Supplementary Materials and Methods

Clinical specimens. We studied surgically resected tissue specimens from two clinically annotated patient cohorts with intrahepatic cholangiocarcinoma (ICC). In addition, we used 22 ICC patient-derived xenograft (PDX) samples previously generated and analyzed by RNA sequencing at the Mayo Clinic Arizona (patient characteristics are listed in **Supplemental Table S3**).

We studied tissue samples from a clinically annotated patient cohort with ICC (n=52). All 52 patients were diagnosed with ICC and underwent surgery at the Department of General, Visceral and Cancer Surgery at the University Hospital of Cologne, Germany. The cohort consisted of 21 females and 31 male patients with a median age of 67.5 years (44–90 years). Median postsurgical follow-up was 24.25 months (0–98 months). We evaluated the expression of PlGF and Nrp1 in stromal or cancer cells using a tissue microarray (TMA). For this, 4 tissue cores from different areas of each tumor were punched out and transferred in a TMA recipient block. TMA construction was performed using standard techniques. In brief, tissue cylinders with a diameter of 1.2mm each were punched from selected tumor tissue blocks using a homemade semi-automated precision instrument and brought into empty recipient paraffin blocks. Four µm sections of the resulting TMA blocks were transferred to an adhesive coated slide system (Instrumedics Inc., Hackensack, NJ). Consecutive sections were used for RNA in situ hybridization (ISH) (for PlGF) and immunohistochemistry (IHC) (for NRP1) in the CLIA-(like)-certified Pathology Laboratory at University of Cologne, Germany.

We also studied 43 consecutive archived cases of intrahepatic ICC, which were surgically resected at Fundeni Clinical Institute, Bucharest, Romania between 1998-2009. Medical records and paraffin-embedded tissues of resected ICC patients were collected and de-identified. Pathological diagnosis was done by two pathologists with expertise in hepato-biliary-pancreatic pathology.

Histological differentiation and tumor staging were based on the 7th edition of Union for the International Cancer Control (UICC) classification.

PlGF ISH using RNA-Scope. The RNA-scope assay was performed according to manufacturer's instructions. In brief, paraffin-embedded TMA blocks were cut in 5µm sections, pretreated according to extended protocol (30min for pretreatment 2 and 3), digested and hybridized at 40°C in the HybEZ oven with human PlGF mRNA probe (NM_001207012) provided by Advanced Cell Diagnostics Europe. Incubation time with hematoxylin was 10 seconds. Target expression was compared to both negative (dapB) and positive (PPIB) controls.

Cells and cell culture. Human cholangiocarcinoma cell lines (HuCCT1, EGL1, TGBC1TKB, and TFK1) were kindly gifted by Dr. Bin Tean Teh (National University of Singapore). Murine ICC cell lines were derived from tumors spontaneously arising in $p53^{KO}$/$Kras^{G12D}$ mice (425 cells) and $Idh2^{R172K}$/$Kras^{G12D}$ mice (SS49 cells) [1, 2]. Human HSC, murine endothelial cell line, HUVEC and NIH3T3 were purchased from ScienCell Research Laboratories (#5300), ATCC (CRL-2299), PromoCell (C-12200) and ATCC (CRL-1658). To expose cells to hypoxic conditions, we placed the cultured cells under 1% CO_2 for 24hr. To generate stable transfectants, the cells were transfected with sh-PlGF and sh-Nrp1 constructs (SHCLNG-NM_002632, 008827 and 003873, Sigma-Aldrich Inc.) according to manufacturer's instructions. Cells were treated with 250ng/ml or 500ng/ml of anti-murine PlGF antibody (5D11D4, obtained under MTA from Oncurious NV, Belgium) for 24hr. Recombinant (r)PlGF was added to the culture media at a concentration of 80ng/ml.

Mouse models of ICC and treatments. Orthotopic ICCs were induced by grafting cells in the liver of C57Bl/6/129 mixed background or SCID mice (for 425 cells), and C57Bl/6 mice for SS49 cells and for 425 cells as a control for $Pgf^{-/-}$/C57Bl/6 mice [3, 4]. All treatments were initiated in

mice with established tumors, when the ICCs reached 5mm in diameter. Tumor growth and treatment response were monitored by high-frequency ultrasound imaging. Anti-mouse PlGF antibody (5D11D4) was provided by Oncurious NV, and gemcitabine and cisplatin were purchased from Pfizer and Fresenius Kabi, respectively. All drug treatments were administered intraperitoneally (i.p.), at doses of 25mg/kg for 5D11D4 thrice weekly, 60mg/kg for gemcitabine twice weekly and 0.3mg/kg for cisplatin twice weekly. All experiments were performed under the MGH IACUC-approved protocol 2014N000083 titled "*Targeted treatments for cholangiocarcinoma*".

Image analysis. For the purpose of quantifying the expression of Nrp1 staining and PlGF RNA ISH, we scanned the whole-slides of the corresponding TMAs using a whole-slide scanner (NanoZoomer S360 Digital slide scanner, Hamamatsu Photonics), corresponding cores were extracted and analyzed by applying a classical watershed algorithm for object detection, while the positivity of Nrp1 was determined by DAB intensity, the cell detection (tumor/stroma) and PlGF RNA ISH was conducted using a classifier that was trained on each slides individually. All results from the analysis were visually confirmed for plausibility by trained GI pathologists (SK, AQ).

Primary culture and isolation of CAFs from murine tumors. Small ICC pieces (approximately 5mm in diameter) were placed in culture in uncoated flasks with DMEM/F12 50:50 medium containing 4% FBS and were incubated at 37°C in 5% CO_2 humidified atmosphere. After several media changes, CAFs grew out in high number from the tissue fragments. After reaching confluence, these cells were passaged to 96-well plate with limited dilution to make single cells per a well. CAF phenotype was confirmed by immunostaining or gene expression analyses for the markers α-SMA, PDGFR-β, and FAP.

Spheroid formation and invasion assay. We used a culture system that closely mimics the

physiological environment of ICC tumor. These cells were seeded on top of 1.5% agarose-precoated matrix with culture medium including 4% FBS. The clear round spheroid can be seen 7 days after seeding. Then we transfer these spheroids on Matrigel-coated well, we measure spheroid and invasion area using Image J and calculate invasion ratio (invasion area divided by total area).

Real-time imaging for tumor invasion assay. For time-lapse 3-*D* culture system, we used an Olympus IX81 Confocal microscope equipped with the Fluoview software (lens:10X air). Slice thickness varied between 1 and 5 μm. Projection of confocal images is automatically produced using the projection-setup of Fluoview software with the loop rest option. The interval between two consecutive images was set to 30min for 145 repeats to cover 72hr (**Supplementary Movies S1** and **S2**).

IHC and immunofluorescence (IF). IHC staining of TMA sections was performed using the BOND MAX from Leica (Leica, Wetzlar, Germany) according to the protocol of the manufacturers. For Nrp1 staining on FFPE samples, clone #EPR3113 was used from Abcam. Briefly, sections of 2-μm thick from a paraffin-embedded tissue blocks were deparaffinized in xylene for 10 min, rehydrated using a graded alcohol series, placed in an endogenous peroxide blocker for 10 min and washed with buffer. The slides were placed in 1mM EDTA at 97 ºC for 20 min for antigen retrieval. Primary antibodies were applied overnight at 4°C and antibody binding was detected using biotinylated anti-goat or anti-rabbit IgG conjugating streptavidin-peroxidase complex (BA-9500, Vector Laboratories, CA, USA) for 60min. Finally, the sections were developed with 3,3′-diaminobenzidine color solution for 3min at room temperature. Then, hematoxylin was used as a chromogen and the slides were consecutively counterstained for 60s.

PlGF expression was evaluated for the extent of staining (percentage of positive tumor cells graded on a scale from 0 to 3) and the intensity of staining (graded on a scale from 0 to 3) using a

previously published scoring method [5, 6]. The Expression Score (ES) varying from 0 to 9 was divided into 4 grades, representing negative (score 0), weak (score 1), moderate (score 2-4), and strong (score 6-9), respectively. Similarly, Nrp1 expression were based on a staining extent scale from 0 to 4 and an intensity score from 0 to 3. The ES of Nrp1 was also divided into 4 grades, representing negative (score 0-2), weak (score 3-5), moderate (score 6-8), and strong (score 9-12), respectively.

For CD31 staining of human primary ICC, we used mouse anti-human CD31 antibody (DAKO, #M0823, clone JC70A). Briefly, the sections were deparaffinized in xylene for 5min x 3 times, rehydrated using a graded alcohol series and placed in generally a citrate buffer at 97ºC for 15min for antigen retrieval. The slides were kept for 1hr at RT, washed with DW and PBS, placed in 3% H_2O_2 for 10 min for blocking with an endogenous peroxide and placed in blocking buffer with normal donkey serum. Primary antibody was applied overnight at 4ºC and peroxidase-conjugated anti-mouse IgG for secondary antibody was applied for 1hr. Finally, the sections were developed with 3,3′-diaminobenzidine color solution for 3 min at room temperature. Then, hematoxylin was used as a chromogen and the slides were consecutively counterstained for 30s.

For IF staining of spheroids, the spheroids were fixed with 4% PFA for 30min, washed with PBS and PBS-T buffers, and placed in blocking buffer with normal donkey serum for 4hr. Anti-Ki-67 primary antibody was applied for 2 days at RT, followed by the reaction with appropriate secondary antibody with DAPI overnight. Spheroids were fixed with 4% PFA for 10 min for protecting antibody binding and wash with PBS. Spheroids were kept in PBS and taken images using a laser-scanning confocal microscope (Olympus, FV-1000). For IF using ICC tissues, frozen sections and paraffin sections were prepared 6 μm thick for subsequent immunostaining. We used antibodies to CK-19 to identify ICC cells, to CD31 for endothelial cells, to Collagen I for this

ECM component, to α-SMA to identify myofibroblast-like cells and to CA-IX for tissue hypoxia. All primary antibodies used in the study are listed in **Table S1**. Secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Frozen sections from OCT-embedded tissue blocks were washed OCT compound with water and treated with normal donkey serum for blocking. Primary antibodies were applied overnight at 4°C, followed by the reaction with appropriate secondary antibodies for 2hr at 24°C. Paraffin sections from paraffin-embedded tissue blocks were deparaffinized in xylene for 10min, rehydrated using a graded alcohol series, boiled in antigen retrieval buffer at 97°C, placed in an endogenous peroxide blocker for 10min and washed with buffer. The slides were blocked endogenous mouse Ig with M.O.M Kit for detecting mouse primary antibodies on mouse tissue (VECTOR, Burlingame, CA). Primary antibodies were applied overnight at 4°C, followed by the reaction with appropriate secondary antibodies for 2hr at 24°C. Analysis was performed five random fields under ×200 magnification in the tumor tissues using a laser-scanning confocal microscope (Olympus, FV-1000). These data were analyzed using ImageJ (US NIH) and Photoshop (Adobe Systems Inc.).

Picrosirius staining. Paraffin sections (3μm thick) from paraffin-embedded tissue blocks were deparaffinized in xylene for 10min and rehydrated using a graded alcohol series. The slides were washed with distilled water, stained with iron hematoxylin for 30sec, washed running water and distilled water for 10min. The tissues were stained with 0.1% Sirius red solution in saturated picric acid for 40min, washed distilled water, dehydrated using a graded alcohol series and xylene. Staining score was determined based on collagen density by picrosirius red. Analysis was performed five fields under x200 magnification in the tumor tissues using Olympus BX40.

DNA transfection and lentivirus transduction. shRNA-knockdown experiments were performed using pLKO.1 puro-based lentiviruses (Sigma). Briefly, 293T cells were seeded ($3x10^5$

cells/well) in 6 well dishes 24hourr prior to transfection. pLKO shRNA-DNA was transfected with psPAX2 packaging and pMD2.G envelop plasmid using Fugene reagent (Promega) according to manufacturer's instructions. Viral supernatant was harvested 48 and 72hr after transfection (filtered through 0.45μm filter). 425 cells were infected with lentivirus expressing-shRNA. After 24 hours, cells were selected by puromycin. In vivo and in vitro experiments were performed 7-10 days after infection.

Cell proliferation assay. To analyze cell proliferation, the cells were seeded onto 96-well plates in N=3 wells, including 3 control wells with cell growth media only. Cells were incubated at 37°C and 5% CO2. Proliferation was assessed 24, 48, 72, and 96hr later based on the colorimetric MTT cell proliferation assay according to the manufacturer's protocol. To evaluate the cell viability in 3-*D* culture conditions, we used the NanoCulture Plates (ORGANOGENIX, Japan). According to the manufacturer's instructions, we seeded the cells (5 x 10^3cells/100μl) in each well under serum starvation. Four days after seeding, treatment was started and one week after treatment, proliferation was assessed using CellTiter-Glo assay (Promega, WI).

ELISA. ELISA measurements for murine-PlGF were performed using a Quantikine MP200 kit (R&D Systems) measured on a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories). For human PlGF, we used Meso-Scale Discovery Vascular Growth Factor array in the CLIA-certified Core of the Steele Laboratories at Massachusetts General Hospital. Plasma levels of PlGF were measured in ICC patients prior to any therapy in previously published studies; samples were collected from control individuals [7], resectable (r)-ICC patients [8], localized (l)-ICC patients [9], and 2 advanced ICC cohorts [8, 10].

Quantitative real-time reverse transcription polymerase chain reaction (qPCR). Total RNA was isolated using RNeasy Mini Kit (Qiagen Inc.) and analyzed by nanodrop. qPCR was carried

out using iTaq Universal SYBR Green Supermix (Bio-Rad, Inc.). GAPDH was used as a housekeeping gene. qPCR was done at the annealing temperature of 60ºC with the primers (**Supplemental Table S3**). The relative amount of mRNA was calculated by the 2−ΔΔCT method.

Protein extraction and Western blotting. The cultured cells were lysed in RIPA buffer. For immunoblotting, the cell lysates were loaded on 8 to 10 % sodium dodecyl sulfate (SDS)-polyacryl-amide gel at equal amounts of protein (20 μg) per well and transferred to PVDF membranes. The membranes were blocked using 2% FBS solution in PBS for 1 hr at room temperature. Then, they were probed with primary antibodies under overnight. The signals were detected by Clarity Western ECL Substrate (Bio-Rad) according to the manufacturer's instructions.

Measurement of Young's modulus (stiffness). Slices of freshly excised tissue, 3-mm in diameter and ~2-mm thick, were placed in an unconfined compression chamber and submerged in physiological saline at room temperature (25°C). The chamber was mounted in a custom-made ultrasensitive servo-controlled materials tester. Each specimen was compressed by 1% of the original height in ramps of 1 sec and allowed to relax for 2min. Twenty successive measurements were performed on each tissue slice. Equilibrium stress (normalized force) has been plotted versus strain (normalized displacement), and a linear regression has been applied to estimate the slope of the stress-strain curve. The Young's modulus has been estimated as the slope of the linear fitted line.

Single-cell RNAseq (scRNASeq)–Droplet encapsulation and library preparation. The single cell suspension was first washed with PBS, labelled with Calcein Green, then resuspended in PBS with 0.01% BSA at a concentration of $3x10^5$ cells/ml. Barcoded poly-T beads were purchased from ChemGenes (cat. MACOSKO-2011-10) and were processed as described in Ref. [11]. The beads

were resuspended in DropSeq Lysis Buffer at 6 x 10^5 beads/mL. The cells and barcoded beads were loaded onto the Dolomite Bio Nadia Instrument with the BioRad Droplet Generation Oil for EvaGreen (cat. 1864006) and an encapsulation protocol was started as directed by the manufacturer. Each cell sample was run on two microfluidic chips (Dolomite Bio) in parallel to general the cell-bead emulsion. Following droplet generation, the emulsion was examined using a fluorescent microscope to ensure the droplets were uniform, the bead encapsulation rate was <10%, the cell encapsulation rate was <5%, and all cells were fully lysed. The emulsion breakage, reverse transcription, exonuclease, and PCR was performed as described in Ref. [11], with 2x10^3 beads per PCR tube. After PCR cleanup, the amplified cDNA samples were evaluated on a TapeStation HS-D5000 screentape to verify the library size distribution. The tagmentation was performed as described in Ref. [11] and the libraries were sequenced to a target depth of 1x10^5 reads per cell by the MGH NextGen Sequencing Core on a HiSeq 2500 (Illumina).

RNA sequencing. Total RNA was extracted from the primary cultured cells of CAFs using Qiagen kits. We sent all samples to Molecular Biology Core Facilities, Dana Farber Cancer Institute (Boston, MA) and they sequenced these RNA data. Biological interpretations of genes were assessed by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database. For the PDX sequencing data, fastq reads were separated into mouse and human by Xenome, Map Mouse/human reads by STAR to mm10/hg38, gene count by featurecounts, normalized to logRPKM by edgeR, respectively. Differential expression levels for *Nrp1* and *PGF* were calculated by subtracting human from mouse logRPKM and illustrated using a box plot.

Single-cell RNAseq – Data analysis. The sequence data was quality checked using the FastQC application (v. 0.11.2) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and the sequence reads were processed as described in Ref. [11] using the DropSeqTools package (v. 1.0),

resulting in a matrix of UMI (Unique Molecular Identifiers) counts. All downstream analysis was performed using R (v. 3.6.2) and the RStudio IDE (https://www.R-project.org/; http://www.rstudio.com/). A "knee" plot was constructed, representing the cumulative sum of reads per cell, ordered by decreasing number of reads. The inflection point was identified, and any cells beyond the inflection point were removed from the analysis due to low coverage. All data were merged into one large matrix and the counts were loaded into the Seurat package (v. 3.1.2) [12]. Cells with abnormally high mitochondrial content or UMI counts were then removed. Cells were split into two datasets, one containing the cultured cell lines, and one containing the heterotypic culture data. We applied Seurat's standard data integration procedure (as implemented in the IntegrateData function) followed by the cell type classification algorithm (as implemented in the TransferData function) to assign each of the heterotypic cells as either CAFs or 425 cells based on the reference profiles generated using the cultured lines. The dataset was then scaled, Principal Components (PCs) were calculated, and cells were projected into tSNE space using the top 30 PCs explaining the most variance in the dataset. We selected the top 30 PCs based on both a scree plot and a jackstraw permutation procedure. Cell clusters were labelled as CAFs or ICC cells based on the identities assigned from the Seurat cell classification algorithm. Transcriptional markers differentiating two clusters of cells were identified based on the Wilcoxon rank-sum test. Multiple hypothesis corrected p-values taken from the Wilcoxon rank-sum test were used to denote significance levels in single-gene violin plots (e.g., **Supplemental Figure S3**). We searched for over-represented KEGG pathways using the ClusterProfiler R package (v. 3.14.3) [13, 14]. Expression values were overlaid onto KEGG pathways of interest using the pathview R package (v. 1.26.0) [15]. To generate a pathway score, we first identified all genes involved in the pathway based on the Broad MSigDB database [16, 17]. We then calculated the score for each cell as the

sum of log-normalized expression scores for all genes in the given pathway (not just the ones identified as markers). Significance values for pathway-level violin plots (e.g., **Figure 2**) were taken from the adjusted p-values calculated from the GSEA algorithm as implemented in the R fgsea package [18]. A proportional representation plot was generated by first calculating the proportion of a group represented by each cell type (e.g., proportion of treated cells contained in CAF_2). These proportions were plotted in a stacked column graph, where each column was scaled to 100%. Note that in this study, the two groups (treatment and control) contained nearly equal numbers of cells, resulting in this plot nearly equating to a standard stacked column plot generated with raw cell numbers as input. However, using group proportions as input yields a more accurate view if group cell numbers are not equal.

Statistical analyses. The χ^2 (Chi-squared) or Fisher's test was used to compare categorical variables and Mann-Whitney u test was to compare two groups with quantitative variables. When experimental cohort includes more than three groups including quantitative variables, one-way ANOVA with Tukey's multiple comparisons test was applied. The Kaplan-Meier method was used to generate survival curves and Cox proportional hazard model, hazard ratio (HR) and 95% CI were calculated for statistical survival analyses for murine models. All analyses were performed using JMP Pro 11.2.0 (SAS Institute Inc., NC, USA) and data presented as mean ± S.E.M. Significant difference between experimental groups was determined as a p-value <0.05.

Supplementary References

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Supplementary Figure Legends

Figure S1. Expression of PlGF in human and murine intrahepatic cholangiocarcinoma (ICC). (A-B) Distribution of PlGF and its receptor Nrp1 expression analyzed by immunohistochemistry (IHC) in the whole archival specimen collection (**A**) and when segregated by tumor stage (**B**) (see also **Table S2**) (n=43). P values from Fisher's test. **(C-D)** Distribution of PlGF analyzed by RNA-in-situ hybridization (ISH) and Nrp1 expression evaluated by IHC in an annotated ICC cohort (n=52). **(E)** Representative image showing tumor blood vessels with lining cells that express PlGF by RNA-ISH, indicated by red dots of PlGF-RNA positivity. **(F)** Plasma levels of PlGF measured in control individuals (n=50), resectable (r)-ICC patients (n=39), localized (l)-ICC patients (n=21) and advanced (a)-ICC patients from 2 studies (n=17 and n=30, respectively). **(G)** Significantly higher expression of the *PGF* gene (encoding for PlGF) gene in ICC samples compared to normal tissues upon examination of the TCGA dataset using the Gene Expression Profile Interactive Analysis (GEPIA) tool (http://gepia.cancer-pku.cn/index.html). (We used a *p*-value cutoff of 0.0001.) **(H)** Expression of *PGF* and *NRP1* genes in human ICC using a published dataset and the Tumor Immune Single-cell Hub (TISCH) search engine. **(I)** Levels of PlGF secreted by ICC cells and primary hepatic stellate cells (HSCs) cultured *in vitro*, measured by ELISA. Note the increased expression in hypoxic conditions, especially in HSCs (n=3 in each group and the bar indicates SD); note the efficient reduction in PlGF secretion in shRNA-knockdown ICC cells. **(J)** Expression of Nrp1 but not Nrp2 or VEGFR1 is high both in human and murine cholangiocarcinoma cells, and comparable to that detected in endothelial cells by Western blotting.

Figure S2: Characterization of carcinoma-associated fibroblasts (CAFs) isolated from murine ICC tissues. (A) Both more quiescent qCAFs (upper panel) and activated (myofibroblast-

like) myCAFs (lower panel) expressed FAP detected by IF evaluation. Bar, 50μm. **(B, C)** myCAFs and qCAFs expressed high levels of profibrotic genes and other fibrosis-related growth factors detected by qPCR (B) and Western blotting **(C)**. **(D, E)** Top ten Gene Ontology (GO) pathway **(D)** and top ten individual genes upregulated in ICC tissue after PlGF blockade using bulk RNA sequencing analysis. **(F)** Representative double-staining for α-SMA and Nrp1 in ICC tissue from 425-bearing mice. Note the expression of Nrp1 in myofibroblast-like CAFs *in vivo*. Bar, 50μm.

Figure S3: Single cell RNA sequencing analysis of CAF and ICC cell subsets. (A) Expression of collagen 1a1 (Col1a1) and Col1a2 genes in CAF subsets and 425 ICC cells cultured from whole tumor lysate. **(B)** Cell cycle phase distribution in these cell subsets. **(C-E)** Transcriptional changes in cluster of cells from whole tumor lysate; proportion of cells expressing the Ki-67 proliferation marker from whole tumor lysate **(C)**; the cluster of cells enriched by PlGF blockade (CAF_2) showed a gene expression pattern associated with high expression of cyclin-dependent kinase inhibitors Cdkn1a and Cdkn1b and low expression of cyclins Ccnd1 and Ccnd2 **(D)**, and low expression of TGF-β2, TGF-β3 and PDGFR-β compared to CAF_1 **(E)**. NS : $p>0.01$; *$p<0.01$; **$p<0.001$; ***$p<0.0001$.

Figure S4: KEGG analysis by treatment in PlGF blockade-responsive CAF_2 subset. (A) Genes upregulated in hypoxia-inducible factor 1 pathway. **(B)** Genes upregulated in glycolysis/glucogenesis pathway.

Figure S5: Inhibition of PlGF significantly reduces the proliferation of murine and human ICC cells and human stellate cells (HSCs) and inhibits Akt signaling. (A) Exposure of HSCs to recombinant (r)PlGF increases the phosphorylation (p) of p65 and Akt and the expression of α-SMA. These effects are prevented by addition of the anti-PlGF Ab 5D11D4 (5D11), PI3K inhibitor

BKM120 or NF-kB inhibitor QNZ. **(B)** Exposure of qCAFs to rPlGF increases the phosphorylation (p) of p65 and Akt and the expression of α-SMA and TGF-β. These effects are prevented in a dose-dependent manner by addition of the anti-PlGF Ab 5D11. **(C)** Association between *PGF* (encoding for PlGF) and *ACTA2* (encoding for α-SMA) in ICC tissues from the TCGA database (n=36), analyzed using the Gene Expression Profile Interactive Analysis (GEPIA) tool (http://gepia.cancer-pku.cn/index.html); R and p values from Spearman correlation test. **(D)** Upper right, validation of genetic inhibition of PlGF using 2 shRNA constructs in 425 cells by Western blotting. Genetic inhibition of PlGF results in a small but significant reduction in the proliferation of SS49 ICC cells. P values from Mann-Whitney test. **(E-F)** PlGF blockade (500ng/ml) did not significantly affect the growth of established 425 murine ICC cell spheroids (n=29) compared to control-treated spheroids (n=45) (**E**) but significantly suppressed invasion in response to CAF-conditioned medium (425: n=8, 425+5D11: n=7; $p<0.0001$ Tukey's test) (**F**). **(G-H)** Both pharmacologic and genetic inhibition of PlGF decreases the expression of phosphorylated Akt in 425 (**G**) and SS49 (**H**) murine ICC cells, and the addition of rPlGF reverses this inhibition. **(I)** Knockdown of Nrp1 expression in human ICC (HuCCT1) cells significantly decreases Akt phosphorylation. **(J)** Stimulation with rPlGF activated Akt in Nrp1 intact cells but not in Nrp1-knocked down cells.

Figure S6: Effects of PlGF inhibition *in vivo*. (A) Effect on 5D11 blocking antibody administration on circulating levels of PlGF in mouse blood measured by ELISA. **(B)** Anti-PlGF treatment using 5D11 antibody delays tumor growth in the *Ihd2/Kras*-mutant (SS49) orthotopic model of ICC. P values from Mann-Whitney test. **(C)** Tumor incidence after orthotopic implantation of murine ICC cells in $Pgf^{+/+}$/C57Bl/6 versus $Pgf^{-/-}$/C57Bl/6 mice (n=10). Tumor take rate in wild-type mice was 70% and dropped to 50% and 30% when using Nrp1-knocked

down cells and PlGF-knocked down cells, respectively. In PlGF-deficient mice, tumor take rate was 20% and dropped to 0% when using PlGF-knocked down cells. **(D)** Representative IHC for CD31 showing vascular collapse in resected human primary ICC tissue. Scale bar, 50µm. **(E)** High magnification IF images of ICC tissue from mice treated with control (left) or anti-PlGF antibody (right). In red, collagen I; in blue, DAPI counterstaining. Bar, 500µm.

Figure S7: Effect of PlGF inhibition on desmoplasia in ICC tissues. (A, B) Representative Picrosirius Red staining and grading in tumors after PlGF inhibition (**A**) and score distribution (**B**). **(C)** Representative IHC for α-SMA and collagen I expression in murine ICC tissues. **(D, E)** PlGF blockade with 5D11 and PlGF knockdown in ICC cells reduced α-SMA+ "myofibroblast-like" CAF density **(D)**, but only PlGF blockade significantly decreased collagen I density **(E)**. P values from chi-square test in a and Tukey's test in **D**, **E**. Bar, 500µm.

Figure S8: Effects of PlGF blockade using a murine blocking antibody 5D11D4 (5D11). PlGF blockade did not significantly change the tumor associated macrophage infiltration or polarization evaluated by flow cytometry in enzymatically digested ICC tissues.

Figure S9: Effects of PlGF and NF-kB inhibition in ICC. (A, B) Effects of therapy with gemcitabine/cisplatin (GC), anti-PlGF antibody (5D11) or their combination in the 425 orthotopic ICC model; Combination therapy significantly decreased the fraction of mice with both bloody ascites **(A)** and pleural effusion **(B)**. P values from chi-square test. **(C)** *In vitro* evaluation of 425 murine ICC cell viability in spheroid, gemcitabine (Gem) induced a dose-dependent cell killing in 425 cells, but Gem cytotoxicity was not affected by concomitant of NF-kB blockade using QNZ.