, MO) using
23 fluorescence microscopy.[10] Terminal deoxynucleotidyl transferase-mediated dUTP nick

end labeling (TUNEL) assays (cell co-culture and rat liver samples) were carried out using the In situ Cell Death Detection kit (Roche, Indianapolis, IN) according to the supplier's protocol and as previously described.[11] Caspase 3/7-activity was quantitated using the ApoONE Homogenous Caspase-3/7 Assay (Promega, Madison, WI) according to manufacturer's recommendations.[10]

Immunoblot analysis. Whole cell lysates were obtained as previously described.[12] For the examination of GLI2 activation, nuclear protein extracts were obtained using the NE-PER Nuclear and Cytoplasmatic Extraction Kit (Thermo Scientific , Barrington , IL; Product no.: 78833). Primary antisera/antibodies used were: Actin (1:2000; Santa Cruz, Santa Cruz, CA; C-11), Lamin B (1:1000; Santa Cruz, Santa Cruz, CA; M-20), PDGFR-β (1:1000; Santa Cruz, Santa Cruz, CA; P-20), phospho-PDGFR-β (Tyr^{857}; 1:1000; Cell Signaling, Danvers, MA; #3170), and GLI2 (R&D Systems, Minneapolis, MN; Antibody Part 965887 from the GLI2 ExactaChIP Kit Catalog no.: ECP3526). Horseradish peroxidase-conjugated secondary antibodies for rabbit (Santa Cruz; sc-2004), goat (Santa Cruz; sc-2020), and sheep (Santa Cruz; sc-2770) were incubated at a dilution of 1:3000 for 1 hr at RT. Proteins were visualized using enhanced chemiluminescence reagents (ECL, Amersham Biosciences, Buckinghamshire, UK) and Kodak X-OMAT films.

Real-time polymerase chain reaction. Total RNA was extracted from cells and liver tissue using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), and was reverse-transcribed with Moloney leukemia virus reverse transcriptase and random primers (Invitrogen, Camarillo, CA). Quantitation of the complementary DNA template was performed with real-time polymerase chain reaction (PCR; LightCycler, Roche,

1 Indianapolis, IN) using SYBR green (Roche) as a fluorophore.[13] Oligonucleotide
2 sequences and expected product sizes for all primer pairs used for quantitative RT-PCR
3 analysis are shown in Suppl. Table 1. As an internal control, primers for 18S rRNA
4 (Ambion, Austin, TX) were employed. Using gel purified amplicons, a standard curve
5 was generated to calculate the copy number/μL. The target mRNA expression level of
6 each sample was calculated as the copy ratio of target mRNA to 18S rRNA and then
7 normalized to the target mRNA expression of controls.
8 **Immunohistochemistry for α-SMA, PDGFR-β, PDGF-BB, and cytokeratin 7.**
9 Immunohistochemistry was performed using formalin-fixed, paraffin-embedded human
10 and rat CCA samples (slides were also stained conventionally with hematoxylin/eosin).
11 Slides were deparaffinized in xylene and rehydrated through sequential graded ethanol
12 steps. For α-SMA-, PDGFR-β- and PDGF-BB-staining, the antigen retrieval was
13 performed by permeabilizing the slides in 0.1% Triton X 100 for 2 min (α-SMA-staining)
14 and incubation in sodium citrate (α-SMA- and PDGFR-β-staining; 0.01 M sodium citrate,
15 0.05% Tween 20; pH 6.0) or Tris-EDTA buffer (PDGF-BB-staining; 0.01M Tris base,
16 1 mM EDTA solution, 0.05% Tween 20, pH 9.0) using a vegetable steamer (30 min for
17 α-SMA- and 60 min for PDGFR-β-/PDGF-BB-staining). After cooling, further steps
18 were carried out according to the protocols of the EnVision+ System-HRP [DAB]
19 detection kits (α-SMA: K4006 [anti-mouse]; PDGFR-β and PDGF-BB: K4010 [anti-
20 rabbit]; Dako, Carpinteria, CA). The primary antiserum against α-SMA 1A4 (MS-113-R7,
21 ready-to-use dilution; NeoMarkers, Fremont, CA) was applied for 60 min at RT
22 (PDGFR-β: P-20, 1:25, applied overnight at 4°C, Santa Cruz, Santa Cruz, CA;
23 PDGF-BB: ab21234, 1:10, applied overnight at 4°C, Abcam, Cambridge, MA). Finally,

1 the slides were counterstained with Mayer's Hematoxylin Solution (Sigma, St. Louis,
2 MO), mounted and examined by light microscopy. For Cytokeratin 7 (CK7)-labeling, the
3 antigen retrieval was performed incubating the slides in deionized water containing 5%
4 urea using a vegetable steamer for 20 min (since the same slides also were labeled for
5 TUNEL-positive cells, an additional antigen retrieval step was performed with sodium
6 citrate followed directly by cooling and application of the TUNEL reaction mix; the
7 TUNEL assay is described in the "Quantitation of apoptosis" section). The primary
8 antibody against CK7 (1:10; Abcam; ab9021) was applied for 30 min at RT. After being
9 washed, the slides were incubated with Texas Red®-X goat anti-mouse IgG (1:1000;
10 Invitrogen, Camarillo, CA; T6390) for 1 hr in the dark. The slides were then washed
11 three times in PBS, one time in water and mounted using Prolong Antifade (also
12 Invitrogen). The slides were analyzed by fluorescent confocal microscopy (LSM 510;
13 Zeiss, Jena, Germany).

14 **Genome-wide mRNA expression analysis.** KMCH-1 cells were treated with
15 vehicle, PDGF-BB (200 ng/ml, 8hrs), or SHH (500 ng/ml, 8hrs) in the presence or
16 absence of cyclopamine (10 μM, 8hrs). After total mRNA extraction (see section real-
17 time polymerase chain reaction) and confirmation of the sample quality by Agilent
18 bioanalysis, 150-500 ng of total RNA per sample were analyzed for 33617 target genes
19 (after MAS5 noise filtering) employing an Affymetrix GeneChip Platform with the
20 Affymetrix Human U133 Plus 2.0 labeling method. Specifically, biotin-labeled cRNA,
21 produced by *in vitro* transcription, was hybridized to the Affymetrix Human Genome
22 U133 Plus 2.0 GeneChips. These experiments were conducted in collaboration with the
23 Advanced Genomics Technology Center Core Mayo Clinic, Rochester, MN.

1 **Statistical analysis.** Data are expressed as the mean ± s.e.m. unless indicated
2 otherwise and represent at least 3 independent experiments. Box-and-whisker plots depict
3 minimum, 25th percentile, median, 75th percentile, maximum, and outliers. Differences in
4 experiments with two groups were compared using the two-tailed Student *t*-test or the
5 χ^2 test for discrete data. Differences in experiments with more than two groups were
6 compared using ANOVA with Bonferroni post hoc correction. Differences were
7 considered as significant at levels of $p < 0.05$. *t*-test-based statistical analysis of the
8 Affymetrix U133 Plus 2.0 GeneChip assay was performed in collaboration with the
9 Division of Biomedical Statistics & Informatics, Mayo Clinic, Rochester, MN.
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1 **SUPPLEMENTAL RESULTS**

2 **Supplemental Figure 1.** PDGFR-β expression in 6 CCA cell lines. mRNA
3 expression levels of PDGFR-β was assessed by qualitative RT-PCR analysis in the 5
4 human CCA cell lines KMCH-1, KMBC, HuCCT-1, TFK-1, and MzChA-1 (left of the
5 100bp-DNA ladder) as well as in the ErbB-2/neu transformed malignant rat
6 cholangiocyte cell line BDEneu (employed in the *in vivo* CCA model; right of the
7 100bp-DNA ladder).

8 **Supplemental Figure 2.** PDGF-BB secreted by MFBs promotes resistance to
9 TRAIL cytotoxicity. (A) KMBC cells were plated alone (monoculture) or together with
10 PDGF-BB secreting LX-2 cells in a transwell insert co-culture system (KMBC cells in
11 the bottom and LX-2 cells in the top wells; 1:1 ratio) for 6 days. Cells were treated as
12 indicated with vehicle (V), rhTRAIL (T; 10ng/mL for 6 hrs on day 6) or rhTRAIL plus
13 anti human PDGF-BB antiserum (T + AB-P; rhTRAIL: 10ng/mL for 6 hrs on day 6; anti
14 human PDGF-BB antiserum: 10μg/mL for 24 hrs on day 5). After rhTRAIL treatment for
15 6 hrs, KMBC cells were stained with DAPI and quantitation of apoptotic nuclei was
16 performed, scoring the nuclei as apoptotic vs. non-apoptotic by fluorescence microscopy.
17 Mean ± s.e.m. (n=5). (B) KMCH-1 cells were plated alone (monoculture) or together
18 with PDGF-BB secreting LX-2 cells (co-culture) as described above. Cells were treated
19 as indicated with vehicle (V), etoposide (E; 25 μM for 48 hrs on day 5 and 6) or
20 etoposide plus anti human PDGF-BB antiserum (E + AB-P; etoposide: 25 μM for 48 hrs
21 on day 5 and 6; anti human PDGF-BB antiserum: 10μg/mL for 48 hrs on day 5 and 6).
22 After etoposide treatment for 48 hrs, KMCH-1 cells were stained with DAPI and
23 quantitation of apoptotic nuclei was performed as described above. Mean ± s.e.m. (n=5).

1 **Supplemental Figure 3.** PDGF-BB has no effect on mRNA expression of members
2 of the Hh signaling pathway. (A) The two human CCA cell lines (serum-starved for 2
3 days) KMCH-1 (upper) and HuCCT-1 (lower) were treated with vehicle or PDGF-BB
4 (500 ng/mL) for 8 hrs followed by quantitative RT-PCR analysis for mRNA expression
5 of the Hh signaling mediators sonic (*SHH*), indian (*IHH*), and desert hedgehog (*DHH*) as
6 well as patched-1 (*PTCH1*), smoothened (*SMO*), and glioma-associated oncogenes
7 (*GLI*) 1-3. 18S ribosomal RNA was used to normalize expression. Mean ± s.e.m. (n=3).
8 (B). Human KMCH-1 cells (serum-starved for 2 days) were treated with vehicle (V) or
9 PDGF-BB (500 ng/mL) for 24 hrs followed by quantitative RT-PCR analysis for mRNA
10 expression of the Hh signaling negative regulators hedgehog-interacting protein (*HIP*)
11 and suppressor of fused (*SUFU*). 18S ribosomal RNA was used to normalize expression.
12 Mean ± s.e.m. (n=3).
13 **Supplemental Figure 4** PDGF-BB promotes SMO translocation to the plasma
14 membrane and stimulates cAMP secretion. (A) KMCH-1 cells were transiently
15 transfected with a plasmid expressing GFP-tagged human SMO (GFP-SMO) and plated
16 alone (monoculture) or together with PDGF-BB-secreting LX-2 cells in a transwell insert
17 co-culture system (transfected KMCH-1 cells in the bottom and LX-2 cells in the top
18 wells; 1:1 ratio) for 3 days. Cells were treated as indicated with vehicle or
19 anti-human PDGF-BB antiserum (AB-P; 10µg/mL for 24 hrs on day 3). GFP-SMO
20 localized at the plasma membrane was analyzed by TIRF microscopy and the fluorescent
21 intensity quantified using image analysis software. Mean ± s.e.m. (n=20). (B)
22 Intracellular cAMP levels (normalized to mg protein) were assessed in the whole cell
23 lysates of vehicle-, PDGF-BB (200 ng/mL, 5 minutes)-, or PDGF-BB (200 ng/mL,

1 5 minutes) plus PDGFR(-β) inhibitor imatinib (10 μM, 5 minutes plus 30 minutes
2 preincubation)-treated HuCCT-1 cells (cultured with media containing 100 μM of the
3 non-specific phosphodiesterase inhibitor IBMX to prevent cAMP degradation) by ELISA.
4 Mean ± s.e.m. (n=3).

5 **Supplemental Figure 5.** BDEneu cells express Hh signaling pathway effectors.
6 mRNA expression levels of the Hh signaling pathway members sonic (*SHH*), indian
7 (*IHH*), and desert hedgehog (*DHH*) as well as patched-1 (*PTCH1*), smoothened (*SMO*)
8 and the transcription factors glioma-associated oncogene (GLI) 1, 2, and 3 were assessed
9 by qualitative RT-PCR analysis in the ErbB-2/neu transformed malignant rat
10 cholangiocyte cell line BDEneu (employed in the *in vivo* CCA model). 18S ribosomal
11 RNA expression is shown in the last lane.
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