Supporting Information for

Original article

A biomimetic liver cancer on-a-chip reveals a critical role of LIPOCALIN-2 in promoting hepatocellular carcinoma progression

Peiliang Shen[a], Yuanyuan Jia[a], Weijia Zhou[a], Weiwei Zheng[a], Yueyao Wu[a], Suchen Qu[a], Shiyu Du[a], Siliang Wang[b,c,*], Huilian Shi[d], Jia Sun[a], Xin Han[a,*]

[a]*Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica, School of Medicine & Holistic Integrative Medicine, Jiangsu Joint International Research Laboratory of Chinese Medicine and Regenerative Medicine, Nanjing University of Chinese Medicine, Nanjing 210023, China*

[b]*Department of Pharmacy, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing 210008, China*

[c]*Nanjing Medical Center for Clinical Pharmacy, Nanjing 210008, China*

[d]*Department of Infectious Diseases, Jiangsu Province Hospital of Chinese Medicine, Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing 210023, China*

Received 2 February 2023; received in revised form 3 April 2023; accepted 15 April 2023

*Corresponding authors.
E-mail addresses: wsl_dth@126.com (Siliang Wang); xhan0220@njucm.edu.cn (Xin Han).

1. Supporting figures

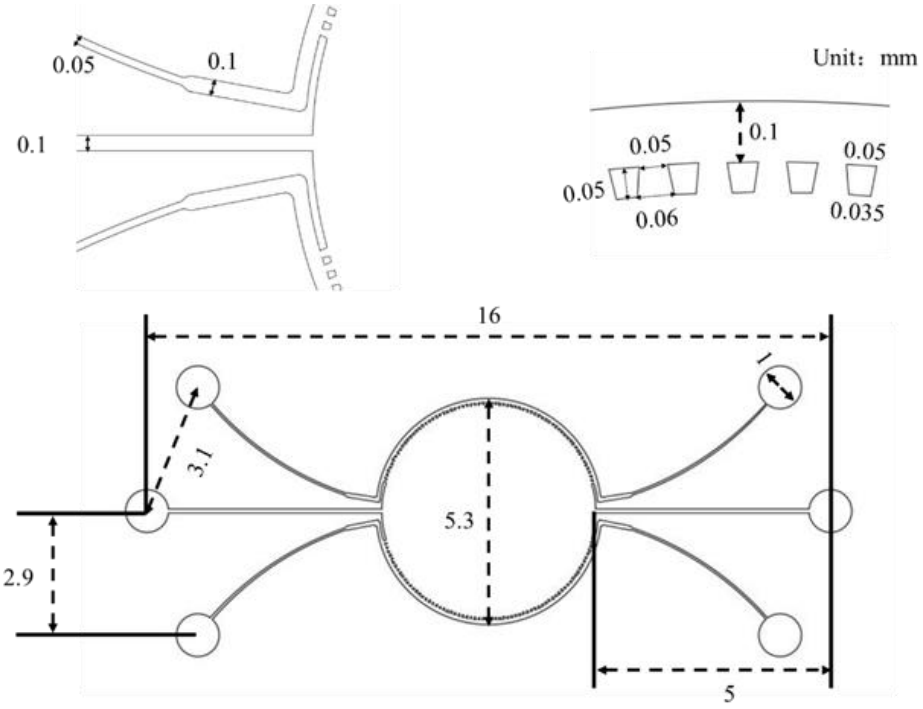

Figure S1 The design of the microfluidic chip. The whole chip is 16 × 7 mm (length × width) in dimension. The central chamber is 5 mm in diameter surrounded by two lateral channels with a width of 100 μm. There are trapezoidal barriers between the chambers and the channels, with the top line of 50 μm, the baseline of 35 μm and a gap from 50 μm to 60 μm. The design of the trapezoidal structure and distance of the gap can trap the Matrigel inside the central chambers without leaking into the side channels during the Matrigel loading process.

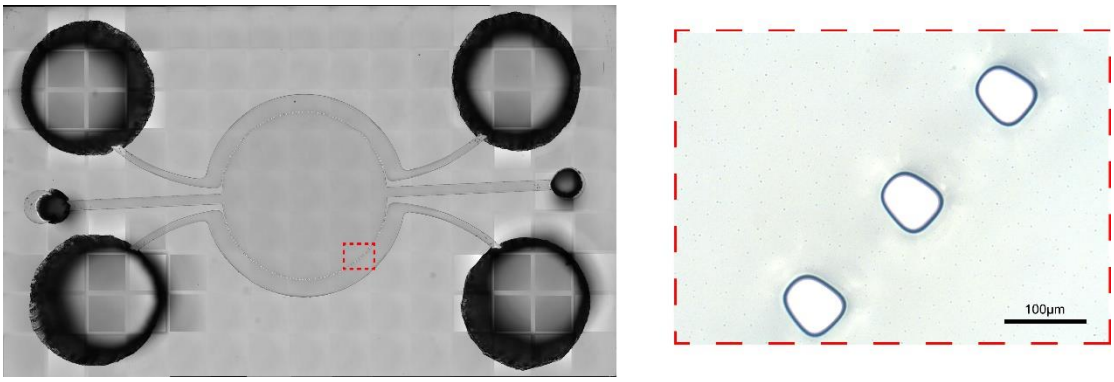

Figure S2 The bright-field images of the whole microfluidic chip. The whole microfluidic chip was scanned by the Thunder wide field high resolution imaging system, and the enlarged picture of indicated area was shown. Scale bar: 100 μm.

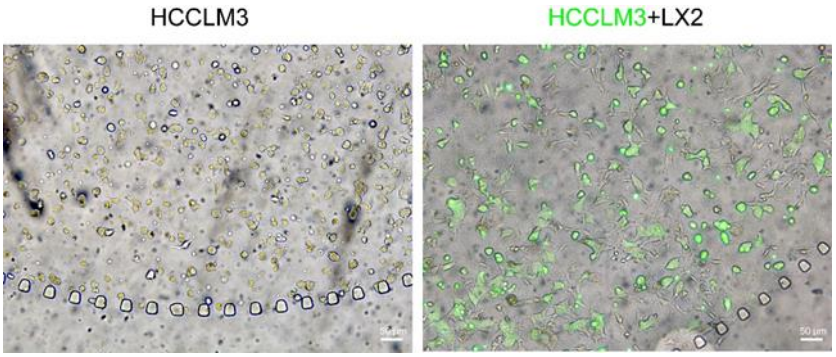

Figure S3 Co-culture of HCCLM3 and LX2 cells in the HCC-on-a-chip. HCCLM3 cells (labeled in green) and LX2 cells (no label) were mixed with the Matrigel at a 1:1 ratio (total density of 10×10^6 cells/ml) and injected into the chip for culture for 3 days. The bright-field images of the chip were captured by an inverted microscope. Scale bar: 50 μm.

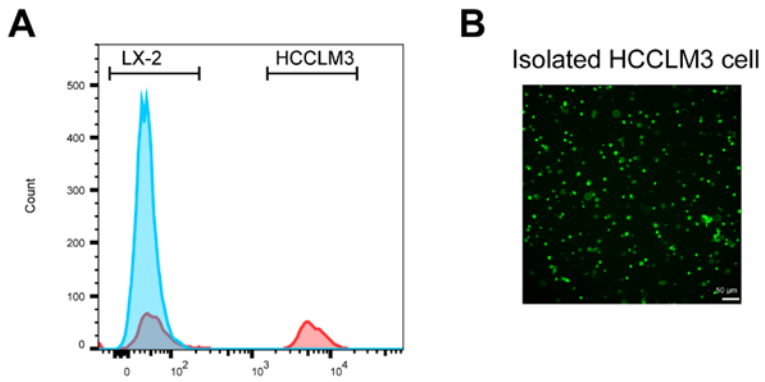

Figure S4 Flow cytometry sorting of cells isolated from the HCC-on-a-chip model. (A) LX2 cells (no label) and HCCLM3 tumor cells (labeled in green) were mixed with the Matrigel at a 1:1 ratio (total density of 10×10^6 cells/ml) and injected into the chip for culture. After 5 days' culture, the hydrogel was degraded and the total cells were isolated. Then HCCLM3 cells were selectively removed which were labeled in green fluorescence for the isolation of pure LX2 cells by flow cytometry and cell sorting. Flow cytometry analysis showed the two groups of cells. (B) Representative fluorescence images showed the isolated HCCLM3 cells (labeled in green) from the HCC-on-a-chip model. Scale bar: 50 μm.

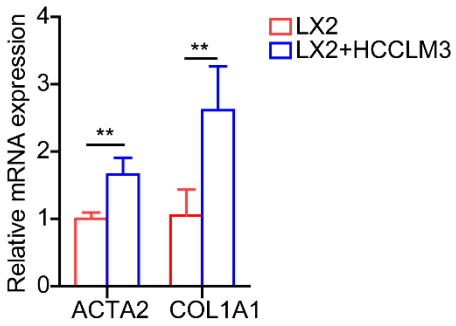

Figure S5 HCCLM3 induced the activation of LX2 cells. After sorting out the cells from the chip, the total RNA was extracted to detect the expression of activated genes in LX2 cells. RT-qPCR analysis revealed the expression levels of ACTA2 and COL1A1 were upregulated in LX2 cells co-cultured with HCCLM3 cells via the chip ($n = 3$ per group). Data were presented as mean ± SEM. $^{**}P < 0.01$.

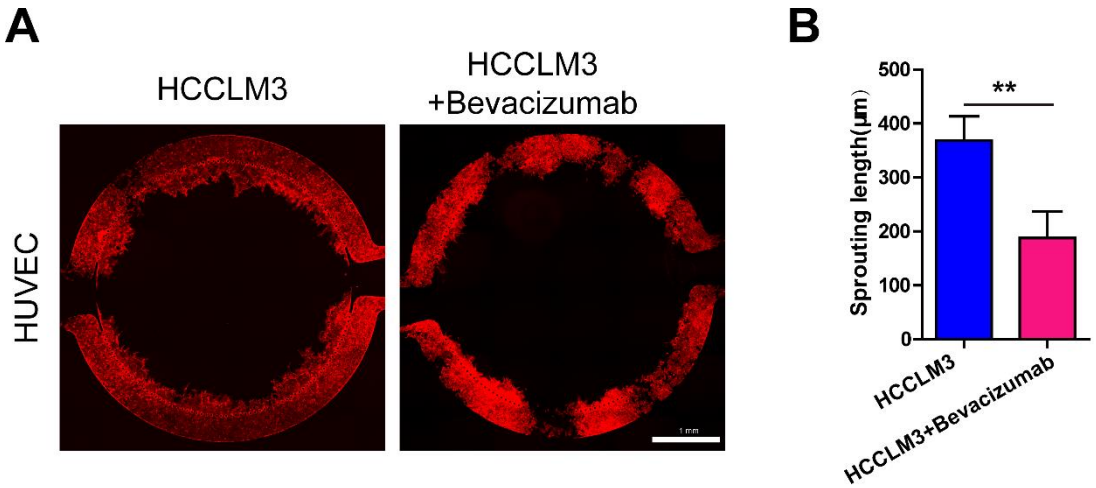

Figure S6 VEGF inhibitor restricted the Endothelial cell invasion. (A) The conditional medium of HCCLM3 cells was mixed with Bevacizumab in the Matrigel and injected into the central chamber. The HUVECs were lined in the lateral channels for 5 days. Fluorescence microscopy images of endothelial cell invasion were shown in indicated groups via chip. Scale bar: 1 mm. (B) Quantification of endothelial cell invasion in indicated groups from A ($n = 3$ per group). Data are presented as mean ± SEM. $^{**}P < 0.01$.

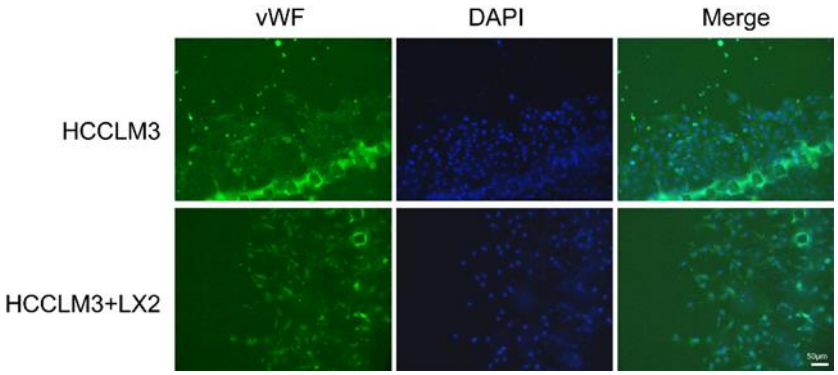

Figure S7 Endothelial properties of HUVECs in the HCC-on-a-chip model. HCCLM3 cells and LX2 cells were mixed with the Matrigel at a 1:1 ratio (total density of 10×10^6 cells/ml) and injected into the chip for 5 days. The conditional medium was collected and mixed with the Matrigel and injected into the central chamber to build the tumor cytokines microenvironments. After Matrigel polymerization, the HUVECs were lined in the lateral channels to mimic the endothelial cell invasion process for 5 days. The HUVECs were fixed and stained with Von Willebrand Factor (vWF) antibody. Representative fluorescence images of vWF staining showed HUVECs invaded towards the tumor cytokines microenvironments, which maintained the significant endothelial properties. Scale bar: 50 μm.

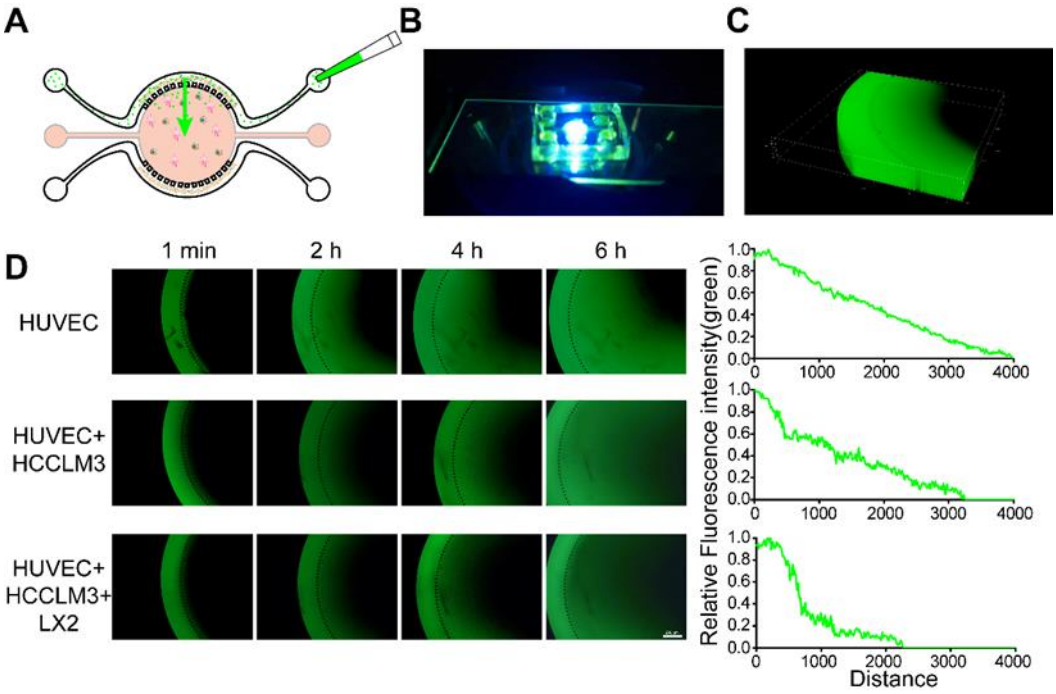

Figure S8 Assessment of the FITC-Dextran permeating. (A) Schematic representation of FITC-Dextran permeated into the central hydrogel. HCCLM3 and LX2 cells were mixed with the Matrigel at a 1:1 ratio (total density of 10×10^6 cells/ml) and injected into the central chamber. Endothelial cells were then lined in the side channels for culture for 3 days. The medium was then removed and FITC-Dextran was induced from one side for 6 h. (B) and (C) Representative fluorescent images of FITC-Dextran permeation were shown. (D) The fluorescence microscopy images of FITC-Dextran were shown from indicated groups in the chip at indicated time points. Scale bar: 200 μm. Intensity versus distance across the chip was measured for quantification.

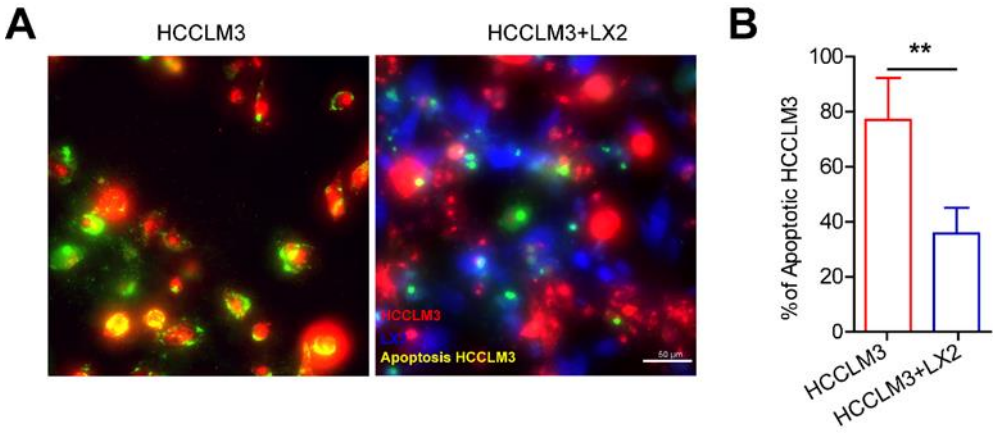

Figure S9 Assessment of Sorafenib resistance in the HCC-on-a-chip model. (A) The HCCLM3 cells (labeled in red) and LX2 (labeled in blue) were mixed with the Matrigel at a 1:1 ratio (total density of 10×10^6 cells/ml) and injected into the chip for 3 days. Then sorafenib was added from one side channel of the chip to investigate the tumor cell killing effects. After 48 h, the SuperView 488 caspase-3 assay kit was used to label the apoptotic cells. Representative fluorescence images were shown from indicated groups in which apoptotic HCCLM3 cells were labeled in yellow. Scale bar: 50 μm. (B) Quantification data of the percentage of apoptotic HCCLM3 cells in indicated groups in the HCC-on-a-chip model ($n = 3$ per group). Data were presented as mean ± SEM. $^{**}P < 0.01$.

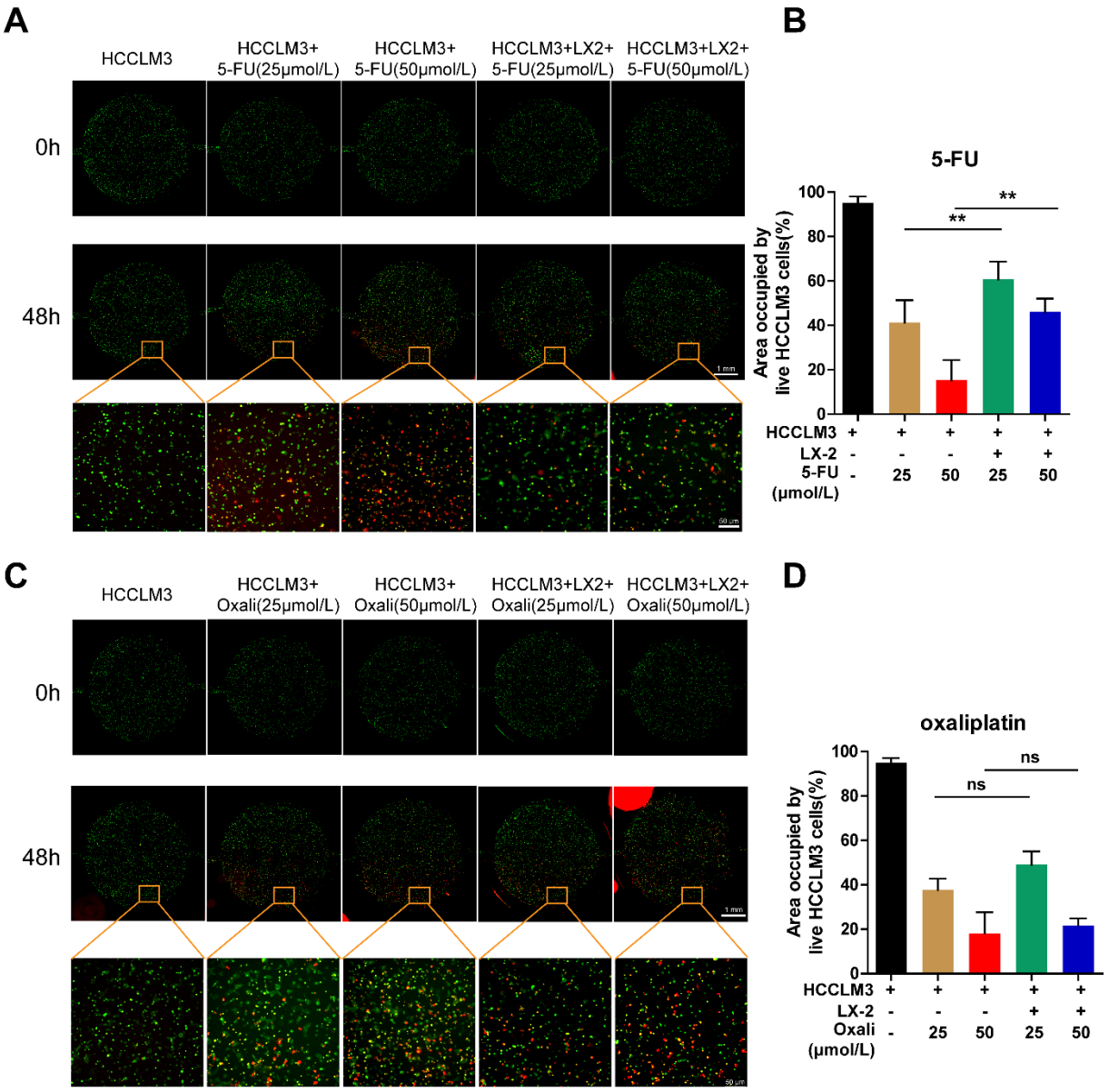

Figure S10 Assessment of cell resistance to chemotherapeutic reagents in the HCC-on-a-chip model. The HCCLM3 cells (labeled in green) and LX2(no label) were mixed with the Matrigel at a 1:1 ratio (total density of 10×10^6 cells/ml) and injected into the chip for 3 days. Then chemotherapeutic reagents were added from one side channel of the chip to investigate the tumor cell killing effects. After 48 h, PI staining was applied to label the dead cells. (A) Representative fluorescence microscopy images of live/dead HCCLM3 cells were shown from indicated groups in the chip after 5-FU treatment were applied for 48h. Scale bar: 1 mm. (B) Quantification of the area occupied by the live HCCLM3 cells (green) in indicated groups from A ($n = 3$ per group). (C) Representative fluorescence microscopy images of live/dead HCCLM3 cells were shown from indicated groups in the chip after oxaliplatin (Oxali) treatment were applied for 48h. Scale bar: 1 mm. (D) Quantification of the area occupied by

the live HCCLM3 cells (green) in indicated groups from C ($n = 3$ per group). Data were presented as mean ± SEM. **$P < 0.01$. ns, not significant.

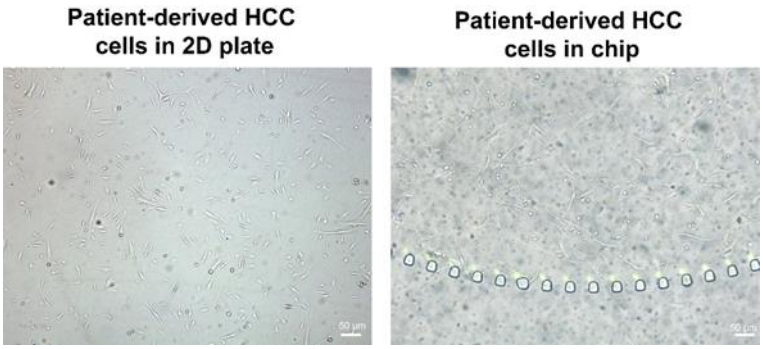

Figure S11 Patient-derived cells culture in the chip. The monolayer patient-derived cells were cultured on the 2D plate which was coated with collagen (left). The patient-derived cells (total density of 10×10^6 cells/ml) were mixed with Matrigel and injected into the chip for 3 days. The bright-field image showed the patient-derived cells cultured in the HCC-on-a-chip model (right). Scale bar: 50 μm.

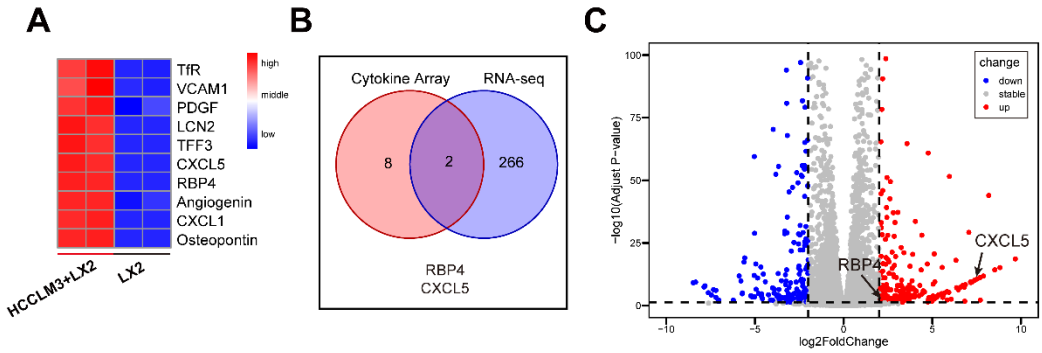

Figure S12 The regulation of gene expression in LX2 cells by the interaction with HCCLM3 cells. The HCCLM3 and LX2 cells (total density of 10×10^6 cells/ml) were mixed with the Matrigel and injected into the chip for 5 days. The conditional medium was then gathered from the chip for cytokine array assessment. (A) Heat map analysis showed significant changed cytokines in secretion in cell co-culture group compared with LX2 cells culture alone. (B) Venn diagram showed the upregulated genes in LX2 cells by the combination of RNA-seq and secretomics analysis results. (C) The Volcano plot analysis of RNA sequencing data in isolated LX2 cells from chip. Two upregulated cytokines were marked which showed an obvious increase of secretion level in the cell co-culture system via chip.

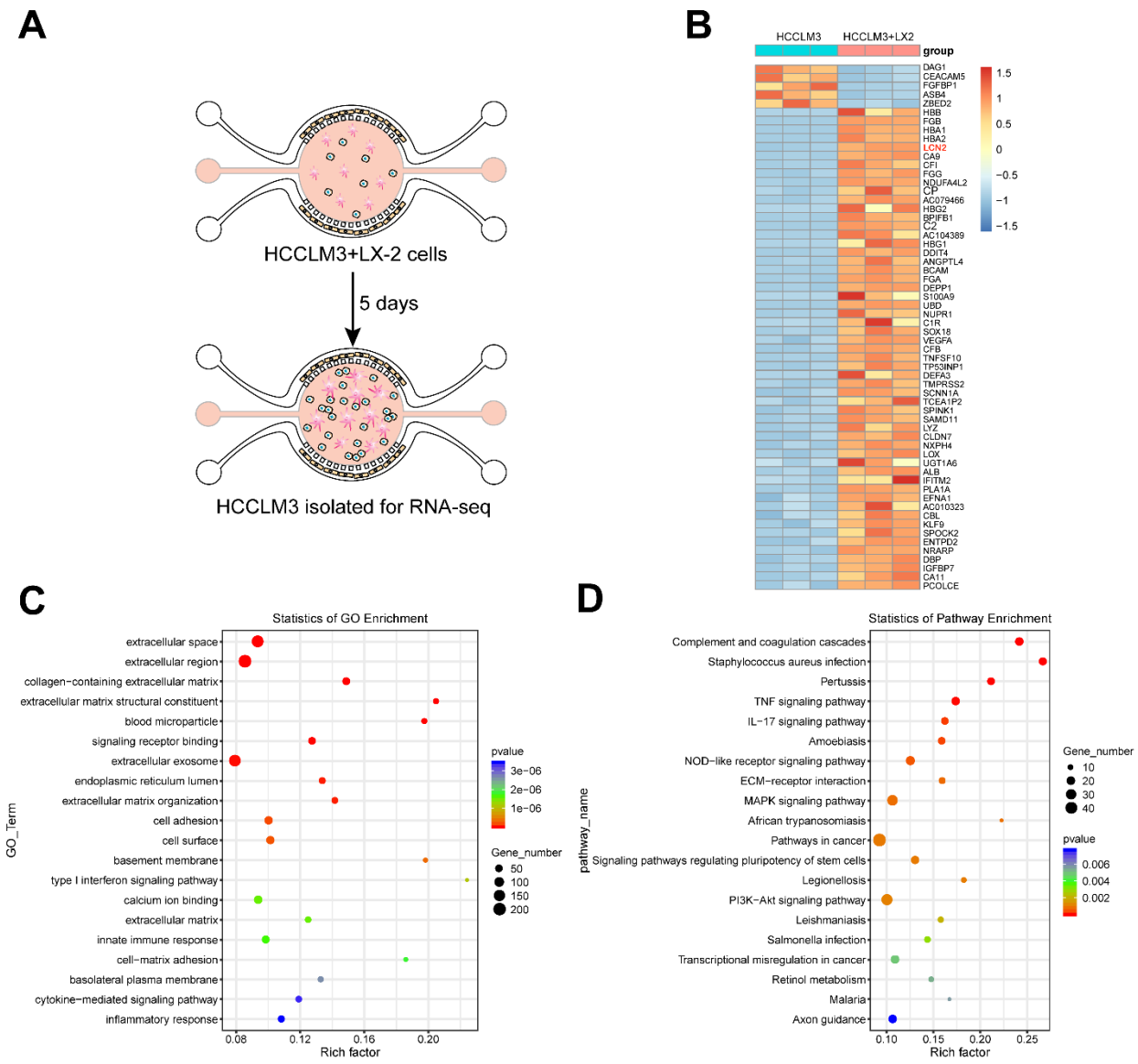

Figure S13 The regulation of gene expression in HCCLM3 cells by the interaction of LX2 cells in the HCC-on-a-chip model. (A) Scheme of HCCLM3 cell isolation from chip. HCCLM3 cells (no label) and LX2 cells (labeled in green) were mixed with the Matrigel at a 1:1 ratio (total density of 10×10^6 cells/ml) and injected into the chip for 5 days. Then the collagen hydrogel was degraded and the total cells were isolated. To obtain pure HCCLM3 cells, LX2 cells, which were labeled with green fluorescence, were selectively removed using flow cytometry and cell sorting. The total RNA of isolated HCCLM3 cells was sent for RNA-seq assessment. (B) Heat map analysis showed significant changed genes in expression from isolated HCCLM3 cells in indicated groups. (C) Gene Ontology enrichment analysis showed the up-regulated genes and enriched biological processes in isolated HCCLM3 cells via the cell co-culture system. (D) A KEGG enrichment analysis indicated the signaling pathway which showed a significant change in isolated HCCLM3 cells via the cell co-culture system.

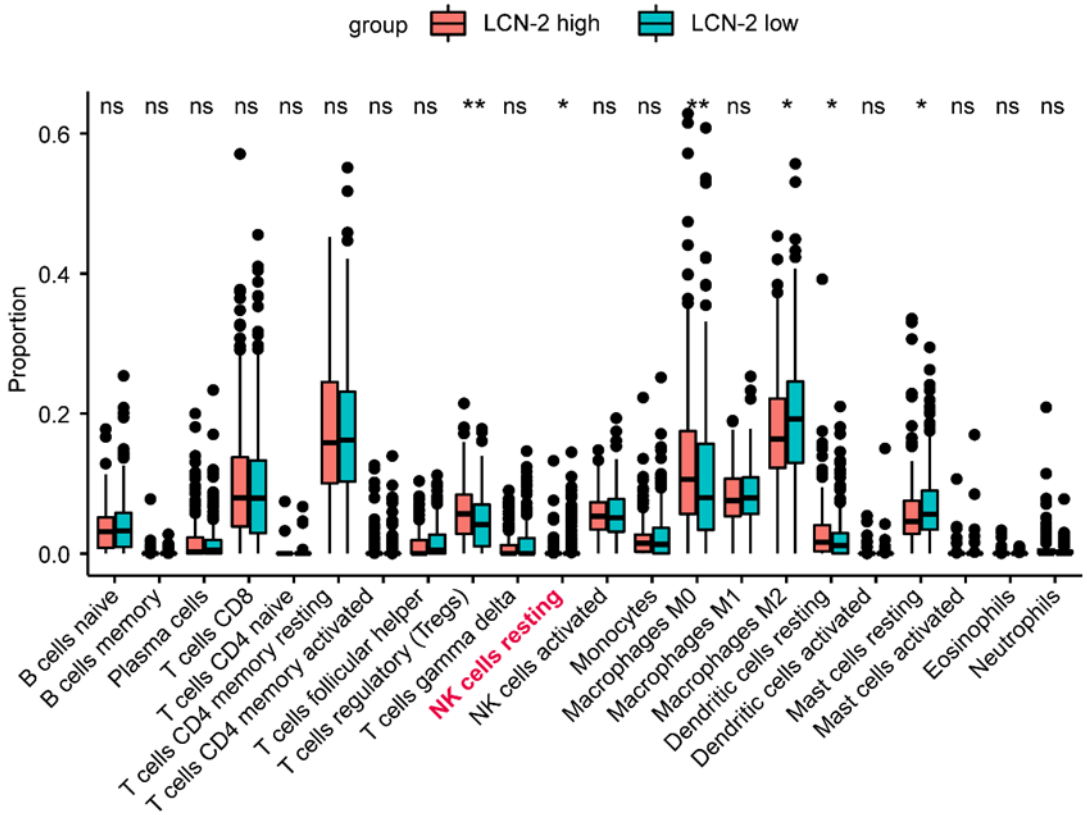

Figure S14 NK cell infiltration was decreased in LCN-2^{high} expression HCC tissues. Immune cell infiltration in HCC tissue was evaluated using the CIBERSORT algorithm. The boxplot graph showed the difference of immune infiltration between LCN-2^{high} and LCN-2^{low} expression HCC tissues from TCGA. *P < 0.05; **P < 0.01.

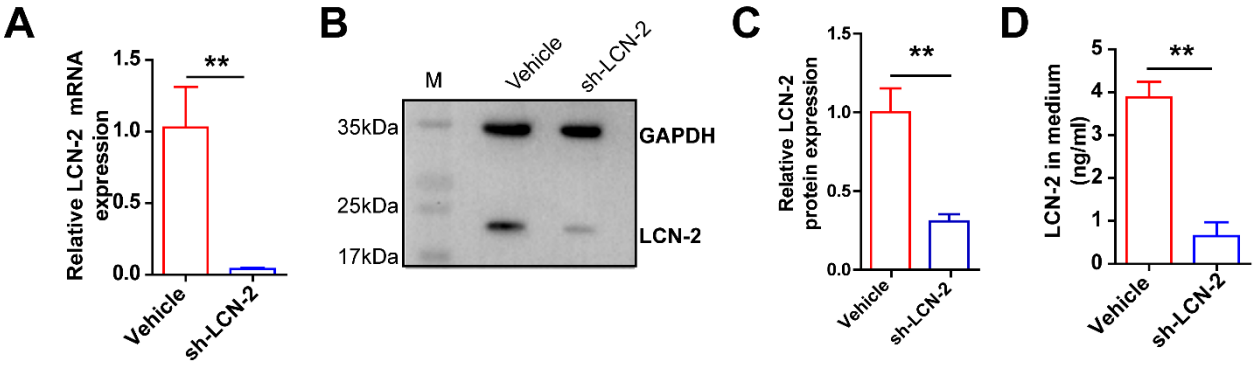

Figure S15 LCN-2 reduction by shRNA knockdown in the HCCLM3 cells. The expression of LCN-2 in HCCLM3 cells was knocked down by a shRNA plasmid transfection. LCN-2 mRNA expression level (A), LCN-2 total protein expression level (B, C) and LCN-2 secretion levels (D) in the medium were detected respectively to show the successful knockdown in HCCLM3 cells ($n = 3$ per group). Data were presented as mean ± SEM. $^{**}P < 0.01$.

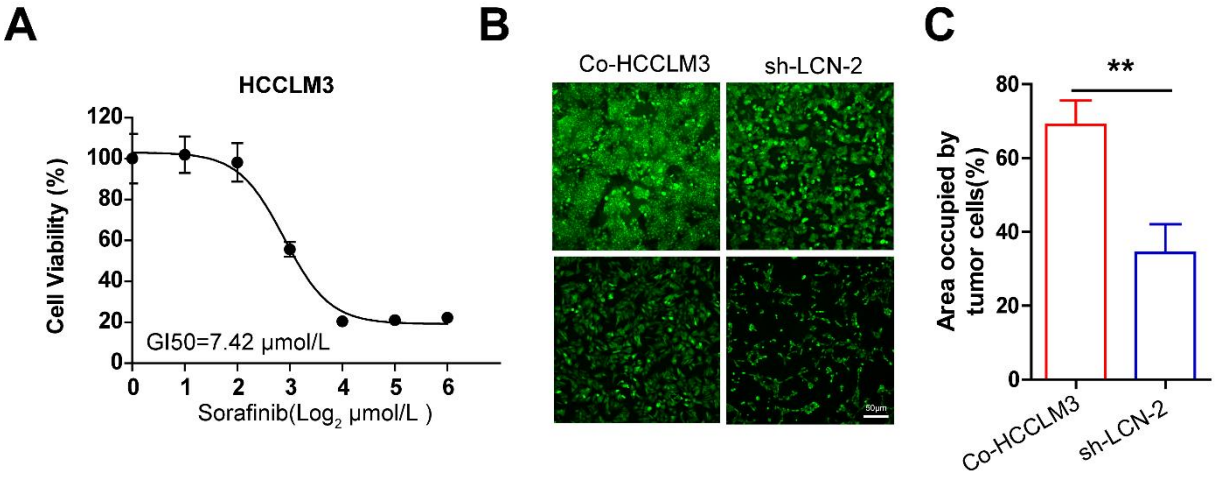

Figure S16 LCN-2 mediated sorafenib resistance. (A) The cell viability of HCCLM3 cells was calculated *in vitro* by using the CCK-8 assay after sorafenib treatment at different concentrations ($n = 6$). (B) The LCN-2 depleted HCCLM3 cells were embedded and cultured with LX2 in the HCC-on-a-chip for 5 days and the culture medium was gathered from side channels of the chip. The conditional medium collected from the chip was used to treat HCCLM3 cells to test cell responses to sorafenib treatment for 48h. Fluorescence microscopy image of the HCCLM3 cells (green) were shown in the indicated groups. Scale bar: 50 μm. (C) Quantitative analysis of area occupied by the HCCLM3 cells in the indicated groups ($n = 3$ per group). Data were presented as mean ± SEM. $^{**}P < 0.01$.

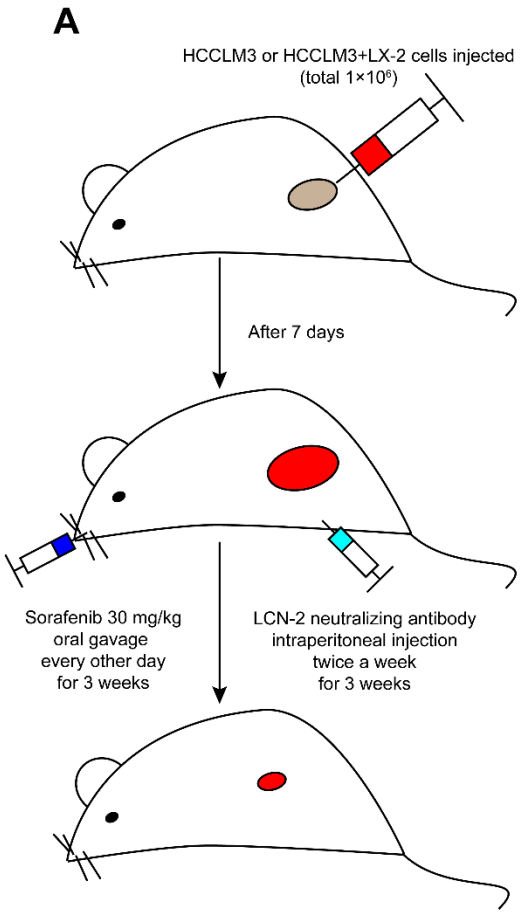

Figure S17 Schematic illustration of the treatment process in the nude mice bearing tumors. The xenografts were established by subcutaneous injection of HCCLM3 cells alone or the mixture of HCCLM3 and LX2 cells into the nude mice. When tumors reached 50-150 mm^3 after 7 days, the mice were treated with sorafenib (30 mg/kg, oral gavage every other day) or with LCN-2 neutralizing antibody (75 μg per mouse, intraperitoneal injection twice a week) for 3 weeks as indicated.

2. Supporting Movies

Supporting Movie 1 Time-lapse showing NK-92 cell invasion in the HCC-on-a-chip model with HCCLM3 cultured alone for 457 min. HCCLM3 cells were mixed with the Matrigel mixture (total density of 2×10^6 cells/ml) and injected into the chip for 3 days. NK-92 cells were then perfused through the side channel at 5×10^6 cells/ml. After NK-92 cells were loaded and stabilized for 2h, images were collected for a total of 457 min.

Supporting Movie 2 Time-lapse showing NK-92 cell invasion in the HCC-on-a-chip model with HCCLM3 and LX2 cells co-cultured for 457 min. HCCLM3 and LX2 cells were mixed with the Matrigel at a 1:1 ratio (total density of 2×10^6 cells/ml) and injected into the chip for 3 days. Then NK-92 cells were perfused through the side channel of the chip at 5×10^6 cells/ml. After NK-92 cells were loaded and stabilized for 2h, images were collected for a total of 457 min.

3. Supporting tables

Table S1 The sequences of primers used for qPCR analysis.

Genes	Sequence (5’ to 3’)
hACTA2-F	AAAAGACAGCTACGTGGGTGA
hACTA2-R	GCCATGTTCTATCGGGTACTTC
hCOL1A1-F	TTTGGATGGTGCCAAGGGAG
hCOL1A1-R	CACCATCATTTCCACGAGCA
hLCN2-F	TCACCTCCGTCCTGTTTAGG
hLCN2-R	CGAAGTCAGCTCCTTGGTTC
hGAPDH-F	GAGTCAACGGATTTGGTCGT
hGAPDH-R	TGGAAGATGGTGATGGGATT