Supplementary Materials and Methods

Huh7 subcutaneous tumor model and treatment

The 5×10^6 Huh7 cells suspended in 100 μL PBS were injected into the right flanks of nude mice. Starting three days after tumor inoculation, mice were randomized into treatment groups and received intratumoral injections of 100 μL CM every other day. For drug treatment groups, mice were administered lenvatinib (4 mg/kg, 5 days/week, by oral gavage) and/or zenocutuzumab (2.5 mg/kg, once weekly, intraperitoneally).

Hepa1-6 subcutaneous tumor model and treatment

Hepa1-6 cells (5×10^6 in 100 μL PBS) were injected subcutaneously into the right flanks of 6–8-week-old male C57BL/6 mice. Tumors were measured with calipers and volumes were calculated as (length×width^2)/2. When tumors reached 80–100 mm^3, mice were randomized to vehicle, lenvatinib (4 mg/kg, oral gavage, 5 days/week), CDX-3379 (5 mg/kg, intraperitoneal, twice weekly), or the combination. Treatment continued until predefined endpoints.

Flow cytometry of tumor-infiltrating CD8$^+$ T cells and TAMs

For flow cytometry, excised tumors were enzymatically and mechanically dissociated, filtered (70 μm), washed, and stained after Fc-block. CD8$^+$ T cells were identified as CD45$^+$CD3$^+$CD8$^+$ and tumor-associated macrophages as CD45$^+$CD11b$^+$F4/80$^+$. Live singlets were gated prior to lineage markers, and samples were analyzed using standard FACS protocols with the antibodies listed in Supplementary Table 1.

Hepatocellular carcinoma (HCC) orthotopic tumor model

The 2×10^6 Huh7 cells suspended in 400 μL PBS were injected into the sub-capsular space of the left liver lobe of BALB/c nude mice. Starting on day 3 post-injection, 100 μL of CM was administered intraperitoneally every other day. Mice in drug treatment groups received lenvatinib (4 mg/kg, 5 days/week, by oral gavage) and/or zenocutuzumab (2.5 mg/kg, once weekly, intraperitoneally) as described above. Mice were euthanized 3–4 weeks later, and tumor burden was assessed by liver-to-body weight ratio and gross inspection of tumor lesions.

Myc/Trp53$^{-/-}$ spontaneous HCC model

For the Myc/TP53-/- spontaneous HCC model, C57BL/6 mice were hydrodynamically (within 3–5 seconds) injected via the lateral tail with a plasmid mixture containing 10 μg sg-p53, 11.4 μg MYC-luc and 3.8 μg SB13 dissolved in normal saline at a final volume of 10% of the body weight of mice. Lenvatinib was administered by oral gavage at 4 mg/kg, 5 days per week. Tumor burden was assessed twice weekly by in vivo bioluminescence imaging. Baseline for response evaluation was the first day of lenvatinib dosing. Initial response (on-treatment shrinkage): ≥30% decrease from baseline on two consecutive measurements. Acquired resistance: subsequent regrowth from the nadir of ≥30% on two consecutive measurements under continuous lenvatinib.

In vivo limiting dilution tumor initiation assay

Huh7 cells resuspended in cold PBS were subcutaneously injected into the right flanks of 4–5-week-old male BALB/c nude mice at serial dilutions of 1×10^6, 1×10^5, and 1×10^4 cells per mouse (100 μL per injection). Each group included at least five mice per dose. After tumor cell inoculation, mice were treated with intratumoral injections of 100 μL CM every other day, following the same regimen as in other in vivo experiments. Tumor formation was monitored twice weekly, and tumors larger than 100 mm^3 within 6–8 weeks were considered positive for tumor initiation. Tumor-initiating frequency was calculated using the Extreme Limiting Dilution Analysis (ELDA) software (http://bioinf.wehi.edu.au/software/elda/), and statistical significance between groups was determined by the built-in chi-square test.

Cell lines and cell culture

Cell lines and cell culture. Human HCC cell lines Huh7 (RRID:CVCL_0336; Guangzhou jenniobio Biotechnology,

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cat. no. JNO-22049), Hep3B (RRID:CVCL_0326; Guangzhou Jenniobio Biotechnology, cat. no. JNO-H0158), human monocytic THP-1 (RRID:CVCL_0006; Guangzhou Jenniobio Biotechnology, cat. no. JNO-H0561), and HEK293T (RRID:CVCL_0063; Guangzhou Jenniobio Biotechnology, cat. no. NO-H0488) were obtained from Guangzhou Jenniobio Biotechnology and were supplied with STR (short tandem repeat) authentication certificates. All cell lines tested negative for mycoplasma contamination; detailed STR authentication reports and mycoplasma test reports are provided in Supplementary Table 2 and uploaded as Supplementary Files. Huh7 and Hep3B cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. THP-1 cells were maintained in RPMI-1640 supplemented with 10% FBS. HEK293T cells were cultured in DMEM supplemented with 10% FBS. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Macrophage differentiation

THP-1 monocytes were differentiated into macrophage-like cells by treatment with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 48 hours. Following differentiation, cells were washed with PBS and maintained in fresh RPMI-1640 medium supplemented with 10% FBS for an additional 24 hours prior to downstream experiments. Differentiation was validated by adherence and morphological changes under phase-contrast microscopy.

Primary human macrophages (MDMs and TAMs)

Peripheral blood and tumor tissues were collected from treatment-naïve HCC patients undergoing surgical resection with informed consent and ethics approval. PBMCs were isolated by Ficoll density-gradient centrifugation, and $CD14^+$ monocytes were enriched magnetically for immediate use. To generate monocyte-derived macrophages (MDMs), purified $CD14^+$ cells were cultured in RPMI-1640 with 10% FBS, antibiotics, and M-CSF (50 ng/mL) for 7 days to allow differentiation. Tumor-associated macrophages (TAMs) were obtained by enzymatic digestion of fresh HCC tissue followed by $CD14^+$ magnetic selection and overnight recovery in macrophage medium. For flow cytometric validation, cells were stained for CD45, CD11b, and CD68, and macrophages were identified as $CD45^+CD11b^+CD68^+$ cells.

CRISPR/dCas9-SAM-Mediated Gene Activation in THP-1 Cells

Briefly, 5×10^5 THP-1 cells were plated in 6-well plates, cultured in growth medium without antibiotics for 24 h, and then transduced with lentiviral supernatant containing dCas9-VP64 (GeneChem Co. Ltd., China) at a final concentration of 8 mg/mL polybrene. Twenty-four hours after transduction, lentiviral supernatant was replaced by selection medium. THP-1 cells were initially transduced with a dCas9-VP64-GFP lentiviral vector (GeneChem Co., Ltd., Shanghai, China) in the presence of 8 μg/mL polybrene. After 48 hours, GFP-positive cells were isolated via fluorescence-activated cell sorting (FACS) using a 488 nm laser and appropriate gating strategies to ensure high-purity selection of transduced cells. Subsequently, cells were transfected with plasmids encoding the MS2-p65-HSF1-sgRNA (Kidan Bio Co. Ltd., China) using Lipo8000 reagent (Beyotime, China), following the manufacturer's protocol. Briefly, plasmid DNA and Lipo8000 were mixed in Opti-MEM medium and incubated for 15–20 minutes at room temperature to allow complex formation. The mixture was then added to THP-1 cells in suspension at a density of 1×10^6 cells/mL. After 6 hours, the transfection medium was replaced with fresh complete medium, and cells were incubated for an additional 48–72 hours prior to downstream analysis. Following confirmation of target gene activation by qRT–PCR, successfully modified THP-1 cells were induced to differentiate into macrophages via treatment with 100 nM PMA for 48 hours. The resulting gene-activated macrophages were then used for downstream assays. The efficient sgRNA sequences of 58 candidate genes are listed in Supplementary Table 3.

Chemicals and recombinant proteins

Lenvatinib (E7080) was purchased from TargetMol (Catalog #T0520). Lenvatinib was diluted to final concentrations as needed, with the final DMSO concentration not exceeding 0.1% (v/v). For in vivo experiments, was administered by oral gavage at 4 mg/kg, 5 days/week. Zenocutuzumab

was obtained from MedChemExpress (Monmouth Junction, NJ, USA) and dissolved in PBS for in vivo use at a concentration of 20 mg/mL. For in vitro experiments, zenocutuzumab was diluted to final concentrations as required. CDX-3379 (anti-HER3 monoclonal antibody; Cat. No. HY-P991240) was purchased from MedChemExpress (Monmouth Junction, NJ, USA). For in vivo experiments, CDX-3379 was administered at 5 mg/kg via intraperitoneal injection twice weekly.

rNRG1 (recombinant human Neuregulin 1) was purchased from MCE (Catalog #HY-P74709, 100 μg) and used at a final concentration of 10 ng/mL for in vitro assays.

Cell proliferation, colony formation, apoptosis, and live/dead staining assays

For the cell proliferation assay, 1,000 Huh7 or Hep3B cells were seeded into 96-well plates and cultured in conditioned medium (CM) derived from either vector- or COLEC12^{OE}-transduced macrophages, with or without lenvatinib (2.5 μM). Cell viability was assessed daily for 5 consecutive days using the Cell Counting Kit-8 (CCK-8; Dojindo, Japan).

For the colony formation assay, 1,000 cells were seeded into 6-well plates and cultured for approximately 10 days in the indicated CM, supplemented with increasing concentrations of lenvatinib (0, 2.5, or 5 μM). At the endpoint, colonies were fixed with 4% paraformaldehyde for 15 minutes, stained with 0.1% crystal violet for 20 minutes, washed with PBS, and air-dried. Colonies containing more than 50 cells were manually counted under a light microscope.

For apoptosis analysis, HCC cells were treated with lenvatinib (2.5 μM) in the indicated CM conditions for 48 hours. Apoptotic cells were stained using Annexin V/APC and 7-AAD (KeyGEN BioTECH, China), following the manufacturer's protocol. Flow cytometric analysis was performed using a BD FACSCanto II cytometer. The gating strategy is illustrated in Supplementary Figure 5.

For live/dead staining, Huh7 cells were co-cultured with gene-activated THP-1-derived macrophages in transwell chambers and treated with lenvatinib (2.5 μM) for 48 hours. Cells were then washed twice with PBS and incubated with freshly prepared Calcein-AM/PI working solution, consisting of 1 μM Calcein-AM and 1 μg/mL propidium iodide (PI) in detection buffer, at 250 μL per well (24-well plate). Staining was performed at 37°C for 30 minutes in the dark, followed by a PBS wash and replacement with fresh serum-free medium. Fluorescence images were captured immediately using an inverted fluorescence microscope. Viable cells fluoresced green (Calcein-AM), and dead cells fluoresced red (PI). All experiments were conducted in triplicate unless otherwise specified.

Genetics of stably expressing cell lines

To generate THP-1-derived macrophages stably overexpressing COLEC12 (COLEC12^{OE}), cells were transduced with lentiviral particles carrying full-length human *COLEC12* under the EF1α promoter (Kidan Bio Co. Ltd., China). For *NRG1* knockdown, THP-1-derived macrophages were transduced with lentiviral particles encoding short hairpin RNAs targeting *NRG1* (shNRG1#1 and shNRG1#2) or a non-targeting scrambled control. Cells were mixed with lentiviral particles and centrifuged at 1,000 *g* for 2 hours at room temperature to enhance infection efficiency. After 48 hours, cells were selected with 1 μg/mL puromycin (A1113803, Invitrogen) for 7–10 days. After 2–3 passages under selection, the resulting cell populations were used directly for downstream experiments without single-cell cloning. Targeting sequences are provided in Supplementary Table 4.

Small interfering RNA for *STAT3*, *JUN*, *RELA*, and *KLF4*

Small interfering RNAs (siRNAs) targeting *STAT3*, *JUN*, *RELA*, and *KLF4* were purchased from GenePharma (Shanghai, China). Reverse transfection was performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 24 hours, the culture medium was replaced with fresh complete medium. Cells were collected 48 hours after transfection, and knockdown efficiency was evaluated by qRT-PCR prior to downstream functional assays. Targeting sequences are provided in Supplementary Table 4.

Tumor sphere-forming assay

For the tumor sphere-forming assay, HCC cells (Huh7 or Hep3B) were plated into ultra-low attachment 6-well plates

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at a density of 1,000 cells per well in serum-free DMEM/F12 medium supplemented with 20 ng/mL epidermal growth factor (EGF), 20 ng/mL basic fibroblast growth factor (bFGF), and B27 supplement (1×; Gibco). Cells were cultured under non-adherent conditions for 7–10 days to allow tumorsphere formation. Where indicated, cells were treated with CM. Tumorspheres were imaged using an inverted microscope, and spheres larger than 100 μm in diameter were counted manually from at least five random fields per well. Sphere-forming efficiency was calculated as the number of spheres formed divided by the number of cells initially seeded. All experiments were performed in triplicate and repeated independently at least three times.

In vitro limiting dilution tumorsphere formation assay

To assess tumor-initiating cell frequency in vitro, a limiting dilution tumorsphere formation assay was performed. HCC cells (Huh7 or Hep3B) were seeded into ultra-low attachment 96-well plates at graded cell densities of 1, 5, 10, 20, 50, and 100 cells per well in serum-free DMEM/F12 medium supplemented with 20 ng/mL EGF, 20 ng/mL bFGF, and B27 supplement (1×; Gibco). For each condition, 10 replicate wells were prepared per cell density.

Cells were cultured under non-adherent conditions for 10–14 days with CM. Wells were scored as positive if they contained at least one tumorsphere larger than 100 μm in diameter. Tumor sphere-forming frequency was calculated using the ELDA software (http://bioinf.wehi.edu.au/software/elda/), and statistical comparisons between groups were performed using the chi-square test provided by the ELDA model. All assays were performed in triplicate and independently repeated with consistent results.

Immunoblotting (IB)

Cells were harvested and lysed in RIPA buffer (Beyotime, China) supplemented with protease and phosphatase inhibitors (TargetMol, USA). Protein concentrations were measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Equal amounts of total protein (20–40 μg) were resolved by SDS–PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% bovine serum albumin (BSA; Beyotime, China) in TBST for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies against the following targets: β-Actin (Absin, Abs830031ss, 1:1,000), CD68 (Proteintech, 66231-2-Ig, 1:1,000), COLEC12 (Affinity, DF10165-100, 1:1,000), NRG1 (Proteintech, 66492-1-Ig, 1:1,000), HER3 (Proteintech, 10369-1-AP, 1:1,000), phospho-HER3 (Tyr228; SAB, SAB243, 1:1,000), HER2 (CST, #2165, 1:1,000), phospho-HER2 (Tyr1221/1222; CST, #2243, 1:1,000), STAT1 (CST, #14994, 1:1,000), phospho-STAT1 (Tyr701; CST, #9167, 1:1,000), phospho-STAT1 (Proteintech, 28979-1-AP, 1:1000), STAT3 (CST, #9139, 1:1,000), phospho-STAT3 (Tyr705; CST, #9145, 1:1000), AKT (CST, #9272, 1:1000), and phospho-AKT (Ser473; CST, #9271, 1:1000). After washing with TBST, membranes were incubated for 1 hour at room temperature with HRP-conjugated secondary antibodies: anti-mouse IgG (CST, #7076S, 1:3000) or anti-rabbit IgG (CST, #7074S, 1:3000). Immunoreactive bands were detected using an enhanced chemiluminescence (ECL) kit (Millipore), and images were acquired using a ChemiDoc MP Imaging System (Bio-Rad). Densitometric analysis was performed using ImageJ software. A detailed list of antibodies is provided in Supplementary Table 5.

Immunoprecipitation (IP) and mass spectrometry analysis of COLEC12 interaction

Cells were transfected with COLEC12-FLAG expression plasmid (purchased from GeneDenovo Biotechnology, Guangzhou, China) and harvested 48 h post-transfection. Cells were lysed with IP lysis buffer (Thermo Fisher Scientific) supplemented with proteinase inhibitor (Sigma-Aldrich), incubated on ice for 15 min, and cleared by centrifugation at 12,000×g at 4 °C for 20 min. After the pre-clearing step with protein G-agarose beads (Thermo Fisher Scientific), cell lysate (2 mg) was subjected to IP with the indicated antibodies (anti-FLAG antibody, Cell Signaling Technology, #14793, 4 μg; Rabbit IgG, Proteintech, B900610, 2 μg) overnight at 4 °C. Then, immune complexes were washed three times in cold lysis buffer. The input and output samples were resolved by SDS-PAGE and detected by immunoblot analysis with the indicated primary antibodies: anti-FLAG antibody (CST, #14793, 1:1,000 dilution) and anti-STAT1 antibody (CST, #14994, 1:1,000 dilution). As a secondary antibody, we used anti-rabbit IgG (Cell Signal-

ing Technology, 7074S, 1:3,000 dilution) (Supplementary Table 4). The uncropped and unprocessed scans of the blots were provided in the Source Data file.

For mass spectrometry analysis, the FLAG immunoprecipitates were separated by SDS-PAGE. Protein bands were excised, digested with trypsin, and subjected to LC-MS/MS. Identified proteins were analyzed using the Mascot search engine and ranked by unique peptide counts.

Immunofluorescent (IF) and immunohistochemical (IHC) staining

For IF analysis of human HCC tissues, we used formalin-fixed paraffin-embedded tissues (FFPE). Tissue sections were deparaffinized, rehydrated, and subjected to antigen retrieval. Sections were permeabilized with PBS containing 0.1% Triton X-100 and blocked with AquaBlock (East Coast Bio, North Berwick, ME). The sections were probed with the following primary antibodies: anti-COLEC12 antibody (Affinity, DF10165-100, 1:100 dilution) and anti-CD68 antibody (Proteintech, 66231-2-Ig, 1:100 dilution). After washing the slides with PBS-T three times, the sections were incubated with Alexa Fluor™ 594 goat anti-rabbit IgG (H+L) (Thermo Fisher Scientific, #R37117, 1:200 dilution) or Alexa Fluor™ 488 goat anti-mouse IgG (H+L) (Thermo Fisher Scientific, #A-11008, 1:200 dilution) secondary antibodies and DAPI-containing mounting solution VECTASHIELD (Vector Laboratories). The slides were visualized by using a Nikon inverted microscope Eclipse Ti-U equipped with a digital camera or a Nikon A1 laser scanning confocal microscope at the Center for Advanced Microscopy/Nikon Imaging Center (CAM).

For IF analysis of mouse subcutaneous tumors, FFPE were used. Sections were processed as described above. Cells were probed with the following primary antibodies: anti-SOX2 antibody (CST, #3579, 1:100 dilution) and anti-OCT4 antibody (Abcam, ab19857, 1:100 dilution); or anti-CD44 antibody (Proteintech, 15675-1-AP, 1:100 dilution) and anti-CD133 antibody (Miltenyi Biotec, 130-090-422, 1:100 dilution). After washing the slides with PBS-T three times, the sections were incubated with Alexa Fluor™ 594 goat anti-rabbit IgG (H+L) or Alexa Fluor™ 488 goat anti-mouse IgG (H+L) secondary antibodies (both 1:200 dilution) and mounted using DAPI-containing VECTASHIELD (Vector Laboratories). Imaging was performed as described above.

For IHC staining, all human tissue research in this study was conducted according to protocols approved by the Sun Yat-sen University Cancer Center, Guangzhou, China. The HCC tissue sections were stained with the following antibody: anti-NRG1 antibody (Proteintech, 66492-1-Ig, 1:200 dilution) as described in Supplementary Table 4.

RNA-seq and quantitative RT real-time PCR (qPCR)

Total RNA was extracted using RNA-Quick Purification Kit (ES Science, Guangzhou, China) and cDNA synthesis using the Prime-Script cDNA synthesis kits (Invitrogen, California, USA) according to the manufacturer's instructions. The reverse-transcribed cDNA products were used for qPCR analysis using SYBR Green PCR kit (Invitrogen, California, USA). Supplementary Table 6 includes detailed information about the sequence of the used primers.

Primary HCC cells and PDO

Freshly resected HCC specimens were immediately immersed in ice-cold Tissue Preservation Solution E (Absin) and delivered on ice to the laboratory within 1 hour. Tissues were minced into 1–3 mm^3 pieces, rinsed three times with ice-cold Primary Culture Buffer B (Absin), and digested in Human Hepatobiliary Cancer Primary Tissue Digestion Solution C (Absin) at 37 °C on an orbital shaker for 15–25 min until abundant single cells or clusters<70 μm were evident. Digestion was stopped by adding three volumes of cold Buffer B; the suspension was passed through a 100 μm nylon strainer and centrifuged for 5 min at 300 *g*, 4°C. Viable cells were counted by trypan-blue exclusion, and primary HCC cells were used for downstream experiments. For PDO culture, 5×10^3–1×10^4 viable cells were mixed with ice-cold Matrigel Basement Membrane Matrix (Absin, abs9495) at a 1 : 25 pellet-to-Matrigel ratio, and 25 μL domes were seeded in pre-cooled 24-well plates, polymerised at 37°C for 10–15 min, and overlaid with 500 μL Human Hepatobiliary Cancer Organoid Medium A (Absin). Cultures were maintained at 37°C, 5% CO_2 with medium changes every 3–4 days. Organoids were passaged 1:3 every 2–3 weeks by mechanical disruption in Organoid Passage Buffer G or brief enzymatic dissociation in Passage Digestion Solution D (both Absin) before re-embed-

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ding in fresh Matrigel.

PDX

Human HCC tumor tissue was acquired from patients by surgery. Fresh tissues were rinsed with PBS and divided into 20–30 mm^3 pieces. The tumor tissues were subcutaneously transplanted into five-week-old NOD/SCID mice. After the tumor volume reached an average size of 50–100 mm^3, mice were divided into groups and daily treated with lenvatinib (4 mg/kg, 5 days/week, by oral gavage) and/or zenocutuzumab (2.5 mg/kg, once weekly, intraperitoneally) as described above. Mice were euthanized 3–4 weeks later, and tumor burden was assessed by liver-to-body weight ratio and gross inspection of tumor lesions.

Mice were euthanized by CO_2 asphyxiation for tumor harvesting after the appearance of tumors with a diameter greater than 1.5 cm in any group. The weights of the excised tumors were recorded.

Immunohistochemistry for pAKT, pHER2, and pHER3 in Huh7 and Hepa1-6 subcutaneous tumors

Immunohistochemistry for phospho-signaling was performed on tumours harvested from Huh7 BALB/c nude and Hepa1-6 C57BL/6J subcutaneous xenograft models at the experimental endpoint. Tumours were excised, fixed in 10% neutral-buffered formalin for 24–48 h, embedded in paraffin, and sectioned at 4 μm. After deparaffinization in xylene and rehydration through graded ethanol, sections underwent heat-induced epitope retrieval in citrate buffer (10 mM, pH 6.0). Endogenous peroxidase activity was quenched with 3% H_2O_2 for 10 min, followed by blocking with 5% bovine serum albumin for 30 minutes at room temperature. Slides were then incubated overnight at 4°C with the following primary antibodies: anti-phospho-AKT (Ser473; Cell Signaling Technology, catalog #9271), anti-phospho-HER2 (Tyr1221/1222; Cell Signaling Technology, catalog #2243), or anti-phospho-HER3 (Tyr228; SAB, catalog SAB243), each diluted 1:200. After washing, sections were incubated with HRP-conjugated secondary antibodies for 30 minutes at room temperature, developed with DAB chromogen, counterstained with hematoxylin, dehydrated, and mounted. Images were acquired under a light microscope at 200× magnification. Staining for p-AKT, p-HER2 and p-HER3 was quantified by H-score. For each tumour, the percentage of cells with 0, 1+, 2+ or 3+ intensity was estimated in multiple high-power fields, and H-scores were calculated as 1×(%1+)+2×(%2+)+3×(%3+) (range 0–300). Individual H-scores were plotted for each mouse in the indicated treatment groups.