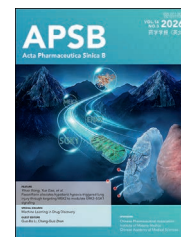




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ORIGINAL ARTICLE

Identification of a small-molecule targeting PLAGL2 DNA-binding domain inhibits extracellular matrix formation and enhances lenvatinib sensitivity in hepatocellular carcinoma



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KEY WORDS

Hepatocellular carcinoma;
Extracellular matrix
remodeling;
Hepatic stellate cells;
Lenvatinib

Abstract The increased stiffness of the extracellular matrix (ECM) is known to promote the progression of hepatocellular carcinoma (HCC). Currently, there are no approved therapies for targeting ECM sensors and remodelers. The objective of this study was to identify the molecular mechanisms underlying the role of Pleomorphic adenoma gene-like 2 (PLAGL2) in HCC ECM remodeling and to formulate compounds that effectively inhibit PLAGL2 transcriptional regulation. Our work revealed that PLAGL2 remodeled the ECM produced by HCC cells *via* an autocrine mechanism and activated HSCs *via* a paracrine pathway. Mechanistically, PLAGL2 functioned as a transcriptional regulator of insulin-like growth factor 2 (IGF2) and insulin-like growth factor 1 receptor (IGF1R). IGF2 enhanced ECM remodeling by HCC cells and activated HSCs through the IGF1R–PI3K–Akt signaling pathway. Furthermore, using a computer-aided drug design strategy, a novel compound, DC218, derived from the chemical evolution of cytosine, has been developed for the first time to exhibit specificity as an inhibitor of the PLAGL2 DNA binding domain. DC218 significantly degraded ECM, overcame lenvatinib resistance, and synergistically inhibited HCC. These findings provide mechanistic insight into the role of PLAGL2 in HCC ECM remodeling, as well as suggest a novel strategy for inhibiting ECM and treating HCC.

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1. Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths globally, posing a significant public health challenge due to its low five-year survival rate. Potential curative treatment options, such as liver transplantation, hepatectomy, and radiofrequency ablation, are available but require early-stage diagnosis. Sorafenib and Lenvatinib have been approved as first-line therapies for advanced HCC¹. Atezolizumab (a PD-L1 inhibitor) combined with bevacizumab (a VEGF inhibitor) showed superior overall and progression-free survival outcomes than sorafenib in patients with unresectable HCC². However, treatment of HCC remains challenging. Therefore, a better understanding of the molecular mechanisms underlying HCC development can help identify new therapeutic targets³.

The extracellular matrix (ECM), a crucial regulator of the tumor microenvironment, dictates cancer development and progression⁴. The ECM is a dynamic system comprising various proteins, including collagen, glycoproteins, proteoglycans, fibronectin, and laminin. The deposition and cross-linking of ECM proteins stiffen tumor tissue, thereby fostering tumor growth and invasion⁵. The expression of various ECM proteins is typically increased in HCC, and both ECM stiffness and viscoelasticity can promote HCC progression^{6,7}. Numerous anticancer strategies have focused on ECM components; however, drug development targeting this therapeutic area has progressed slowly. Cancer-associated fibroblasts (CAFs) are regarded as the main producers of ECM in tumor tissues⁸. Hepatic stellate cells (HSCs) and tumor cells that synthesize multiple ECM proteins are key producers in HCC⁹. Understanding ECM protein regulation and identifying key mediators are vital for ECM-targeted tumor progression inhibition. Targeting the genes that promote ECM formation is a promising therapeutic approach.

Pleomorphic adenoma gene-like 2 (PLAGL2), PLAG1, and PLAGL1 belong to the PLAG gene family of zinc finger transcription factors. PLAGL2 is overexpressed in multiple tumors and is crucial for tumor progression^{10–13}. Oligonucleotide microarray analysis identified PLAG1 target genes that encode many

ECM proteins¹⁴. PLAGL2 and PLAG1 share high homology, but ECM regulation by PLAG proteins remains unclear.

In the present study, we revealed that PLAGL2 is involved in ECM deposition/remodeling and HSC activation. PLAGL2 acts as a transcriptional regulator of IGF2 and IGF1R in HCC cells. HCC cells release IGF2 into their local microenvironment. The IGF1R–PI3K/Akt pathway is activated *via* autocrine and paracrine IGF2 in HCC and LX-2 cells, respectively, which in turn promotes the secretion of multiple ECM proteins. Therefore, targeting the PLAGL2 oncoprotein to inhibit ECM formation is a novel strategy for treating HCC. Following multiple rounds of screening, DC218, obtained from the chemical evolution of cytosine, was developed for the first time to exhibit specificity as an inhibitor of the PLAGL2 DNA-binding domain. DC218 significantly inhibited HCC ECM production and exhibited a synergistic effect with lenvatinib in inhibiting HCC progression, thus providing a new potential therapeutic strategy for HCC.

2. Materials and methods

2.1. Hepatocellular carcinoma (HCC) tissue samples

HCC tissue samples used for qRT-PCR were surgically resected from patients at Nanjing Drum Tower Hospital (Nanjing, China). Fresh surgical specimens were immediately frozen in liquid nitrogen and stored at -80°C for analysis. An ethics permit was obtained from the Nanjing Drum Tower Hospital ethics committee (approval No. 2022-713-02), and informed consent was obtained from all patients involved in the study. Information on the human tissue samples is summarized in Supporting Information Tables S1 and S2.

2.2. Hydrodynamic injection mouse HCC model and *in vivo* drug studies

For HCC induced by hydrodynamic tail vein injection (HTVi), C57BL/6 mice aged 6–7 weeks were used. To generate the Nras-driven HCC model ($n = 6$), for each mouse, 20 μg of pT3-c-Myc, 20 μg of pT3-NRasV12, 20 μg of pT3-mPLAGL2-T2A-

EGFP/pT3-EGFP (20 µg), and with SB100 × transposase plasmids (in a ratio of 25:1 of the total plasmid mass) were dissolved in 2 mL saline (0.9% NaCl), filtered through 0.22 µm filter (Millipore, Billerica, MA, USA), 2 mL of the plasmid mixture was hydrodynamically injected (7–8 s) through the tail vein to the liver *in vivo*.

For *in vivo* drug studies, C57BL/6 mice at 6–7 weeks were used for *in vivo* drug studies. To generate the Nras-driven HCC model, ALT and AST levels were measured, mice were grouped according to serum ALT and AST levels and subjected to treatment with vehicle ($n = 6$), lenvatinib (4 mg/kg/day) ($n = 6$), DC218 (60 mg/kg/day, i.p.) ($n = 6$), or lenvatinib (4 mg/kg/day) combined with DC218 (60 mg/kg/day, i.p.) ($n = 6$).

All animal experimental protocols were approved by the Animal Ethics Committee of the Center for New Drug Evaluation and Research, China Pharmaceutical University (Nanjing, China) (approval no. B20190624-1).

2.3. Human cytokine array analysis

A cytokine antibody array containing 440 human cytokines (GSH-CAA-440, RayBiotech, Peachtree Corners, GA, USA) was utilized to detect cytokines that were differentially secreted in the serum-free medium of Huh-7-pLvz-zsGreen and Huh-7-pLvz-PLAGL2 cells. Supernatant from the same number of cultured cells was collected after 48 h, and an equal amount of culture supernatant was collected. The cytokine array analysis was performed according to the manufacturer's protocols.

2.4. Xenografted tumor model and *in vivo* drug studies

For *in vivo* drug studies, 5-week-old male athymic BALB/c nude mice were purchased from GemPharmatech Co., Ltd. (Nanjing, China). Animals were assigned a group designation and weighed. A total of 24 animals were divided into 4 different groups (6 animals per group). Each animal was assigned a temporary random number within the weight range group. HCCLM3 cells transfected with shCtrl and shPLAGL2 or transfected with pLvz-zsGreen and pLvz-PLAGL2 were applied in animal experiments. 1×10^6 transfected HCCLM3 cells were injected into the right posterior flank of the 5-week-old-male BALB/c nude mice ($n = 6$), when the tumor volume reached approximately 100 mm³ in size, animals were randomized after using a computer based random order generator, and subjected to the treatments with vehicle, lenvatinib (4 mg/kg/day) or DC218 (30 mg/kg/day, and 60 mg/kg/day, i.p.). Tumor size was measured every 2 days, and tumor volume was calculated by Eq. (1):

$$\text{Tumor volume} = 0.5 \times (\text{Long diameter}) \times (\text{Short diameter})^2 \quad (1)$$

At the end of the study, mice were sacrificed and tumors were isolated, photographed, and weighed. H&E and IHC staining were performed on paraffin-embedded xenograft tumors following the standard protocols. All animal experimental protocols were approved by the Animal Ethics Committee of the Center for New Drug Evaluation and Research, China Pharmaceutical University (Nanjing, China) (B20201025-1).

2.5. Liver metastasis model in BALB/c nude mice

HCCLM3-pLvz-zsGreen-shCtrl-mCherry, HCCLM3-pLvz-PLAGL2-shCtrl-mCherry, or HCCLM3-pLvz-PLAGL2-shIGF2-mCherry cells (1×10^6) were injected into the spleen of

5–6-week-old male BALB/c nude mice ($n = 5$) (GemPharmatech Co., Ltd.). Approximately 3 weeks later, the mice were euthanized, and the liver was removed. The livers were fixed in 4% paraformaldehyde and subjected to H&E and IHC staining.

2.6. Adeno-associated virus and *in vivo* studies

SiPLAGL2 and siCtrl were cloned into the AAV-32 pscAAV-U6-CMV-EGFP vector according to the manufacturer's instructions. pAAV-RC8 and pHelper vectors were co-transfected with the genomic plasmid into HEK293 cells to pack the Adeno-associated virus, and the particles were further amplified and purified.

BALB/c mice were orthotopically implanted with H22 tumors. For knockdown of PLAGL2 in H22 tumor, 100 µL of 1×10^{11} active viral particles of adeno-associated virus expressing shPLAGL2 or shCtrl were injected into BALB/c mice. After 14 days, mice were sacrificed. H22 orthotopic tumors were isolated for subsequent testing.

To generate the Nras-driven HCC model, 2 weeks later, mice were grouped according to serum ALT and AST levels, and 100 µL of 1×10^{11} active viral particles of adeno-associated virus expressing shPLAGL2 or shCtrl were injected into C57BL/6 mice. After 3 weeks, the mice were sacrificed. Nras-driven orthotopic tumors were isolated for subsequent testing.

Human HCC patient-derived xenografts (PDX) were injected into NCG immunodeficient mice. For knockdown of PLAGL2 in PDX, 100 µL of 1×10^{11} active viral particles of adeno-associated virus expressing shPLAGL2 or shCtrl were injected into the tumor. At the end of the study, mice were sacrificed. PDX tumors were isolated for subsequent testing.

2.7. Patient-derived xenograft (PDX) model and *in vivo* drug studies

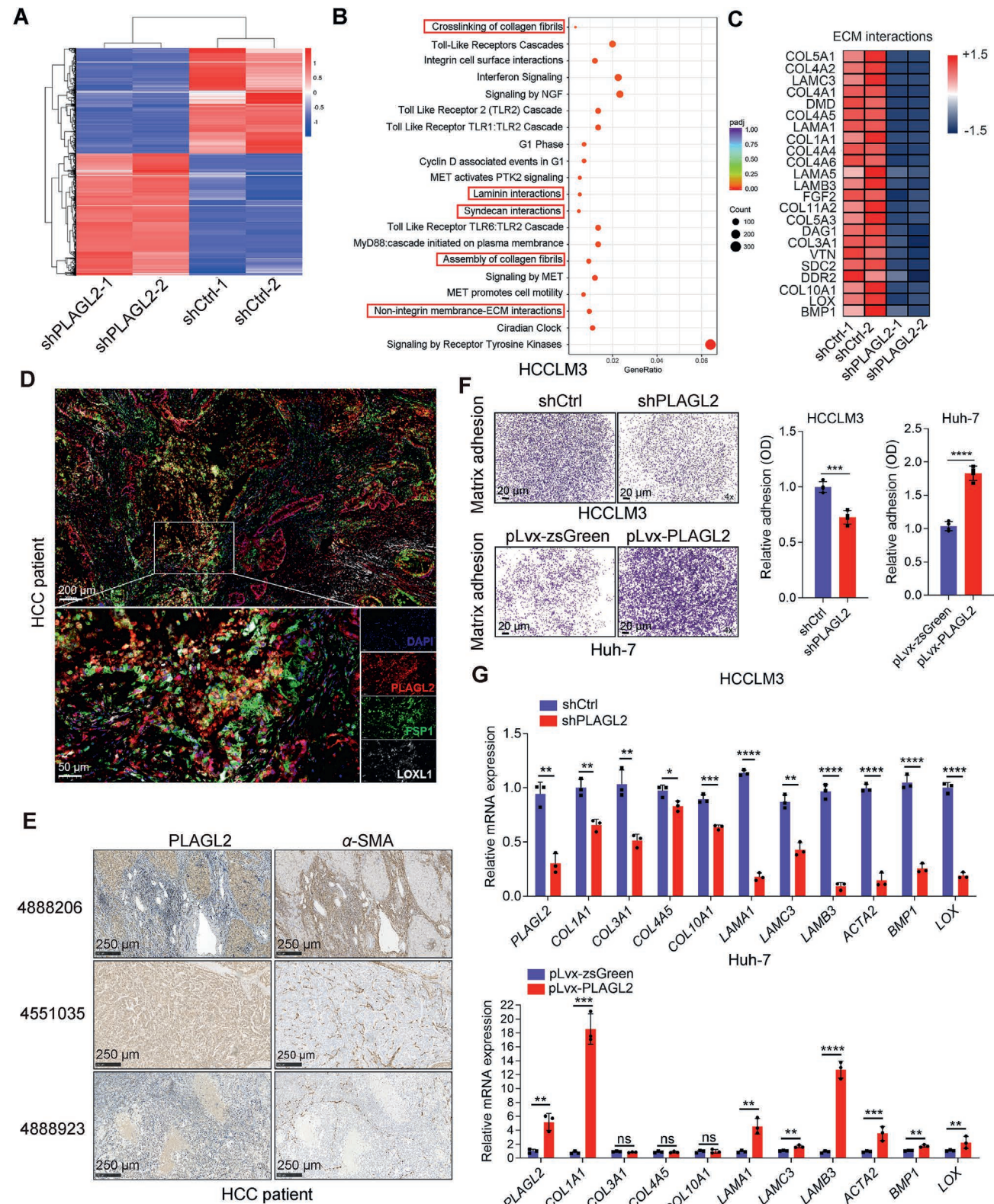
The patient-derived xenograft (PDX) model was established according to previous studies. Briefly, 1 cm × 1 cm × 0.5 cm tumors were collected from the patients, cut into 20–30 mm³ slices, and immediately implanted subcutaneously by using an 18-gauge trocar into the flanks of 6–8-week-old female NOD-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/Nju (NCG) mice (GemPharmatech Co., Ltd.). After 3 serial passages *in vivo*, a tumor with a volume of 1 mm × 1 mm × 4 mm was grafted into the flanks of NCG mice. After 3 days, PDXs were treated with vehicle or DC218 (60 mg/kg/day, i.p.). At the end of the study, mice were sacrificed and tumors were isolated, photographed, and weighed. H&E and IHC staining were performed on paraffin-embedded xenograft tumors following the standard protocols.

2.8. DEN/CCl₄-induced hepatocarcinoma model

DEN (25 mg/kg) was injected intraperitoneally into 14-day-old PLAGL2^{fl/fl} ($n = 5$) and PLAGL2^{fl/fl} Alb-cre male mice ($n = 5$), followed by intraperitoneal injection of 10% CCl₄ (5 mL/kg) dissolved in corn oil (Sigma–Aldrich, St Louis, MO, USA) weekly for a total of 17 weeks. Mice were sacrificed and processed for further studies at 5 months of age.

2.9. Efficacy of targeted drugs on tumor organoids

The sensitivity of tumor organoids to DC218 and Lenvatinib was evaluated *in vitro*. Organoids of tumors were digested and seeded in Matrigel droplets at a density of 2×10^3 – 5×10^3 cells. On Day



7, a series of experimental concentrations of each drug compound was added to the cultures. On the seventh day of drug treatment, the organoids were photographed, and their diameters were measured. The results were normalized to the DMSO control. All experiments were performed in triplicate.

2.10. Patient-derived tumor organoid (PDO) culture

Upon fresh resected tissues' arrival, approximately 1–2 mm³ pieces of tumor tissue were minced and incubated at 37 °C for 0.5–1 h in a digestion solution. In the digestion solution, 0.125 mg collagenase IV (Sigma–Aldrich) and 0.1 mg DNase (Sigma–Aldrich) were added. After filtering through a nylon cell strainer, the suspension was centrifuged for 3 min at 450 rpm. After washing the pellet, it was mixed with DMEM/F12 (Invitrogen, Carlsbad, CA, USA) and seeded into 24-well plates pre-warmed with Matrigel (CORNING, New York, NY, USA) at a concentration of 3×10^3 cells to 5×10^3 cells. Tumor organoid culture medium consisted of the following agents: advanced DMEM/F-12 (Invitrogen) supplemented with B-27 (1:50, Invitrogen), 10 mmol/L HEPES (Invitrogen), 1% penicillin/streptomycin (Invitrogen), 1% glutamax (Invitrogen), 1:100 N₂ supplement (Invitrogen), *N*-acetyl-L-cysteine (1.25 mmol/L, Sigma–Aldrich), Nicotinamide (10 mmol/L, Sigma–Aldrich), recombinant human epidermal growth factor (EGF, 50 ng/mL, PeproTech, Cranbury, NJ, USA), recombinant human fibroblast growth factor (FGF10, 100 ng/mL, PeproTech), recombinant human hepatic growth factor (HGF, 25 ng/mL, PeproTech), R-spondin 1 (100 ng/mL, PeproTech), Noggin (100 ng/mL, PeproTech), Wnt3a (100 ng/mL, Fitzgerald, New York, NY, USA), Forskolin (10 μmol/L, Selleck, Houston, TX, USA), and A8301 (5 μmol/L, Selleck). The culture medium was changed twice a week. When the cultures became dense, the organoids were split at a 1:3 ratio and subsequently stored in liquid nitrogen.

2.11. Chromatin immunoprecipitation (ChIP) assay

To measure the binding activity of PLAGL2 *in vivo*, Hep3B cells were used for the ChIP assay, which was conducted with the EZ-Magna ChIP A/G (Millipore) according to the manufacturer's protocol. In brief, 1×10^7 cells were crosslinked with 4% paraformaldehyde, lysed with sodium dodecyl sulfate buffer, and sonicated. After sonication, fragmented chromatin was added to the ChIP dilution buffer and incubated overnight with anti-PLAGL2 polyclonal antibody (GTX32095, GeneTex, Irvine, CA, USA) or normal Rabbit IgG (Santa Cruz, Dallas, TX, USA) was added as a negative control antibody. Immunoprecipitated products were collected after incubation with Protein A + G-coated magnetic beads. The bound chromatin was eluted and digested with proteinase K, and then the DNA was purified. To quantitatively analyze relative levels of precipitated chromatin, q-PCR was used with primers directed against specific fragments of IGF-1R and IGF2 genes (the primers are listed in Supporting Information Table S3).

2.12. Single-cell RNA sequencing

We performed single-cell RNA sequencing (scRNA-seq) on a hydrodynamic injection mouse HCC model. Tumor tissues from 3 mice per group were mixed for sequencing. Fresh tissues were stored in the GEXSCOPE™ tissue preservation solution. Fresh tissues were washed with phosphate-buffered saline PBS multiple times and minced into small pieces in Hank's balanced salt solution. The tissue pieces were digested with sCellLive tissue dissociation solution (Cat#1020012, Singleron, Nanjing, China) at 37 °C for 20 min, filtered through a 70 μm sterile cell strainer (Cat#352340, BD, Franklin Lakes, NJ, USA), supernatants were discarded, and the cell pellet was resuspended with PBS/DMEM, the cell viability was assayed by trypan blue staining. Single-cell suspensions (2×10^5 cells/mL) were loaded onto a microwell chip using the Singleron matrix single cell processing system. Single-cell suspensions were barcoded to generate scRNA-seq libraries using the Chromium Single Cell 3' and 5' Library. RNA from the barcoded cells was reverse-transcribed, the cDNA was amplified by PCR, and then sequencing adapters were ligated to the cDNA. The library was generated and sequenced on GEXSCOPE single-cell RNA library kits (Singleron). Individual libraries were sequenced on Illumina Novaseq 6000 with 150 bp paired-end reads. Raw sequencing reads of scRNA-seq samples were processed to generate gene expression matrices using an internal pipeline. The cell types were clustered using signature gene sets, including liver cells, including hepatocytes, endothelial cells (ECs), B cells, plasma cells, T cells, NK cells, neutrophils, mononuclear phagocytes (MPs), plasmacytoid dendritic cells (pDCs), and fibroblasts.

2.13. CUT&Tag-sequence assay

The CUT&Tag assay was performed as previously described. Briefly, 10^5 fresh cells were collected and incubated with equilibrated ConA Magnetic Beads II (TransGen Biotech, Beijing, China) at room temperature. After washing, the cell-bead complexes were resuspended and incubated overnight at 4 °C with a primary antibody (Genetex) against PLAGL2 (1:1000). Subsequently, the samples were treated with a species-matched secondary antibody. Following antibody incubation, the bead-bound cells were washed and subjected to tagmentation using the TransNGS CUT&Tag library prep kit for Illumina (KP172, TransGen Biotech). The transposition reaction was performed according to the manufacturer's protocol, followed by DNA elution using the provided elution buffer. The final libraries were amplified by PCR, size-selected, and quantified before high-throughput sequencing on an Illumina platform.

2.14. Chemical synthesis

N-(4-Cyanobenzyl)-3-((2,5-dimethoxyphenyl)sulfonamido)-4-((1*R*,5*S*)-8-oxo-1,5,6,8-tetrahydro-2*H*-1,5-methanopyrido[1,2-*a*][1,5]diazocin-3(4*H*)-yl)benzamide (compound 218). m.p. = 149.3–154.3 °C. ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.96 (t, *J* = 6.1 Hz, 1H), 7.76 (d, *J* = 7.9 Hz, 2H), 7.58 (d, *J* = 2.0 Hz, 1H), 7.50 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.43 – 7.34

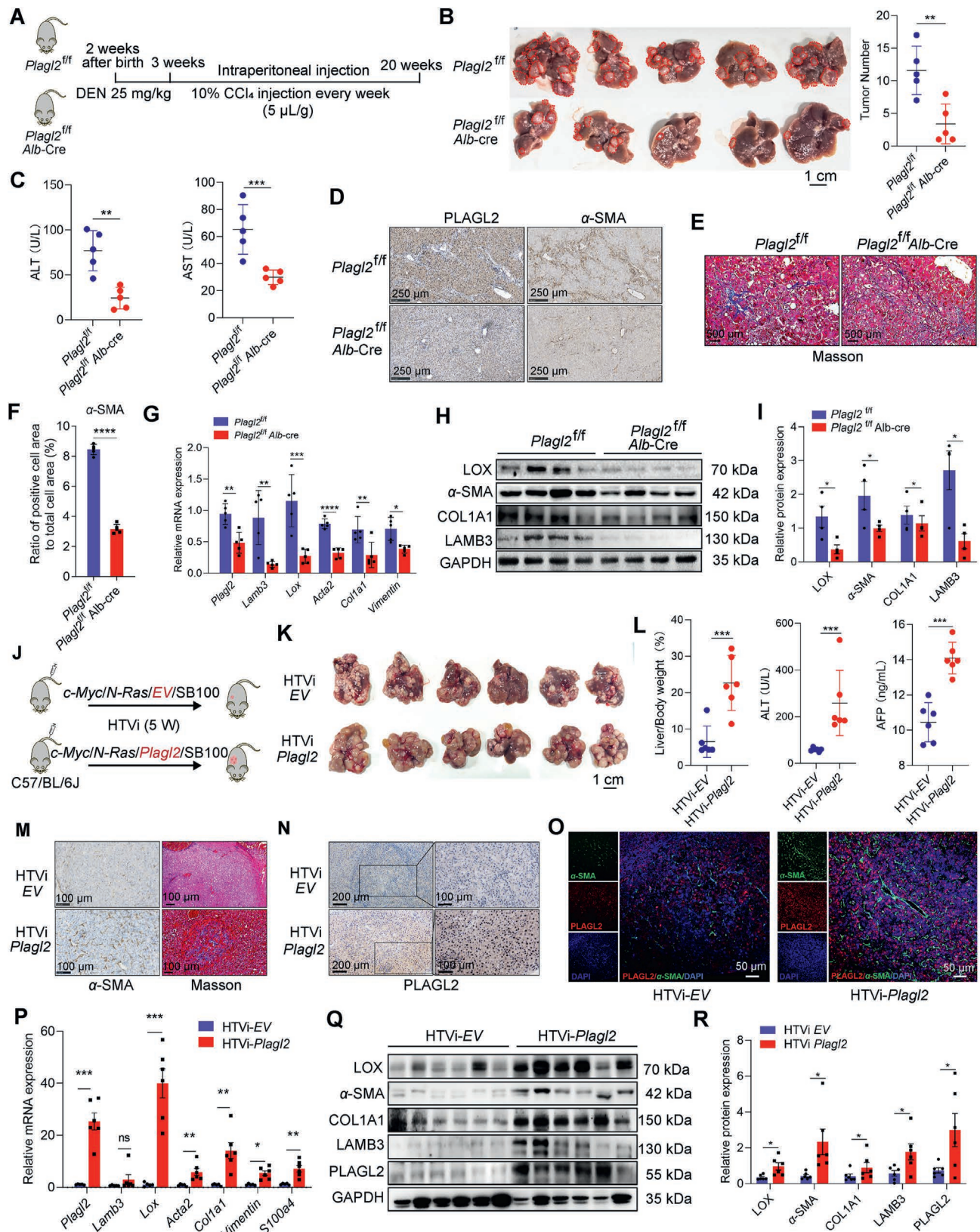


Figure 2 PLAGL2 remodels HCC ECM *in vivo*. (A) Schematic diagram of the DEN/CCl₄-induced HCC model. (B) Morphological characteristics and tumor number of the whole liver ($n = 5$ per group). (C) Serum levels of hepatotoxicity indicators, AST and ALT activity, were quantified ($n = 5$ per group). (D) IHC analysis of α-SMA and PLAGL2 in tumors from PLAGL2-knockdown DEN/CCl₄-induced HCC model. (E) Masson staining of tumor tissue collagen in tumors from PLAGL2-knockdown DEN/CCl₄-induced HCC model. (F) Quantitative analysis of α-SMA-positive cell area ($n = 5$ per group). (G) qRT-PCR analysis of the mRNA expression of ECM-related genes in tumors from

(m, 4H), 7.22 (d, $J = 8.4$ Hz, 1H), 7.15 (dd, $J = 9.1, 3.2$ Hz, 1H), 7.08 – 7.00 (m, 2H), 6.30 (d, $J = 9.1$ Hz, 1H), 6.21 (d, $J = 6.8$ Hz, 1H), 4.49 – 4.38 (m, 2H), 4.00 – 3.92 (m, 1H), 3.88 (dd, $J = 15.6, 6.5$ Hz, 1H), 3.73 (s, 3H), 3.64 (s, 3H), 3.25 (d, $J = 11.0$ Hz, 1H), 3.19 (s, 1H), 3.10 (d, $J = 11.1$ Hz, 1H), 3.04 (d, $J = 11.1$ Hz, 1H), 2.84 (d, $J = 11.1$ Hz, 1H), 2.60 (s, 1H), 1.99–1.88 (m, 2H). ^{13}C NMR (150 MHz, DMSO- d_6) δ 165.89, 162.78, 152.69, 150.85, 150.60, 146.08, 144.13, 139.72, 132.69, 131.75, 130.25, 128.33, 126.28, 122.57, 121.88, 121.16, 119.40, 116.72, 116.66, 115.34, 115.12, 109.92, 105.12, 59.67, 57.67, 57.04, 56.25, 49.75, 42.86, 34.87, 27.77, 25.24. HRMS (ESI) (m/z) calculated for $\text{C}_{34}\text{H}_{32}\text{N}_5\text{O}_6\text{S}$ [$\text{M}-\text{H}$] $^-$: 638.2079, found: 638.2093. Retention time: 3.048 min, eluted with 65% methanol/35% water.

2.15. Surface plasmon resonance assay

Surface plasmon resonance (SPR) assay for evaluating the interaction between compound 218 or its analogs and PLAGL2 DNA-binding domain using Biacore T200 system (GE Healthcare, Chicago, IL, USA). The purified recombinant human PLAGL2 DNA-binding domain was immobilized on a carboxymethylated 5 sensor chip using a standard amine coupling method with an amino coupling kit. Various concentrations of Compound 218 or its analogs in 1.05 PBS-P running buffer were injected as analytes. Data were analyzed with the Biacore evaluation software (T200 Version 2.0) and processed using standard double-referencing and fit to a 1:1 binding model to determine the association rate (K_{on} , $(\text{mol/L})^{-1}\cdot\text{s}^{-1}$), dissociation rate (K_{off} , s^{-1}), estimate of error (SE), Chi-Square (χ^2), and statistic and maximum response (R_{max} ; response units (RU)). The equilibrium dissociation constant (K_d) was calculated from the relationship Eq. (2):

$$K_d = K_{\text{off}}/K_{\text{on}} \text{ (mol/L)} \quad (2)$$

2.16. Statistical analysis

All quantitative *in vitro* and *in vivo* data are presented as mean $\pm$ standard deviation (SD) unless otherwise noted. All studies were repeated at least 3 times with similar results. Unless otherwise stated, unpaired two-sided (CI of 95%). A two-tailed Student's *t*-test was used to compare the differences between the 2 groups. Pearson's correlation analysis was used to identify correlations between 2 variables (GraphPad Software 7.0). All tests were two-tailed, and a value < 0.05 was considered statistically significant.

2.17. Data availability

All data associated with this study are present in the paper or the Supporting Information Materials. RNA-seq was deposited at Gene Expression Omnibus database with the accession number GSE121072, and scRNA-seq data were deposited at the National Genomics Data Center with the accession number subCRA032108. The data, analytical methods, and some other information will be made available on request to other researchers for reproducing the results or replicating the procedure. Requests for data should be made to the corresponding author.

3. Results

3.1. PLAGL2 is involved in ECM remodeling

Previously, we showed that PLAGL2 is upregulated in HCC samples¹⁵. TIMER analysis confirmed that PLAGL2 was overexpressed in the HCC tissues (Supporting Information Fig. S1A). Single-cell sequencing data from HCC clinical samples analysis confirmed that PLAGL2 was predominantly expressed in hepatocellular carcinoma cells, NK/T cells, and myeloid cells, while exhibiting minimal expression in fibroblasts. Therefore, our research focused on the indirect activation of HSCs by PLAGL2 through the paracrine pathway of HCC cells, rather than the direct regulation of HSCs by PLAGL2 (Fig. S1B). A microarray comparing HCCLM3-shCtrl with HCCLM3-shPLAGL2 *via* reactome-enriched pathways revealed downregulation of genes implicated in ECM interactions (non-integrin, collagen assembly, syndecan, laminin, and collagen crosslinking) (Fig. 1A and B). ECM-related genes were markedly downregulated in shPLAGL2-transfected cells (Fig. 1C). Furthermore, TCGA data obtained *via* GEPIA revealed a positive correlation between PLAGL2 and ECM-related gene expression (Fig. S1C). Immunofluorescence showed that a large number of fibroblasts were recruited around tumors with high PLAGL2 expression (Fig. 1D). PLAGL2 expression was positively correlated with α -SMA expression in HCC samples (Fig. 1E and Supporting Information Fig. S2A and S2B). Matrix adhesion assays showed reduced adhesion to collagen upon PLAGL2 knockdown, but enhanced adhesion upon overexpression (Fig. 1F and Supporting Information Fig. S3A and S3B). Quantitative RT-PCR validated the microarray results in HCC cells and xenograft tumors. PLAGL2 suppression decreased ECM-related gene expression, whereas overexpression increased it (Fig. 1G and Fig. S3C and S3D). Masson's trichrome staining and immunohistochemical staining (IHC) for α -SMA were used to visualize the ECM in xenograft tumors. PLAGL2 knockdown

PLAGL2-knockdown DEN/CCl₄-induced HCC model ($n = 6$ per group). (H, I) Western blot and quantitative analysis of ECM-related proteins in tumors from PLAGL2-knockdown DEN/CCl₄-induced HCC model ($n = 4$ per group). (J) Schematic diagram of PLAGL2-overexpression Nras-driven orthotopic HCC model. (K) Morphological characteristics of whole-liver ($n = 6$ per group). (L) Liver/weight ratio, serum levels of ALT, and AFP activity were quantified ($n = 6$ per group). (M, N) IHC analysis of α -SMA, PLAGL2, and Masson staining in Nras-driven orthotopic HCC model. (O) Immunofluorescence staining of PLAGL2 and α -SMA in a tumor from a PLAGL2-overexpression Nras-driven orthotopic HCC model. (P) qRT-PCR analysis of the mRNA expression of ECM-related genes in tumors from the Nras-driven orthotopic HCC model ($n = 6$ per group). (Q, R) Western blot and quantitative analysis of ECM-related proteins in tumors from PLAGL2-overexpression Nras-driven orthotopic HCC model ($n = 6$ per group). Data are presented as mean $\pm$ SD, and differences were tested by the Student's *t*-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$; ns, no significance. HCC, hepatocellular carcinoma; ECM, extracellular matrix; AST, aspartate aminotransferase; ALT, alanine transaminase; AFP, alpha fetoprotein; qRT-PCR, quantitative real-time polymerase chain reaction; IHC, immunohistochemistry; H&E, hematoxylin-eosin; EV, Empty vector; HTVi, hydrodynamic tail vein injection.

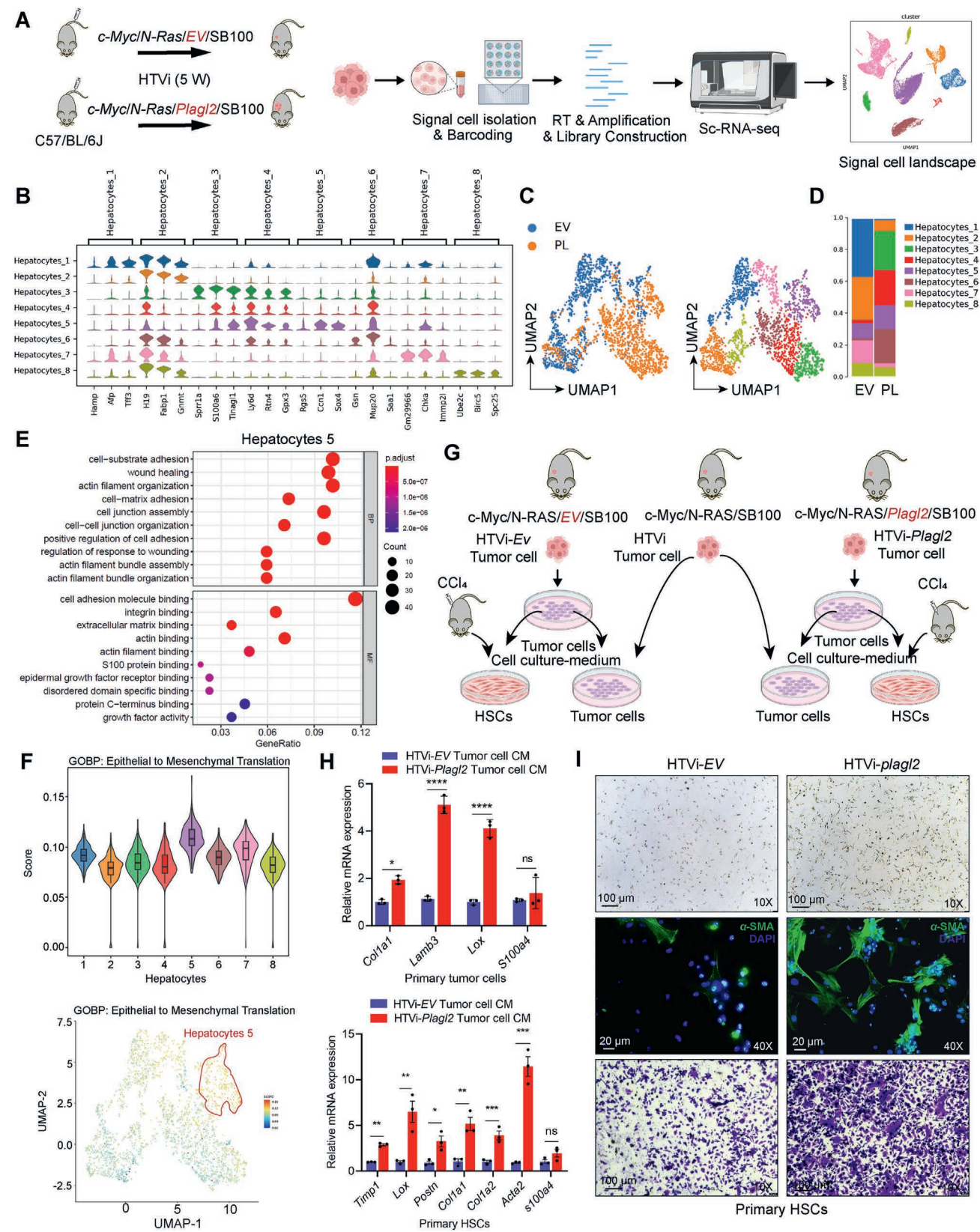


Figure 3 Single-cell transcriptome analysis reveals that *PLAGL2* is involved in the regulation of ECM in HCC. (A) Workflow of sample collection and data analysis. (B) Dot plot showing the expression of the top three subtype-specific gene markers in each hepatocyte subtype. (C) UMAP visualization of the EV and PL group hepatocyte cells. (D) UMAP showing the subtypes of hepatocyte and the percentage distribution of hepatocyte subtypes. (E) GO pathway enrichment analysis was performed for differential genes in cluster 5 hepatocytes. (F) Violin plots and UMAP showing the gene set activities of EMT in the hepatocyte cells of eight subclusters (AUCell score). (G) *In vitro* co-culture model of

reduced collagen and α -SMA expression, while overexpression increased these levels (Fig. S3E and S3F). Collectively, these results implied that PLAGL2 was involved in regulating the expression of ECM-related genes *in vitro*.

3.2. PLAGL2 remodels HCC ECM *in vivo*

To demonstrate that PLAGL2 is involved in the regulation of HCC ECM, hepatocyte-specific PLAGL2 knockout mice (*Plagl2^{flf}* Alb-cre) were generated to assess its pathophysiological role. The loss of PLAGL2 in hepatocytes was confirmed by polymerase chain reaction (PCR), Western blot, and IHC (Supporting Information Fig. S4A–S4C). Serological aspartate aminotransferase (AST), alanine transaminase (ALT), and hematoxylin and eosin (H&E) analyses showed no spontaneous liver injury in *Plagl2^{flf}* and *Plagl2^{flf}* Alb-cre mice (Fig. S4D and S4E). No significant difference in ECM-related gene expression was observed among the mice (Fig. S4F–S4H).

An *N*-nitrosodiethylamine (DEN)/carbon tetrachloride (CCl₄)-induced HCC mouse model¹⁶ was employed to determine the role of PLAGL2 in HCC. The HCC fibrosis model was induced in 14-day-old mice using DEN (25 mg/kg, i.p.) and repeated low-dose CCl₄ (5 μ L/g, i.p.) from 3 weeks to 5 months (Fig. 2A). Hepatocyte-specific PLAGL2 knockout inhibited liver tumorigenesis (Fig. 2B and C) and significantly reduced AST and ALT levels (Fig. 2D). IHC for α -SMA revealed ECM in DEN/CCl₄-treated mice, and hepatocyte-specific PLAGL2 knockout reduced α -SMA expression in tumors (Fig. 2E and F). The mRNA and protein expression of ECM-related genes was significantly decreased in tumors from *Plagl2^{flf}* Alb-cre mice (Fig. 2G–I).

To further evaluate the specific role of PLAGL2 in HCC, we established a genetically engineered mouse model in which transposable elements containing *c-Myc/N-Ras* and *Plagl2* were delivered into the livers of wild-type C57/BL/6J mice through hydrodynamic tail-vein injection (HTVi) (Fig. 2J). Morphological observations of the liver proved that mice with overexpression of PLAGL2 had a higher degree of malignancy of orthotopic liver tumors (Fig. 2K). The liver-to-body weight ratio and serum ALT and AFP levels were markedly increased in the HTVi *Plagl2* as compared to the HTVi EV group (Fig. 2L). Masson staining, IHC, and immunofluorescence (IF) detection for α -SMA and PLAGL2 showed increased expression in the HTVi *Plagl2* group (Fig. 2M–O). Both the mRNA and protein expression of ECM-related genes were significantly increased in tumors from HTVi *Plagl2* mice (Fig. 2P–R).

Two orthotopic HCC tumor models were established, and a loss-of-function approach was performed using adeno-associated virus serotype 8 encoding shPLAGL2 (AAV8-shPLAGL2) and AAV8-shCtrl (Supporting Information Figs. S5A and S6A). The effects of PLAGL2 knockdown on H22- and *Nras*-driven orthotopic tumor growth were assessed, and PLAGL2 knockdown significantly limited tumor growth (Figs. S5B and S6B), while Ki-67 and α -SMA levels were remarkably reduced (Figs. S5C and

S6C), as were the mRNA levels of ECM-related genes (Figs. S5D and S6D).

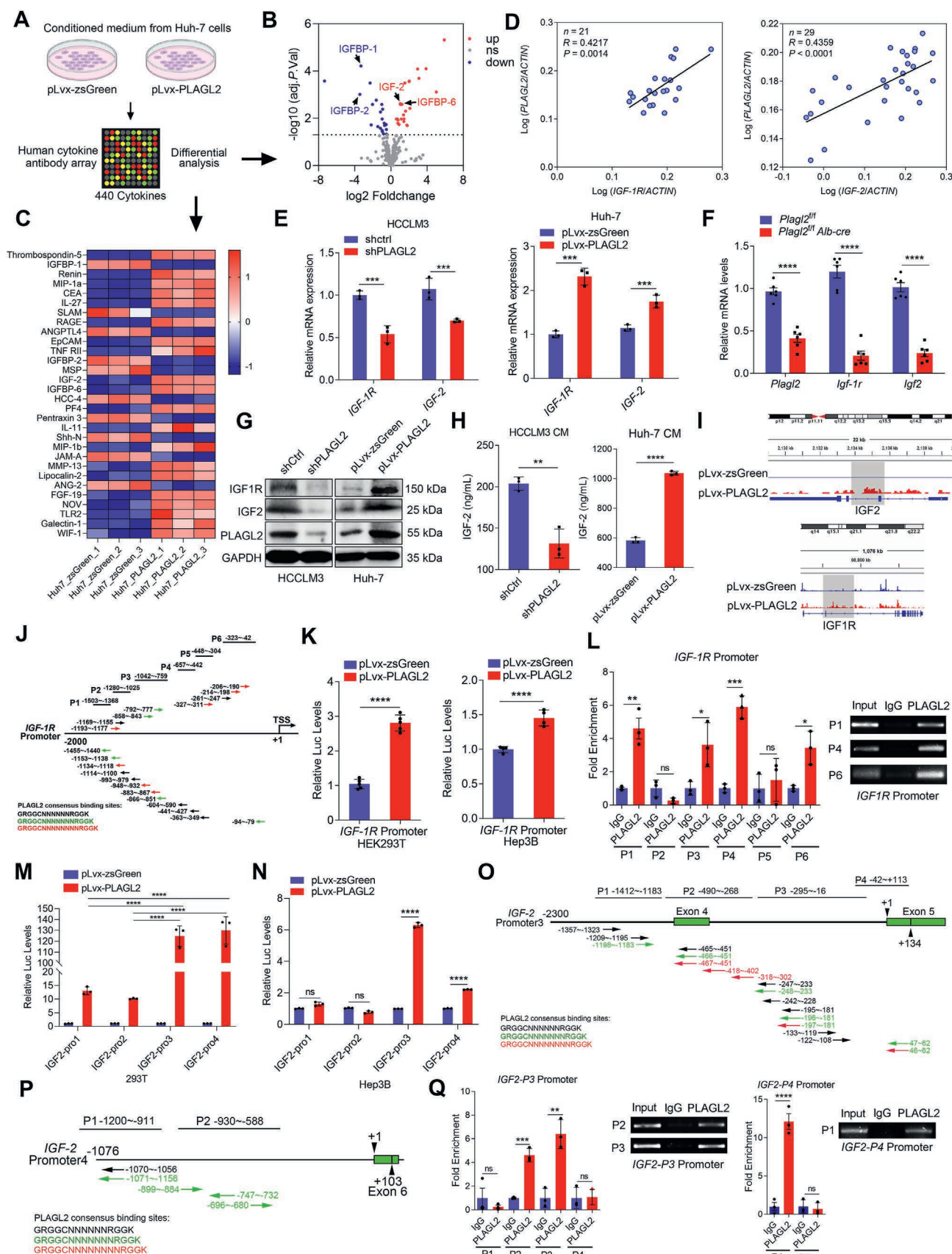
An HCC PDX mouse model was established (Supporting Information Fig. S7A), with 2 patients expressing high levels of PLAGL2 and one expressing low levels of PLAGL2 (Fig. S7E). 1×10^{11} GCs of AAV8-shPLAGL2 and AAV8-shCtrl were injected intratumorally into tumor-bearing mice. Although tumor volume and weight did not decrease significantly (Fig. S7B–S7D), significant reductions in Ki-67 and α -SMA levels were observed after PLAGL2 knockdown in individuals with high PLAGL2 expression. Masson staining also revealed a significant downregulation of collagen deposition in the AAV8-shPLAGL2 group (Fig. S7E). These results further suggested that PLAGL2 knockdown significantly impaired ECM proteins and inhibited HCC progression.

3.3. Single-cell analysis shows PLAGL2 regulates ECM in HCC

Single-cell RNA sequencing (scRNA-seq) was performed to analyze changes in the tumor microenvironment (TME) in a PLAGL2-overexpressed HCC mouse model (Fig. 3A). scRNA-seq identified 28,590 liver cells, including hepatocytes, endothelial cells (ECs), B cells, plasma cells, T cells, NK cells, neutrophils, mononuclear phagocytes (MPs), plasmacytoid dendritic cells (pDCs), and fibroblasts (Supporting Information Fig. S8A–S8C). To uncover the cellular heterogeneity in HCC, we analyzed the subpopulation of hepatocytes separately. UMAP1 clustering identified multiple hepatocyte populations (Clusters 1–8) (Fig. 3B and C). Clusters 3, 4, 5, and 6 of hepatocytes were significantly increased in the PLAGL2 overexpression group (Fig. 3D). Gene Ontology (GO) pathway enrichment analysis of cluster 5 hepatocyte differential genes revealed a rebound towards cell-substrate adhesion, collagen-containing extracellular matrix, and cell adhesion molecular binding (Fig. 3E). Furthermore, enrichment of positive epithelial-mesenchymal transition (EMT) was observed in cluster 5 hepatocytes (Fig. 3F). EMT plays a pivotal role in enhancing the plasticity of cancer cells, enabling them to evade chemotherapies and targeted therapies¹⁷. In summary, our data indicate that PLAGL2 promotes ECM production in HCC cells.

To demonstrate the potential crosstalk between tumor cells and other cells, we conducted a cell-to-cell interaction analysis. The cell-Chat algorithm identified significantly stronger interactions between cluster 5 hepatocytes (with themselves) and fibroblasts (Supporting Information Fig. S9A). Activated HSCs trans-differentiate into collagen-secreting myofibroblasts, thereby boosting ECM production. Vimentin, an intermediate filament protein in myofibroblasts, is correlated with advanced tumor stage, lymph node metastasis, and patient survival¹⁸. S100 calcium-binding protein A6 (S100A6) was a universal marker of activated myofibroblasts¹⁹. Lumican (LUM) is a proteoglycan secreted by the ECM that regulates collagen fibrillogenesis²⁰. THY1⁺ myofibroblasts highly express profibrogenic genes, and THY1 is correlated with ECM organization and promotes HSC

primary isolated mouse cells. (H, I) Primary tumor cells and HSCs were treated with CM from tumor cells in EV or PL groups. qRT-PCR analysis of the mRNA expression of ECM-related genes in primary tumor cells and HSCs (H), morphological observation, immunofluorescent staining, and cell migration assay of HSCs were tested (48 h) (I). Data are presented as mean $\pm$ SD, $n = 3$, and differences were tested by the Student's *t*-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$; ns, no significance. EV, empty vector; UMAP, Uniform Manifold Approximation and Projection; GO, gene ontology; CM, conditioned medium; ECM, extracellular matrix; HSC, hepatic stellate cells; qRT-PCR, quantitative real-time polymerase chain reaction; HTVi, hydrodynamic tail vein injection.



activation²¹. MARCKS is involved in myofibroblast activation and promotes the progression²². By comparing gene expression in fibroblasts between the Ctrl (empty vector, EV) and PLAGL2 overexpression (PL) groups, fibroblast activation genes were found to be significantly upregulated in the PL group (Fig. S9B). GO enrichment analysis of the upregulated genes revealed enrichment of terms associated with actin cytoskeletal organization, collagen-containing extracellular matrix, and cell-substrate junction (Fig. S9C). These results indicated that PLAGL2 participates in the paracrine pathway to regulate fibroblast activation in HCC cells.

3.4. PLAGL2 remodels HCC ECM through the autocrine and paracrine pathways

The role of the autocrine pathway in HCC cell proliferation was explored; the conditioned medium (CM) of HCCLM3-shPLAGL2 and Huh-7-PLAGL2 had no significant effect on parental cell growth (Supporting Information Fig. S10A). The role of the autocrine pathway in HCC migration, invasion, and apoptosis was examined. HCCLM3-shPLAGL2 CM notably suppressed HCCLM3 migration, invasion, and apoptosis, and Huh-7-PLAGL2 CM significantly enhanced Huh-7 migration, invasion, and apoptosis (Fig. S10B–S10D). ECM proteins in HCC cells were analyzed, and HCCLM3-shPLAGL2 CM suppressed ECM protein expression, whereas Huh-7-PLAGL2 CM had the opposite effect (Fig. S10E and S10F).

Because HSCs produce ECM and promote HCC, we explored PLAGL2's regulation of HSCs *via* the paracrine pathway. HCCLM3-shPLAGL2 CM inhibited LX-2 proliferation and migration, whereas Huh-7-PLAGL2 CM promoted it (Supporting Information Fig. S11A and S11B). PLAGL2 regulates LX-2 apoptosis *via* paracrine signaling. HCCLM3-shPLAGL2 CM promoted LX-2 apoptosis, while Huh-7-PLAGL2 CM inhibited apoptosis (Fig. S11C and S11D). ECM-related proteins were detected in LX-2 cells; HCCLM3-shPLAGL2 CM suppressed ECM expression in LX-2 cells, whereas Huh-7-PLAGL2 CM had the opposite effect (Fig. S11E–S11H). The morphology of LX-2 cells was then determined. HCCLM3-shPLAGL2 CM hindered the formation of LX-2 dendrites, whereas Huh-7-PLAGL2 CM significantly enhanced it (Fig. S11I). IF analysis validated that HCCLM3-shPLAGL2 CM significantly inhibited α -SMA

expression in LX-2 cells, whereas Huh-7-PLAGL2 CM significantly promoted its expression (Fig. S11J).

To validate these results, mouse primary HCC and HSC cells were isolated from the liver (Fig. 3G). The supernatant from PLAGL2-overexpressing HCC cells boosted ECM gene expression in both HCC and HSC cells, and significantly activated HSCs (Fig. 3H and I). Taken together, these results suggest that PLAGL2 regulates ECM *via* autocrine and paracrine pathways.

3.5. PLAGL2 promotes IGF2 and IGF1R expression

Microarray analysis of differential mRNA expression between DEN/CCL₄-treated *Plagl2*^{fl/fl} and *Plagl2*^{fl/fl} Alb-cre mice revealed downregulated genes in ECM organization, degradation, and IGF receptor signaling *via* reactome analysis (Supporting Information Fig. S12A). The cytokine array on PLAGL2-overexpressed Huh-7 supernatants showed enrichment in growth factors and IGF I/II binding cytokines (Fig. S12B). In the supernatant of PLAGL2-overexpressed Huh-7 cells, IGFBP-1 and IGFBP-2 expression levels significantly decreased, whereas IGF2 and IGFBP-6 expression levels significantly increased (Fig. 4A–C). TCGA data from GEPIA and LinkedOmics showed that the expression of PLAGL2 was positively correlated with IGF2 and IGF-1R (Supporting Information Fig. S13A and S13B). Furthermore, the expression of PLAGL2 was positively correlated with IGF2 and IGF1R expression in HCC patients, as confirmed by interactive gene expression profiling analysis (Fig. 4D). PLAGL2 promoted the transcription of IGF2 and IGF1R in HCC cells (Fig. 4E and Fig. S13C). IGF2 and IGF-1R mRNA levels were significantly downregulated in the liver of hepatocyte-specific PLAGL2-knockout mice (Fig. 4F). PLAGL2 promoted IGF2 and IGF-1R protein expression in HCC cells (Fig. 4G and Fig. S13D). PLAGL2 promoted IGF2 secretion in HCC cells (Fig. 4H). Overall, the present results suggest that PLAGL2 is involved in the regulation of the expression of both IGF2 and IGF1R.

Cut&Tag-seq data analysis shows that the PLAGL2 protein specifically binds to the transcriptional start site (TSS) regions of the IGF2 and IGF1R genes (Fig. 4I). PLAGL2 binds to the GRGGC(N)6–8RGGK site in target gene regulatory regions to activate transcription, and 20 PLAG consensus binding sites were identified in the IGF1R promoter (–2000 to +1 from the translation start site) (Fig. 4J). Overexpression of PLAGL2 in

Figure 4 PLAGL2 promotes IGF2 and IGF1R expression. (A) Screening of cytokines from conditioned media of control and PLAGL2-overexpressed Huh-7 cells. (B) The volcano plots display secreted proteins upon PLAGL2 overexpression in Huh-7 cells. (C) Heat map displays the top 30 differentially secreted proteins with a Log₁₀*P*-value <0.05. (D) Correlation of PLAGL2 with IGF1R and IGF2 expression in clinical HCC tumors was determined by qRT-PCR. (E) The transcript levels of IGF1R and IGF2 in PLAGL2-knockdown (HCCLM3) and PLAGL2-overexpression (Huh-7) cell lines were analyzed by qRT-PCR (*n* = 3). (F) The transcript levels of PLAGL2, IGF1R, and IGF2 in the liver of hepatocyte-specific PLAGL2 knockout mice were analyzed by qRT-PCR (*n* = 6 per group). (G) The protein expression levels of PLAGL2, IGF1R, and IGF2 in PLAGL2-knockdown (HCCLM3) and PLAGL2-overexpression (Huh-7) cell lines were analyzed by Western blot. (H) IGF2 concentrations in supernatant of PLAGL2-knockdown (HCCLM3) and PLAGL2-overexpression (Huh-7) cell lines were analyzed by ELISA (*n* = 3). (I) Cut&Tag-Seq binding peaks for PLAGL2 within the TSS of IGF2 and IGF1R (UCSC genome browser). (J) PLAGL2 consensus binding sites were identified in the IGF1R promoter (–2000 to +1 from the translation start site). The PLAGL2 consensus binding sites are identified as GRGGC(NNNNNN)RGGK (black arrow), GRGGC(NNNNNNN)RGGK (green arrow), and GRGGC(NNNNNNNN)RGGK (red arrow). (K) The regulation of PLAGL2 on the IGF1R promoter was detected by Dual-Luciferase Reporter assay (*n* = 5 in 293T, *n* = 4 in Hep3B). (L) The enrichment of PLAGL2 in the IGF1R promoter region was determined by ChIP (*n* = 3). (M and N) The regulation of PLAGL2 on 4 IGF2 promoters was detected by Dual-Luciferase Reporter assay (*n* = 3). (O and P) PLAGL2 consensus binding sites were identified in the IGF2 promoter 3 and 4. (Q) The enrichment of PLAGL2 in the IGF2 promoter 3/4 region was determined by ChIP (*n* = 3). Data are presented as mean ± SD, and differences were tested by the Student's *t*-test, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001; ns, no significance. ChIP, chromatin immunoprecipitation; qRT-PCR, quantitative real-time polymerase chain reaction; TSS, Transcription Start Site.

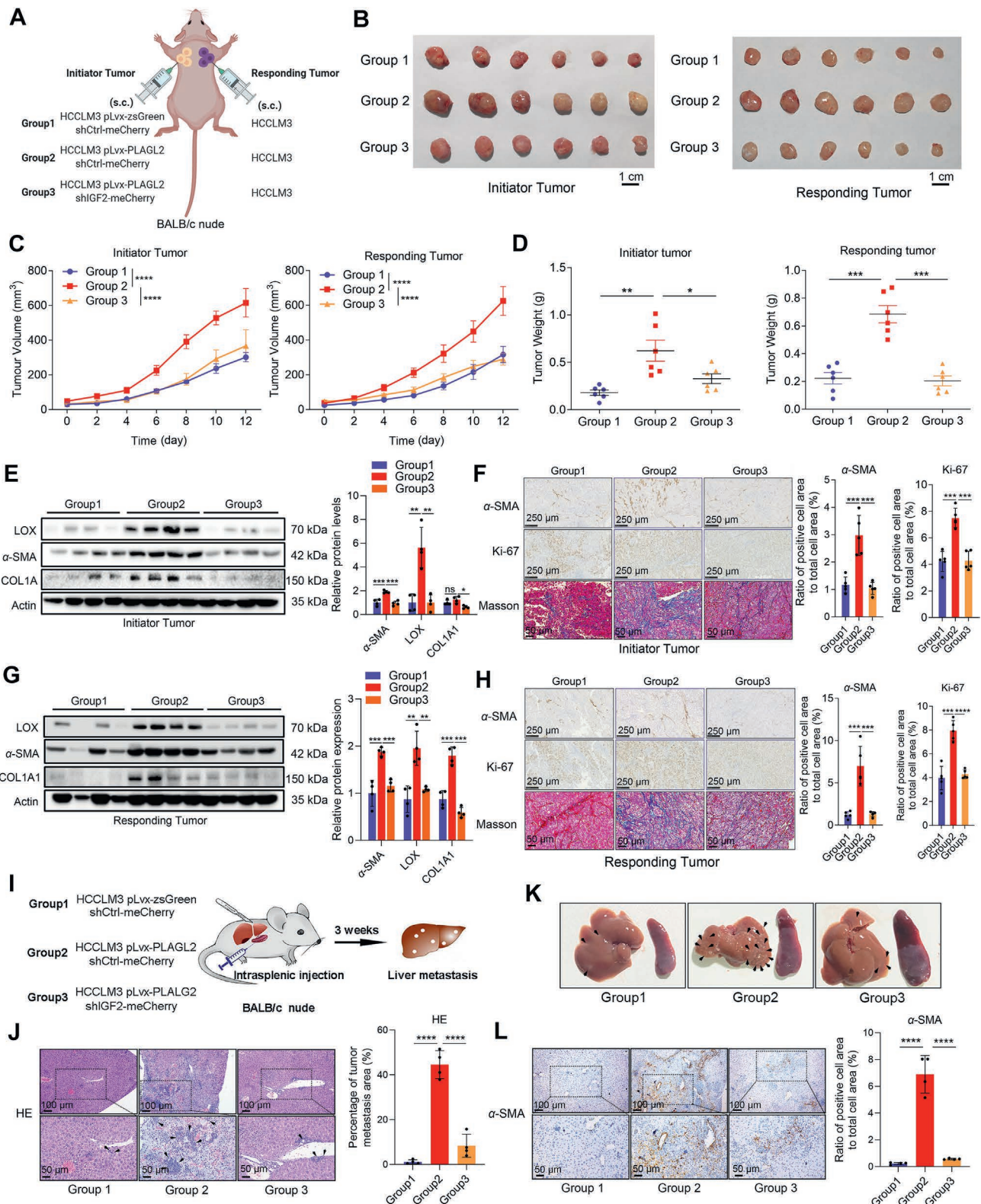


Figure 5 Effects of PLAGL2-induced IGF2 on the tumorigenicity and metastasis of HCC cells in nude mice. (A) Experimental model and groups. (B–D) Tumor pictures (B), tumor growth curves (C), and tumor weight (D) for the initiator and responding tumor xenograft experiments ($n = 6$ per group). (E) Comparison of ECM-related proteins in the initiator tumors by Western blot analysis ($n = 4$ per group). (F) Masson staining of tumor tissue collagen, comparison of Ki-67 and α -SMA in the initiator tumors by IHC, and quantitative analysis of positive cell area ($n = 5$ per group). (G) Comparison of ECM-related proteins in the responder tumors by Western blot analysis ($n = 4$ per group). (H) Masson staining of tumor tissue collagen, comparison of Ki-67 and α -SMA in the responder tumors by IHC, and quantitative analysis of positive cell area ($n = 5$ per group). (I) A schematic diagram of the liver metastasis model. (J) Representative light microscopic images of H&E staining in liver

HEK293T and Hep3B cells significantly increased IGF1R promoter-driven luciferase activity (Fig. 4K). Quantitative chromatin immunoprecipitation (qChIP) with the PLAGL2 antibody in HCCLM3 cells demonstrated specific binding to the IGF1R promoter sites: -1503 to -1368, -657 to -442, and -323 to -42 (Fig. 4L). IGF2 has 4 promoters (P1-P4) (Fig. S12C). To confirm which IGF2 promoter is directly regulated by PLAGL2, overexpression of PLAGL2 in HEK293T and Hep3B cells using 4 IGF2 promoter-luciferase reporter plasmids significantly increased luciferase activity for IGF2 promoter 3 and IGF2 promoter 4 (Fig. 4M and N). Additionally, 18 PLAG consensus binding sites were identified in IGF2 promoter 3, and 5 in IGF2 promoter 4 (Fig. 4O and P). The qChIP assay demonstrated that PLAGL2 specifically binds to IGF2 Promoter 3 (-490 to -268) and Promoter 4 (-1200 to -991) regions (Fig. 4Q). These findings establish that PLAGL2 acts as a transcriptional regulator of IGF1R and IGF2.

3.6. PLAGL2 remodels HCC ECM via IGF2/IGF1R-PI3K-AKT pathway

The phosphoinositide 3-kinase (PI3K)/AKT and RAS/RAF/MAPK signaling pathways are the main downstream pathways of IGF2/IGF1R (Supporting Information Fig. S14A). The down-regulation of PLAGL2 markedly reduced the phosphorylation of IGF1R (*p*-IGF1R Y1131) and AKT (*p*-AKT T308, *p*-AKT S473), whereas its upregulation enhanced both. Conversely, the levels of ERK phosphorylation remained unaffected (Fig. S14B and S14C), indicating that the AKT signaling pathway plays a significant role in PLAGL2-mediated remodeling of the HCC ECM. The IGF1R inhibitor NVP-AEW541 significantly suppressed the effects of PLAGL2 on both IGF1R and AKT phosphorylation and significantly inhibited the effects of PLAGL2 on the expression of ECM-related proteins (Fig. S14D and S14H). These results indicate that PLAGL2 remodels HCC cell-produced ECM primarily through the IGF2/IGF1R-AKT signaling pathway.

Several upstream regulators of AKT signaling, including PI3K, phosphoinositide-dependent kinase-1 (PDK1), and mTOR complex 2 (mTORC2), were examined to identify the downstream mechanism by which IGF1R mediates the effects of PLAGL2 on AKT activation and ECM remodeling in HCC (Inhibitor information is provided in Supporting Information Table S4). LY294002 (a PI3K inhibitor) reduced AKT (Thr308 and Ser473) activation induced by PLAGL2 (Fig. S11E). Additionally, AKT (Ser473) activation was significantly decreased by KU-0063794 (an mTORC2 inhibitor) (Fig. S14F), and AKT (Thr308) activation was notably suppressed by OSU-03012 (a PDK1 inhibitor) (Fig. S14G). LY294002, KU-0063794, and OSU-03012 also markedly inhibited the effects of PLAGL2 on ECM-related protein expression (Fig. S14I). These findings suggest that PLAGL2 primarily remodels the ECM produced by HCC cells through the IGF2/IGF1R-PI3K-(PDK1 or mTORC2)-AKT signaling pathway.

We have previously established in prior experiments that HCC and LX-2 cells are regulated by PLAGL2 through autocrine and

paracrine pathways. In addition, PLAGL2 participates in the secretion of IGF2 in HCC cells. However, the role of IGF2 in ECM production in HCC and LX-2 cells remains unclear. IGF2 increased the expression of ECM-related proteins in a dose-dependent manner (Fig. S14J). Inhibition of IGF1R expression by its pharmacological inhibitor NVP-AEW541 or siRNA knock-down significantly inhibited IGF2 effects on the expression of ECM-related proteins in HCCLM3 cells (Fig. S14K and S14L). Additionally, inhibition of PDK1, PI3K, and mTORC2 activities by OSU-03012, LY294002, and KU-0063794 significantly inhibited IGF2 effects on the expression of ECM-related proteins in HCCLM3 cells (Fig. S14M). IGF2 promoted AKT phosphorylation at the T308 and S473 sites, as well as ECM-related protein expression, in LX2 cells in a dose-dependent manner (Fig. S14N and S14O). The IGF1R inhibitor NVP-AEW541 significantly inhibited the effect of IGF2 on ECM-related protein expression in LX-2 cells (Fig. S14P). Likewise, LY294002, KU-0063794, and OSU-03012 significantly inhibited the effect of IGF2 on the expression of ECM-related proteins in LX-2 cells (Fig. S14Q). These results suggest that IGF2 contributes to the modulation of the ECM produced by LX-2 and HCC cells.

3.7. IGF2 mediates PLAGL2-driven ECM remodeling in HCC

To further prove the role of IGF2 in mediating PLAGL2-promoted proliferation and metastasis of HCC cells, *in vitro* and *in vivo* experiments were performed. HCCLM3-pLvz-zsGreen-shCtrl-mCherry (Group 1), HCCLM3-pLvz-PLAGL2-shCtrl-mCherry (Group 2), and HCCLM3-pLvz-PLAGL2-shIGF2-mCherry (Group 3) cells were acquired by flow cytometry sorting (Supporting Information Fig. S15A), and the secretion and expression of IGF2 were validated by ELISA and Western blot (Fig. S15B and S15C). For *in vitro* experiments, the conditioned medium (CM) from Group 1, Group 2, and Group 3 cells had no significant impact on the proliferation of HCCLM3 cells (Supporting Information Fig. S16A). In contrast, CM from Group 2 significantly promoted the proliferation of LX-2 cells, but CM from IGF2 knockdown in Group 3 attenuated the proliferation of LX-2 cells (Fig. S16B). Huh-7-PLAGL2 CM promoted the migration of Huh-7/LX-2 cells, and the invasion of Huh-7 cells was suppressed by the application of IGF2 antibody (Fig. S16C and S16D). For *in vivo* experiments, cells from Groups 1, 2, and 3 were subcutaneously inoculated into the left flank of nude mice, and parental HCCLM3 cells were inoculated into their right flank (Fig. 5A). The overexpression of PLAGL2 in Group 2 resulted in significantly larger initiator and responding tumors than those in the empty vector control group (Group 1). However, IGF2 knockdown in Group 3 attenuated the growth of initiator and responding tumors compared with the PLAGL2 overexpression group (Group 2), as represented by the tumor growth curves (Fig. 5B), tumor volume (Fig. 5C), and final tumor weight (Fig. 5D). Overexpression of PLAGL2 in Group 2 significantly promoted the expression of ECM-related proteins and the proliferation marker Ki-67 in the initiator and responding tumors compared to the empty vector control group (Group 1), but knockdown of IGF2 in

paraffin section from each group and quantitative analysis of metastasis area ($n = 4$ per group). (K) Representative images of liver metastasis. (L) Comparison of α -SMA in paraffin sections of the liver metastasis nodules by IHC and quantitative analysis of positive cell area ($n = 4$ per group). Data are presented as mean $\pm$ SD, and differences were tested by the Student's *t*-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. HCC, hepatocellular carcinoma; ECM, extracellular matrix; IHC, immunohistochemistry.

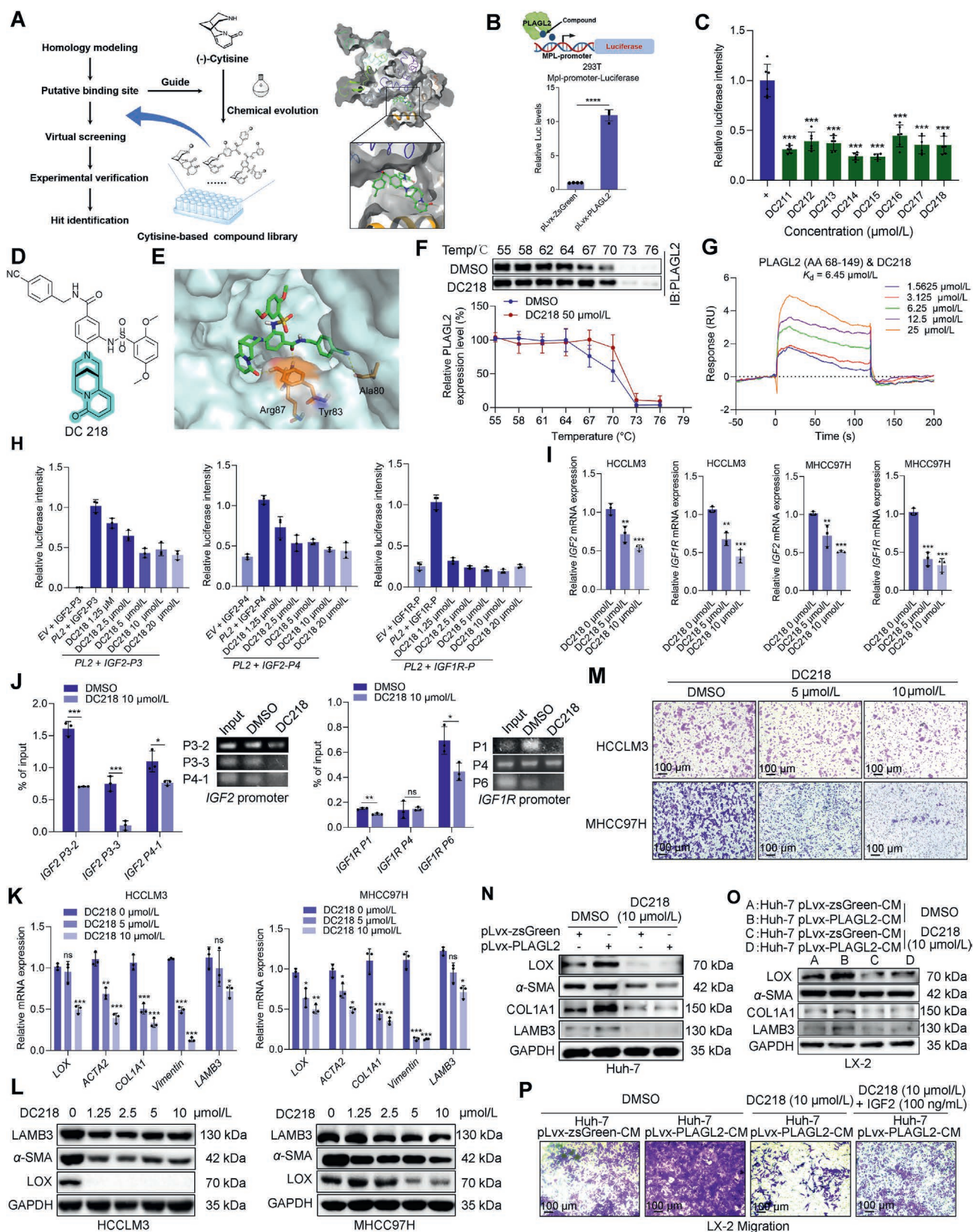


Figure 6 Discovery of a novel PLAGL2 transcriptional inhibitor, DC218. (A) Schematic workflow of the docking-based virtual screening for small-molecule inhibitors of PLAGL2 transcriptional regulation. (B) A transcription-regulated MPL promoter luciferase reporter system ($n = 4$). (C) Dual-Luciferase Reporter assay screening for small molecules that inhibit PLAGL2 transcriptional regulation ($n = 6$). (D) Chemical structure of the top hit small-molecule DC218. (E) Predicted binding mode of DC218 to PLAGL2 (homology modelling). (F) CETSA was used to evaluate the binding between DC218 and PLAGL2 at thermodynamic levels. (G) SPR assay for the affinity between DC218 and purified PLAGL2 (AA68-

Group 3 significantly inhibited the expression of ECM-related proteins and the proliferation marker Ki-67 in the initiator and responding tumors compared to the PLAGL2 overexpression group (Group 2) (Fig. 5E–H). The concentrations of IGF2 in the serum and tumor were measured using ELISA. Overexpression of PLAGL2 in Group 2 increased IGF2 levels in the serum and tumors compared to the empty vector control group (Group 1); however, knockdown of IGF2 in Group 3 decreased IGF2 levels in the serum and tumors compared with the PLAGL2 overexpression group (Group 2) (Supporting Information Fig. S17A and S17B). In order to prove that IGF2 is the most important secretory factor in this context, we evaluated whether IGF2 receptor (IGF1R) knockout in responder tumors can block the effect of IGF2 in PLAGL2 overexpressing cells in initiator tumors. HCCLM3-pLvz-zsGreen and HCCLM3-pLvz-PLAGL2 cells were subcutaneously injected into the left flank of different groups of nude mice. HCCLM3-shCtrl and HCCLM3-shIGF1R cells were injected subcutaneously into the right side (Supporting Information Fig. S18A). Overexpression of PLAGL2 in Groups 3 and 4 resulted in a significantly larger initiator than that in the empty vector control group (Groups 1 and 2). The overexpression of PLAGL2 in Group 3 resulted in the development of significantly larger responding tumors than the empty vector control group (Group 1). Knockdown of the IGF1R in response tumors in groups 2 and 4 significantly blocked the growth-promoting effect of PLAGL2 overexpression in initiating tumors on response tumors, as represented by the tumor growth curves (Fig. S18B), tumor volume (Fig. S18C), and final tumor weight (Fig. S18D). This assay demonstrated that knockdown of the IGF2 receptor could block the effect of IGF2 in PLAGL2 overexpression cells in initiator tumors. The liver metastasis model in nude mice also showed that overexpression of PLAGL2 in Group 2 significantly increased the number of metastatic nodules on the surface of the liver and the expression of α -SMA compared to the empty vector control group (Group 1). However, knockdown of IGF2 in Group 3 attenuated the metastatic nodules on the surface and the expression of α -SMA in the liver compared with the PLAGL2 overexpression group (Group 2) (Fig. 5I–L and Fig. S17D). These results, therefore, suggest that IGF2 plays a significant role in mediating the PLAGL2-promoted proliferation and metastasis of HCC cells.

3.8. Compound DC218 is screened as a novel PLAGL2 transcriptional inhibitor

PLAGL2 regulates the ECM in HCC and is a potential target; thus, we propose a computer-aided drug design strategy to screen for novel PLAGL2 transcriptional inhibitors. This strategy involves homology modelling, molecular dynamics simulation, virtual

screening, and experimental validation (Fig. 6A). As the precise crystal structure of mammalian PLAGL2 has not yet been reported, I-TASSER, a protein structure homology model, was employed to produce the three-dimensional structure of human PLAGL2 based on the structure of other zinc finger family members. After analysis of the putative binding site of PLAGL2, we found that the natural product (–)-cytisine could occupy the sub-pocket. Therefore, through the binding site-guided chemical evolution of cytisine, we successfully constructed a cytisine-based natural product library for the discovery of PLAGL2 inhibitors. PLAGL2 has been reported to be involved in transcriptional regulation of the thrombopoietin receptor MPL. We constructed a transcription-regulated MPL promoter-luciferase reporter system (Fig. 6B). After multiple rounds of docking and screening, eight compounds in the natural product library were preliminarily selected to inhibit PLAGL2 transcriptional regulation (Fig. 6C). Surface plasmon resonance (SPR) was performed to measure the binding ability of 8 compounds with human recombinant PLAGL2 DNA-binding domain (Supporting Information Fig. S19A and S19B). Compounds DC211, DC214, and DC218 showed strong binding affinity to human recombinant PLAGL2 DNA-binding domain with the estimated equilibrium dissociation constant at 1.8, 4.37, and 6.45 $\mu\text{mol/L}$, respectively (Fig. S19C). DC218 inhibited the transcriptional regulation of MPL by PLAGL2 in a dose-dependent manner (Supporting Information Fig. S20A). We further evaluated the ability of the 3 compounds to inhibit HCC ECM *in vitro*. DC218 significantly inhibited the ECM of HCC cells and inhibited the activation of LX-2 cells by HCC cells through paracrine signaling (Fig. S20B–S20D). The above screening results indicate that DC218, featuring a cytisine scaffold, is a potentially active small molecule (Fig. 6D).

The binding mode of DC218 with PLAGL2 (Fig. 6E) indicated that DC218 was well fitted in the active pocket of the DNA-binding domain. The cytisine fragment forms a key hydrogen bond with the Tyr83 residue, and the amide fragment forms a hydrogen bond with Arg87. In addition, the cyan-benzyl group extended to the hydrophobic pocket and formed a hydrogen bond with the Ala80 residue. To determine whether DC218 directly bound to PLAGL2, drug affinity responsive target stability and cellular thermal shift assays were performed. DC218 increased the thermal stability of PLAGL2 (Fig. 6F) and showed strong binding affinity to the human recombinant PLAGL2 DNA-binding domain (residues 68–149 aa) with an estimated equilibrium dissociation constant at 6.45 $\mu\text{mol/L}$ (Fig. 6G). Next, we explored whether DC218 inhibits the transcription of IGF2 and IGF1R by interfering with the binding of PLAGL2 to its response element on these two gene promoters. The results showed that DC218 inhibited IGF2/IGF1R transcription in a dose-dependent manner (Fig. 6H and I). We performed ChIP-qPCR analysis using an anti-

149) protein. (H, I) The dual luciferase reporter system and qRT-PCR analysis verified that DC218 inhibited the transcriptional regulation of IGF2 and IGF1R by PLAGL2 in a dose-dependent manner. (J) ChIP-qPCR analysis of equal amounts of chromatin-immunoprecipitated DNA from HCC cells treated with DC218. (K, L) qRT-PCR and Western blot analysis verified that DC218 inhibited ECM-related proteins in a dose-dependent manner. (M) The Transwell assay verified that DC218 inhibited HCC cell migration in a dose-dependent manner. (N) Western blot analysis verified that DC218 inhibited the regulation of ECM-related proteins by PLAGL2 in Huh-7-PLAGL2 cells. (O) Western blot analysis verified that DC218 inhibited the activation of LX-2 cells by Huh-7-PLAGL2 cells. (P) Transwell assay verified that DC218 inhibited the migration of LX-2 cells by Huh-7-PLAGL2 cells or IGF2. Data are shown as the mean $\pm$ SD, $n = 3$, differences were tested by the Student's *t*-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$; ns, no significance. SPR, surface plasmon resonance; CETSA, cellular thermal shift assay; EV, empty vector; PL, PLAGL2; IGF2-P3, IGF2 promoter3; IGF2-P4, IGF2 promoter4; IGF1R-P, IGF1R promoter; qPCR, quantitative polymerase chain reaction; qRT-PCR, quantitative real-time polymerase chain reaction.

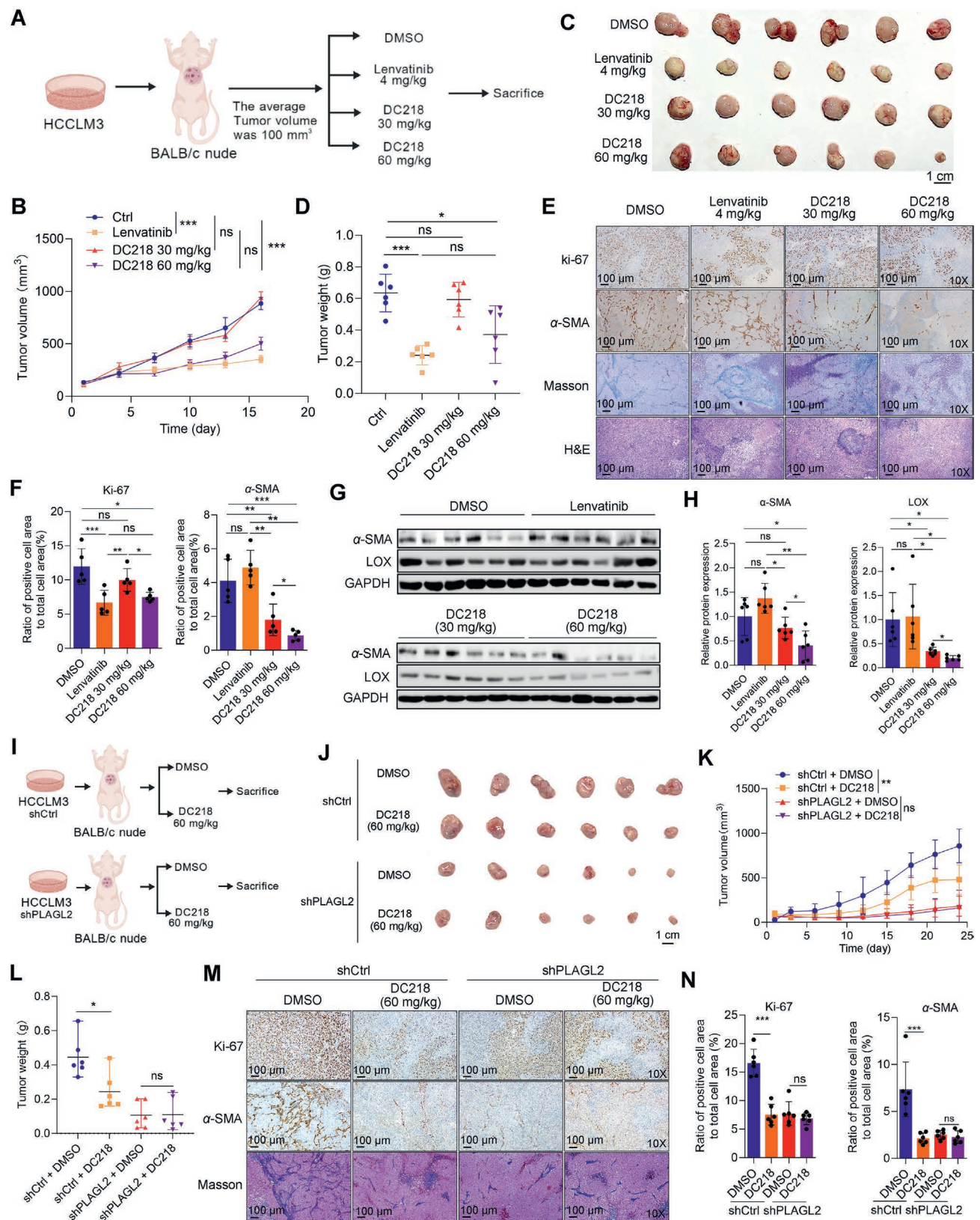


Figure 7 Compound DC218 significantly inhibited ECM production *in vivo* models. (A) Schematic diagram of DC218 administration in subcutaneous tumor model ($n = 6$ per group). (B) Statistical diagram of tumor growth curve. (C) Tumor pictures. (D) Tumor Weight Statistics ($n = 6$ per group). (E, F) IHC and quantitative analysis of Ki-67 and α -SMA in tumors ($n = 5$ per group), Masson staining of tumor tissue collagen. (G, H) Western blot and quantitative analysis of ECM-related proteins in the tumor ($n = 6$ per group). (I) Representative images of xenografts derived from nude mice subcutaneously injected with HCCLM3-shCtrl and HCCLM3-shPLAGL2 cells administered with DC218 or

PLAGL2 antibody. This assay confirmed that IGF2 and IGF1R levels were significantly lower after DC218 treatment than after vehicle treatment (Fig. 6J). Taken together, given the important roles of IGF2 and IGF1R in regulating HCC ECM, we can deduce that DC218 exerts its function by directly targeting the PLAGL2 DNA-binding domain, resulting in the downregulation of IGF2 and IGF1R transcription.

Furthermore, we explored whether DC218 inhibited HCC progression *in vitro*. DC218 inhibited the expression of ECM-related proteins and inhibited HCC cell migration in a dose-dependent manner (Fig. 6K–M and Supporting Information Fig. S21A and S21B) and exhibited no notable impact on the proliferation, cell cycle progression, or apoptosis of HCC cells (Supporting Information Fig. S22A–S22F). DC218 inhibited the regulation of ECM-related proteins by PLAGL2 in HCC cells (Fig. 6N) and inhibited the activation and chemotaxis promotion of LX-2 cells caused by high expression of PLAGL2 in HCC cells; this effect was rescued by IGF2 supplementation (Fig. 6O and P). Given that DC218 inhibits the transcriptional regulation of PLAGL2, we performed RNA sequencing (RNA-seq) analysis of HCCLM3 cells with or without DC218 treatment, and GO pathway enrichment analysis revealed that DC218 treatment significantly inhibited genes related to ECM (Fig. S21C and S21D). These results demonstrate the efficacy of DC218 in modulating ECM formation, thereby highlighting its potential as a therapeutic agent for the treatment of HCC.

To evaluate whether DC218 could inhibit HCC progression *in vivo*, we treated an HCCLM3 xenograft tumor model with different doses of DC218 administered intraperitoneally (Fig. 7A). Lenvatinib, a commercial drug used to treat HCC, was included as a control to compare the effects of DC218. Compared with the control treatment with DMSO, treatment with DC218 at doses of 60 mg/kg markedly inhibited tumor growth and decreased tumor weight (Fig. 7B–D). Masson staining and quantification of Ki-67 and α -SMA IHC staining showed a significant decrease in DC218 expression at a dose of 60 mg/kg (Fig. 7E and F). ECM-related protein expression was suppressed by DC218 at a dose of 60 mg/kg (Fig. 7G and H). These results demonstrated that DC218 did not inhibit HCC proliferation as significantly as lenvatinib, but significantly inhibited ECM compared to lenvatinib. It has been proposed that the combination of DC218 with Lenvatinib has the potential to enhance the therapeutic efficacy of lenvatinib.

To evaluate whether the inhibition of HCC progression by DC218 was dependent on PLAGL2, the protective effect of DC218 was evaluated using HCCLM3-shCtrl and HCCLM3-shPLAGL2 cells. The results demonstrated that PLAGL2 knock-down alleviated the inhibitory effects of DC218 on ECM-related proteins and HCC cell migration (Supporting Information Fig. S23A and S23B). In the xenografted tumor model (Fig. 7I), as shown by the liver imaging (Fig. 7J), tumor volume and weight measurement (Fig. 7K and L), Ki-67, α -SMA, Masson, and HE staining (Fig. 7M), the administration of DC218 (60 mg/kg) significantly inhibited the progression of HCC in the shCtrl group, but did not have an additional tumor and ECM-related protein

suppressive effect in the shPLAGL2 group. These results demonstrate that DC218 inhibits HCC progression mainly through PLAGL2.

3.9. DC218 treatment enhances the antitumor activities of lenvatinib

Our previous study has demonstrated that PLAGL2 functions as a transcriptional regulator of EGFR¹⁵. Another study found that EGFR activation limits the response of HCC to Lenvatinib^{23,24}. We speculated that inhibition of PLAGL2 transcriptional regulation might also sensitize HCC cells to lenvatinib. We found that inhibiting PLAGL2 expression significantly enhanced the sensitivity of HCC cells to lenvatinib (Supporting Information Fig. S24A–S24D). We further explored whether DC218 treatment enhances the efficacy of lenvatinib. Suppression of PLAGL2 transcriptional regulation in combination with lenvatinib markedly inhibited the proliferation of HCCLM3 and MHCC97H cells (Fig. 8A and B, Supporting Information Tables S5–S8). To further validate the effects of DC218 and Lenvatinib on HCC growth, patient-derived organoids (PDOs) were collected. These results also demonstrated that DC218 significantly promoted tumor organoid dispersion. DC218 and Lenvatinib synergistically inhibited the growth of organoids (Fig. 8C). These results confirmed that DC218 cooperates with lenvatinib to inhibit HCC *in vitro*.

The Nras-driven HCC model was used to evaluate whether DC218 treatment enhanced the efficacy of lenvatinib *in vivo* (Fig. 8D). AFP is a highly specific marker for HCC. The serum AFP levels were significantly reduced in all treatment groups, with the combination group exhibiting the most pronounced effect (Fig. 8E). Liver imaging showed that combination treatment significantly reduced the number of liver tumors (Fig. 8F). Masson staining demonstrated that collagen in tumor tissues was significantly downregulated in the DC218 administration group. Meanwhile, in the combination group of DC218 and Lenvatinib, the expression of collagen-related proteins was also significantly downregulated compared with that in the Lenvatinib monotherapy group (Fig. 8G). IHC analysis revealed that the DC218 treatment group significantly inhibited the expression of α -SMA, whereas the lenvatinib group exhibited a large number of CAFs. IHC analysis revealed a significant reduction in Ki-67-positive cells in the combination group, whereas HE staining indicated a corresponding decrease in tumor size (Fig. 8G and H). DC218 treatment at a dose of 60 mg/kg alone displayed no notable difference in antitumor activity, comparable to that of lenvatinib at a dose of 4 mg/kg. However, the combination of DC218 and Lenvatinib exerted significantly stronger antitumor activity than either therapy alone. These results demonstrate that combination treatment with DC218 improved the antitumor efficacy of lenvatinib *in vivo*.

The potential toxicity of DC218 and Lenvatinib was assessed, and the results indicated no significant effect on the body weight of the experimental mice (Supporting Information Fig. S25A). The combination of DC218 and Lenvatinib significantly reduced

with a vehicle control ($n = 6$ per group). (J) Tumor pictures. (K) Statistical diagram of tumor growth curve. (L) Tumor weight statistics ($n = 6$ per group). (M, N) IHC and quantitative analysis of Ki-67 and α -SMA in tumors ($n = 6$ per group), Masson staining of tumor tissue collagen. Data are presented as mean $\pm$ SD, and differences were tested by the Student's *t*-test; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; ns, no significance. ECM, extracellular matrix; IHC, immunohistochemistry.

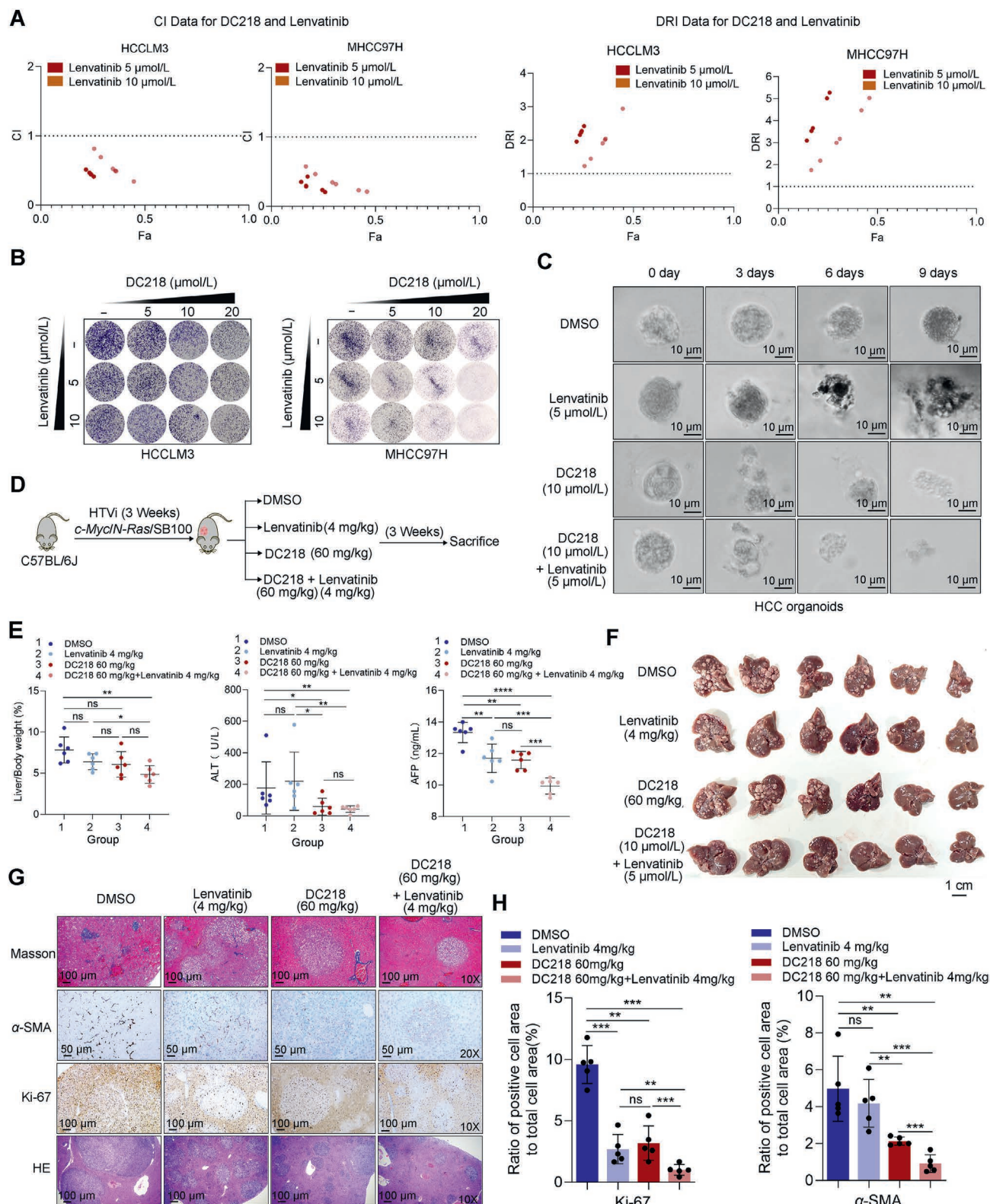


Figure 8 Compound DC218 treatment enhances the antitumor activities of Lenvatinib. (A) The synergy between DC218 and Lenvatinib was quantified using CompuSyn software, which uses the Chou-Talalay algorithm to calculate CI and DRI value. In the present study, CI values < 1 or DRI values > 1 were considered to be synergistic ($n = 6$). (B) A clone formation assay was used to evaluate the combination effect of DC218 and Lenvatinib ($n = 3$). (C) HCC organoids were treated with lenvatinib, DC218 at corresponding time points. (D) Schematic diagram of DC218 and Lenvatinib administration in *Nras*-driven orthotopic HCC model ($n = 6$ per group). (E) Liver/body weight ratio, serum levels of ALT, and AFP activity were quantified ($n = 6$ per group). (F) Representative images of mouse liver tissue from the indicated groups at 6 weeks. (G, H) Masson staining of tumor tissue collagen, IHC, and quantitative analysis of Ki-67 and α -SMA in *Nras*-driven orthotopic HCC model ($n = 5$ per group). Data are

the serum levels of ALP, CHOL, TBIL, and TGL, thereby markedly restoring liver function (Fig. S25B). H&E staining revealed that DC218 and Lenvatinib did not cause heart, spleen, lung, or kidney injury in the mice (Fig. S25C). Taken together, these findings suggest that inhibition of PLAGL2 transcriptional regulation could potentially serve as a promising strategy for the treatment of HCC.

4. Discussion

In the present study, we discovered that PLAGL2 promotes IGF2 secretion and IGF-1R expression, and that IGF2 subsequently enhances ECM production in HCC and HSCs *via* autocrine and paracrine mechanisms. Our findings reveal a novel role for IGF2 in mediating the interplay between HCC cells and HSCs, emphasizing the pivotal role of the IGF2/IGF1R signaling pathway in PLAGL2-mediated ECM remodeling in HCC.

Previous work from our group initially identified high expression of PLAGL2 in hepatocellular carcinoma (HCC), where it promotes tumorigenesis and progression. We demonstrated that the PLAGL2–EGFR–HIF-1/2 α signaling axis drives HCC advancement, underpinning PLAGL2's crucial role in HCC cell proliferation, metastasis, and erlotinib insensitivity¹⁵. Subsequent investigations elucidated the molecular basis for PLAGL2 upregulation in HCC, revealing that the neurotransmitter epinephrine (EPI) deubiquitinates and stabilizes PLAGL2 *via* USP10, thereby elevating PLAGL2 expression to facilitate HCC development²⁵. Building on these findings, the current study further delineates the molecular mechanisms through which PLAGL2 promotes HCC pathogenesis. This research reflects an updated understanding of PLAGL2's oncogenic functions and facilitates the development of small-molecule drugs targeting PLAGL2, with the potential to benefit HCC patients.

ECM stiffness enhances the survival of cancer cells while inhibiting their response to anticancer drugs. To date, some promising therapeutic approaches targeting the ECM have not yet been proven to be effective in clinical settings. Examples include targeting fibroblast activation protein- α (FAP), LOX, LOXLs, MMP2/9, and TGF- β pathway²⁶. CAFs are considered the primary source of ECM in tumors, where they are activated by cancer-derived factors during tumorigenesis, differentiation, and secretion of ECM components. Therefore, targeting the genes that promote ECM formation in malignant cells is a promising therapeutic approach. In this study, we found that PLAGL2 remodels HCC cell-produced ECM *via* an autocrine mechanism and induces HSC activation *via* a paracrine pathway. Therefore, targeted inhibition of PLAGL2 function is a promising strategy for inhibiting ECM formation.

The IGF2/IGF1R axis is a key driver in the pathogenesis of HCC^{27,28}. Recent studies implicate IGF2 overexpression and reduced SRSF3 splicing activity as primary contributors to DNA damage and hepatocarcinogenesis²⁹. Furthermore, the role of this axis in conferring drug resistance to HCC therapies has garnered significant research interest in recent years. Enhanced IGF/IGF1R signaling confers targeted drug resistance in HCC. For instance, IGF/IGF1R signaling causes sorafenib resistance by increasing

cancer stemness in cancer cells³⁰. IGF/IGF1R signaling pathways antagonize the inhibitory effects of regorafenib on pro-apoptotic proteins and activate growth signaling proteins³¹. It has also been reported that IGF1R inhibition can activate the EGFR–HER3–Akt pathway, and EGFR activation also confers potent resistance to IGF1R inhibitors. The EGFR inhibitor gefitinib, combined with the IGF1R inhibitor AVE1642, can synergistically inhibit the proliferation of HCC³². IGF1R/EGFR cross-talk contributes to gefitinib resistance in HCC³³. Altogether, these findings suggest that combined IGF1R and EGFR blockade can suppress resistance to single-receptor blockade. In cholangiocarcinoma, treatment with the EGFR inhibitor erlotinib stimulates the secretion of IGF2 in CAFs and activates the IGF2/IR/IGF1R pathway in tumor cells, which mediates resistance to erlotinib by CAF³⁴. Lenvatinib is an oral tyrosine multi-kinase inhibitor authorized for the first-line treatment of HCC. However, the overall clinical response rate for lenvatinib is limited, highlighting the urgent need for new combination therapy strategies to improve the clinical benefits of lenvatinib-based therapies for HCC³⁵. Several studies have identified the molecular mechanisms underlying the insensitivity of HCC cells to lenvatinib; for example, inhibition of EGFR overcomes acquired lenvatinib resistance driven by STAT3–ABCB1 or PAK2–ERK5 signaling axis^{23,24}. CAF-derived secreted phosphoprotein 1 contributes to HCC resistance to sorafenib and lenvatinib³⁶. Our previous and current studies have demonstrated that PLAGL2 is involved in the transcriptional regulation of EGFR and IGF1R¹⁵. Specifically, PLAGL2 promotes the activation of HCC CAFs *via* paracrine IGF2 signaling. Therefore, targeted inhibition of PLAGL2 function is a promising strategy to enhance drug sensitivity in HCC.

Another important finding of this work was the discovery of DC218 as a novel PLAGL2 transcriptional inhibitor through a computer-aided drug design and chemical evolution of the cytosine strategy. To the best of our knowledge, this is the first small molecule that targets the PLAGL2 DNA-binding domain. DC218, featuring a cytosine scaffold, showed a strong binding affinity to human recombinant PLAGL2 DNA-binding domain. DC218 significantly inhibited HCC ECM production both *in vitro* and *in vivo* and significantly enhanced the sensitivity of HCC cells to lenvatinib, thus providing a new potential therapeutic strategy for HCC.

5. Conclusions

To summarize, the transcription factor PLAGL2 facilitates the expression of ECM in HCC cells and activates HSCs. PLAGL2 directly regulates IGF2 and IGF1R expression in HCC and activates the IGF1R–PI3K/Akt pathway in HCC cells and HSCs through autocrine and paracrine IGF2, respectively. A novel small molecule, DC218, which inhibits PLAGL2 transcriptional regulation, was successfully developed. DC218 is an effective therapeutic agent for inhibiting ECM and exhibits a synergistic effect with lenvatinib in inhibiting HCC progression. These findings provide mechanistic insights into the role of PLAGL2 in ECM remodeling, as well as suggest a novel strategy for inhibiting ECM and treating HCC.

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Author contributions

Weiwei Hu: writing - original draft, supervision, visualization, project administration, investigation, formal analysis, data curation, conceptualization. Jiaping Ni, Shufang Zheng, Xiaohui Wei, Xueqing Shao: validation, investigation, formal analysis, data curation. Dongqing Zhai, Jinglin Qi, Xinqi Ye, Mingyin Lu, Hanrui Bian, Jintong Li, Yumeng Shen, Jingwei Jiang, Weijun Zhao, Shihao Xu: investigation. Yong Yang, Hong Liu, Xuewu Liang: supervision, resources, project administration, investigation, conceptualization.

Conflicts of interest

Authors declare that they have no competing interests.

Appendix A. Supporting information

Supporting information to this article can be found online at <https://doi.org/10.1016/j.apbsb.2025.12.016>.

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