Activated HSCs promote progression of post-heat residual HCC from autophagic survival to proliferation

Rui Zhang, Xia-Hui Lin, Hua-Hua Liu, Min Ma, Jie Chen, Jun Chen, Dong-Mei Gao, Jie-Feng Cui, Rong-Xin Chen*

Liver Cancer Institute, Zhongshan Hospital, Fudan University and Key Laboratory of Carcinogenesis and Cancer Invasion, Ministry of Education, Shanghai, China.

*Correspondence to: Rong-Xin Chen, Fax: +86-21-64037181, Tel: +86-21-64041990-2043, E-mail: chen.rongxin@zs-hospital.sh.cn

Doc S1. Supplementary Materials and Methods

Cell cultures, Conditioned Medium (CM) Collection, Heat treatment and Cell transfections

Human HCC cell lines MHCC97H, HCCLM3 (Liver Cancer Institute of Fudan University, Shanghai, China), SMMC7721 (Cell bank of Chinese Academy of Sciences, Shanghai, China), HepG2 (American Type Culture Collection, USA), Huh7 (Japanese Cancer Research Bank) were routinely maintained in Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Primary human hepatic stellate cells (pHSCs) (Sciencell, USA) and LX2 cells (a kind gift from S. Friedman) were cultured in the provided media and DMEM supplemented with 2% FBS, respectively. All cell cultures were performed in a 37 ℃ incubator containing 5% CO_2. Cell line authentications have been carried out by STR profiling.

CM from activated HSC (HSC-CM) was collected as previously described [1]. Briefly, CM of LX-2 or pHSCs was collected from confluent LX-2 or pHSCs in 25-cm^2 flasks by 24 hours of incubation in 2% FBS DMEM. The same culture medium without cells was served as the control medium.

According to our prior descriptions, HCC cells were heated at gradient temperatures and cell viabilities were assessed using WST-1 assay (Roche, Germany). IT50 (the temperature of inducing a 50% reduction of cell viability) was determined to simulate sublethal thermal condition.

Lentivirus expressing short hairpin RNA (shRNA) of ATG5, BECN1 and negative control were produced by Genechem (Shanghai, China). For the transfection, cells were seeded in 6-well plates at 40%-50% confluence and infected with the lentivirus with a multiplicity of infection (MOI) of 10 and 5 μg/ml polybrene. After 24 h

of lentiviral transfection, transfecting medium was replaced and cells were harvested for passage or experiment when they were grown to 80% confluence.

Flow Cytometry for Cell Apoptosis and Cell Cycle

Cell apoptosis analysis was performed using annexin V/propidium iodide (PI) staining kit (Invitrogen, California, USA) in accordance with manufacturer's instructions. As described previously, cells were maintained in the prepared media for 24 h, and subsequently trypsinized, washed, and incubated with annexin V and PI staining for desirable time. FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) was used to analyze the stained cells. For cell cycle distribution analysis, cells were analyzed on the flow cytometer after fixed and incubated with PI/RNase staining buffer at room temperature for 15 min.

Cell proliferation

Proliferation was assessed using a WST-1 assay (Roche, Germany). Cells (1×10^4 cells per well) were seeded onto 96-well plates and cultured in the prepared media for desirable time. The absorbance was measured with a Microplate Spectrophotometer.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated using Trizol reagent (Ambion, CA, USA) and reversely transcribed with RevertAid First Strand cDNA synthesis kit (Thermo Scientific, Waltham, Mass). PCR amplification was performed using Maxima SYBR Green qPCR Master Mix kit (Thermo Scientific, Waltham, Mass). Quantitative analysis of target gene expression was determined with the $2^{-\Delta\Delta Ct}$ method using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) Ct values for normalization.

Western blotting analysis

Total proteins were extracted with RIPA lysis buffer (Beyotime biotechnology, China) supplemented with protease and phosphatase inhibitors (Roche, Mannheim, Germany). Proteins were quantified using BCA Protein Assay Kit (Thermo Scientific, Waltham, Mass), separated by SDS-PAGE and then electro-transferred to polyvinylidene difluoride membranes (Millipore, MA, USA). Membranes were blocked with 5% non-fat milk, incubated with primary antibody, and probed with horseradish peroxidase-conjugated secondary antibody. The immuno-reactive bands were detected using enhanced chemiluminescence (Millipore, MA, USA).

Immunohistochemistry

Immunohistochemistry staining was performed according to the manufacturer's protocol. Briefly, paraffin-embedded tissue sections were deparaffinized, hydrated, and then subjected to microwave antigen retrieval. After incubated with primary and secondary antibodies, slides were developed using DAB and counterstained with Mayer's hematoxylin. Images were photographed with an Olympus BX51 microscope (under ×100 magnification).

Immunofluorescence

Cells (pHSCs and LX2) were seeded onto 24-well plates for 24h. After that, cells were washed, fixed and permeabilized. Then, the cells were blocked with 5% bovine serum albumin (BSA) for 30 min at 37 °C and incubated with antibody overnight at 4°C. The nuclei were stained with DAPI. Images were captured with an Olympus BX51 microscope (under ×200 magnification).

Cytokine antibody array and Enzyme-linked immunosorbent assay (ELISA)

The cytokine profiles of conditioned media from activated HSCs were analyzed with a semiquantitative human cytokine antibody array (Raybiotech, Norcross GA) that detects 507 cytokines in one experiment. In brief, a panel of antibodies is immobilized on the surface of an array glass slide. Samples were biotinylated, dialyzed, hybridized to the array and incubated overnight at 4 °C. After labelled with Cy3-Conjugated streptavidin, the signals were detected using a GenePix 4000 scanner and analyzed with GenePix Pro 6.0 (Molecular Devices, Sunnyvale, CA). ELISA verification of cytokine was conducted in accordance with the manufacturer's instructions.

Autophagy analysis

Autophagy was assessed by microtubule-associated protein light chain 3 (LC3) redistribution and electron microscopy. To detect the occurrence of autophagy flux, MHCC97H or HepG2 cells reached 60%-70% confluence were transfected with mCherry-GFP-LC3B lentiviral vector (GenePharma Co., Ltd., Shanghai, China) at an MOI of 20 for 24 h. In this system, autophagosomes are colored yellow spots (LC3II dot or puncta) as they express both GFP and mCherry. When autophagosome is fused with lysosome, autolysosomes are colored red spot as they express mCherry alone. Representative images were captured with a fluorescence microscope (Olympus BX51, Tokyo, Japan).

Cells and tumor tissues were immediately fixed with 2.5% glutaraldehyde containing 0.1 mol/L sodium cacodylate and stored at 4 °C before embedding. Samples were fixed with 1 % osmium tetroxide followed by an increasing gradient dehydration step using ethanol and propylene oxide, embedded in araldite and then cut into ultrathin sections (50-60 nm). Images were acquired with a JEM-1200 electron microscope after samples were stained with 3% lead citrate-uranyl acetate.

Microarray analysis

Total RNA was extracted from heat-exposed residual MHCC97H cells treated with HGF (0, 2, 10 ng/mL) using miRNeasy Mini kit (QIAGEN, Germany). Genome-wide expression profiling was determined using Affymetrix GeneChip Human Genome U133 Plus 2.0 Array according to the manufacturer's instructions. Briefly, total RNA samples were amplified, labelled and purified to obtain biotin-labeled cRNA using GeneChip 3'IVT PLUS reagent Kit (Affymetrix, CA, US). The arrays were hybridized, washed and stained using the GeneChip Hybridization Wash and Stain Kit, and then scanned with a GeneChip Scanner 3000 (Affymetrix, CA, US). The gene expression data were processed using MAS 5.0 algorithm, Affy packages in R. Differentially expressed genes were selected based on fold change ≥ 2 or ≤ 0.5 ($P < 0.05$). Analyses of KEGG pathway and K-means clustering were conducted for the differentially expressed genes. The microarray data have been deposited into Gene Expression Omnibus (GEO) database (GSE112349).

TCGA dataset

Gene expression profiles of HCC cohorts were obtained from public database of the Caner Genome Atlas project (TCGA dataset, https://tcga-data.nci.nih.gov/tcga/) for analyses. The expression enrichment of a gene signature was assessed using the pre-ranked GSEA (gene set enrichment analysis). The associations between two parameters was evaluated using Spearman's correlation test.

Animal experiments

All animal experiments were performed in accordance with the guidelines of Shanghai Medical Experimental Animal Care Commission. The protocols of animal experiments were approved by Zhongshan Hospital Research Ethics Committee of Shanghai, China**.**

Male nude mice aged 6 weeks were purchased from Shanghai Slack Laboratory Animal Co., Ltd. MHCC97H orthotopic nude mouse model was developed as previously described [2]. Briefly, approximately 5×10^6 MHCC97H cells were re-suspended in serum-free DMEM and injected subcutaneously into the right flanks of each nude mice. After 4 weeks, tumor samples were dissected and cut into slice of 2

mm^3. Then, the slice was orthotopically re-implanted into the left hepatic lobe of each mouse. When tumor size reached about 1.5cm in diameter, mice were subjected to partial RFA or sham ablation. As we described previously [3], the ablation was conducted using Cool-tip RFA needle (Covidien, Inc. Boulder, CO, USA) with 1-cm active tip. In brief, the mouse anesthetized with pelltobarbitalum natricum (50 mg/kg, i.p.) was placed on a conductive metal plate. After laparotomy, the tumor was inserted using a 17-gauge RFA needle and ablated at a power output of 5 W for 30 s. Ablation was not turned on for the sham-ablated mouse. After ablation, skin incision was sutured with 5-0 non-absorbable sutures. Mice were euthanized at 24 h post-ablation and tumors were harvested.

To investigate the therapeutic effects of dual blocking autophagy and HGF/c-Met signaling, mice bearing orthotopic tumor were subjected to incomplete RFA and randomized into the following treatments started from 24 h after ablation (n=5 for each group): (i) Control group, saline treatment (0.9% sodium chloride, i.p.); (ii) Chloroquine treatment group (60mg/kg, i.p., 4 times per week and maintained for one week); (ii) c-Met inhibitor treatment group (PHA665752, 0.83 mg/kg, i.p., daily, continued for two weeks); (iii) Combined treatment group (60mg/kg, i.p., 4 times per week and maintained for one week, and PHA665752, 0.83 mg/kg, i.p., daily, continued for two weeks). Animals were sacrificed 24 h after the last treatment administration and tumors were removed, photographed, fixed and analyzed.

References

1. Zhang R, Yao RR, Li JH, Dong G, Ma M, Zheng QD, et al. Activated hepatic stellate cells secrete periostin to induce stem cell-like phenotype of residual hepatocellular carcinoma cells after heat treatment. Sci Rep. 2017;7: 2164.

2. You A, Cao M, Guo Z, Zuo B, Gao J, Zhou H, et al. Metformin sensitizes sorafenib to inhibit postoperative recurrence and metastasis of hepatocellular carcinoma in orthotopic mouse models. J Hematol Oncol. 2016; 9:20.

3. Zhang R, Ma M, Lin XH, Liu HH, Chen J, Chen J, et al. Extracellular matrix collagen I promotes the tumor progression of residual hepatocellular carcinoma after heat treatment. BMC Cancer. 2018;18(1):901.

Table S1. Lists of Antibodies.

Name	Cat No.	Methods	Supplier
anti-LC3B	3868	Western blots/ immunohistochemistry	Cell Signaling Technology, Danvers, MA
anti-cleaved Caspase3	9664	Western blots/ immunohistochemistry	Cell Signaling Technology, Danvers, MA
anti-P62	88588	Western blots/ immunohistochemistry	Cell Signaling Technology, Danvers, MA
anti-Cyclin D1	2978	Western blots	Cell Signaling Technology, Danvers, MA
anti-Atg5	12994	Western blots/ immunohistochemistry	Cell Signaling Technology, Danvers, MA
anti-Beclin1	3495	Western blots	Cell Signaling Technology, Danvers, MA
anti-p44/42 MAPK (Erk1/2)	4695	Western blots	Cell Signaling Technology, Danvers, MA
anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/tyr204)	4370	Western blots/ immunohistochemistry	Cell Signaling Technology, Danvers, MA
anti-c-Met	8198	Western blots	Cell Signaling Technology, Danvers, MA
anti-phospho-c-Met (Try1234/1235)	3077	Western blots/ immunohistochemistry	Cell Signaling Technology, Danvers, MA
anti-Bcl-2	Ab59348	Western blots/ immunohistochemistry	Abcam, Cambridge, UK
anti-α-SMA	Ab195889	Immunocytochemistry	Abcam, Cambridge, UK
anti-α-SMA	BM0002	Immunohistochemistry	Boster Biological Technology, China
anti-HGF	DF6326	Immunohistochemistry	Affinity Biosciences, USA
anti-Ki-67	Ab16667	Immunohistochemistry	Abcam, Cambridge, UK

anti-actin	AA128	Western blots	Beyotime biotechnology, China
anti-GAPDH	AG019	Western blots	Beyotime biotechnology, China

Table S2. Primers for quantitative RT-PCR.

Gene	Sequence
ATG7	Forward-5'-CTGTGGCATCTGCTGACCTC-3' Reverse-5'-AGGTCCGGTCTCTGGTTGAA-3'
ATG5	Forward-5'-TGGACAGTTGCACACACTAGGA-3' Reverse-5'-TCAGATGTTCACTCAGCCACTG-3'
Beclin1	Forward-5'-CACATCTGGCACAGTGGACA-3' Reverse-5'-GGAGCAGCAACACAGTCTGG-3'
P62	Forward-5'-TGGCCATGTCCTACGTGAAG-3' Reverse-5'-AGCCATCGCAGATCACATTG-3'
LC3B	Forward-5'-GGCGCTTACAGCTCAATGCT-3' Reverse-5'-GATTGGTGTGGAGACGCTGA-3'
GAPDH	Forward-5'-GGAGCGAGATCCCTCCAAAAT-3' Reverse-5'-GGCTGTTGTCATACTTCTCATGG-3'

Table S3. The changes of autophagy-related genes in heat-treated surviving MHCC97H cells in response to HGF at 0, 2, 10 ng/mL.

Gene	Fold change in heat-treated residual MHCC97H cells		
	HGF (ng/ml) 2 vs. 0	10 vs 2	10 vs 0
ATG13	1.22	0.98	1.19
ULK2	1.42	0.8	1.13
ATG14	1.58	0.74	1.18
ATG12	1.72	0.93	1.59
ATG4B	1.41	0.77	1.08
BECN1	2.62	0.72	1.88
ATG5	4.83	0.63	3.02
ATG101	1.22	0.84	1.03
ATG7	1.3	0.9	1.17
ATG16L1	2.74	0.65	1.79
ATG10	1.01	0.77	0.78
MAP1LC3B2	3.06	0.76	2.32
ULK1	1.66	0.81	1.34

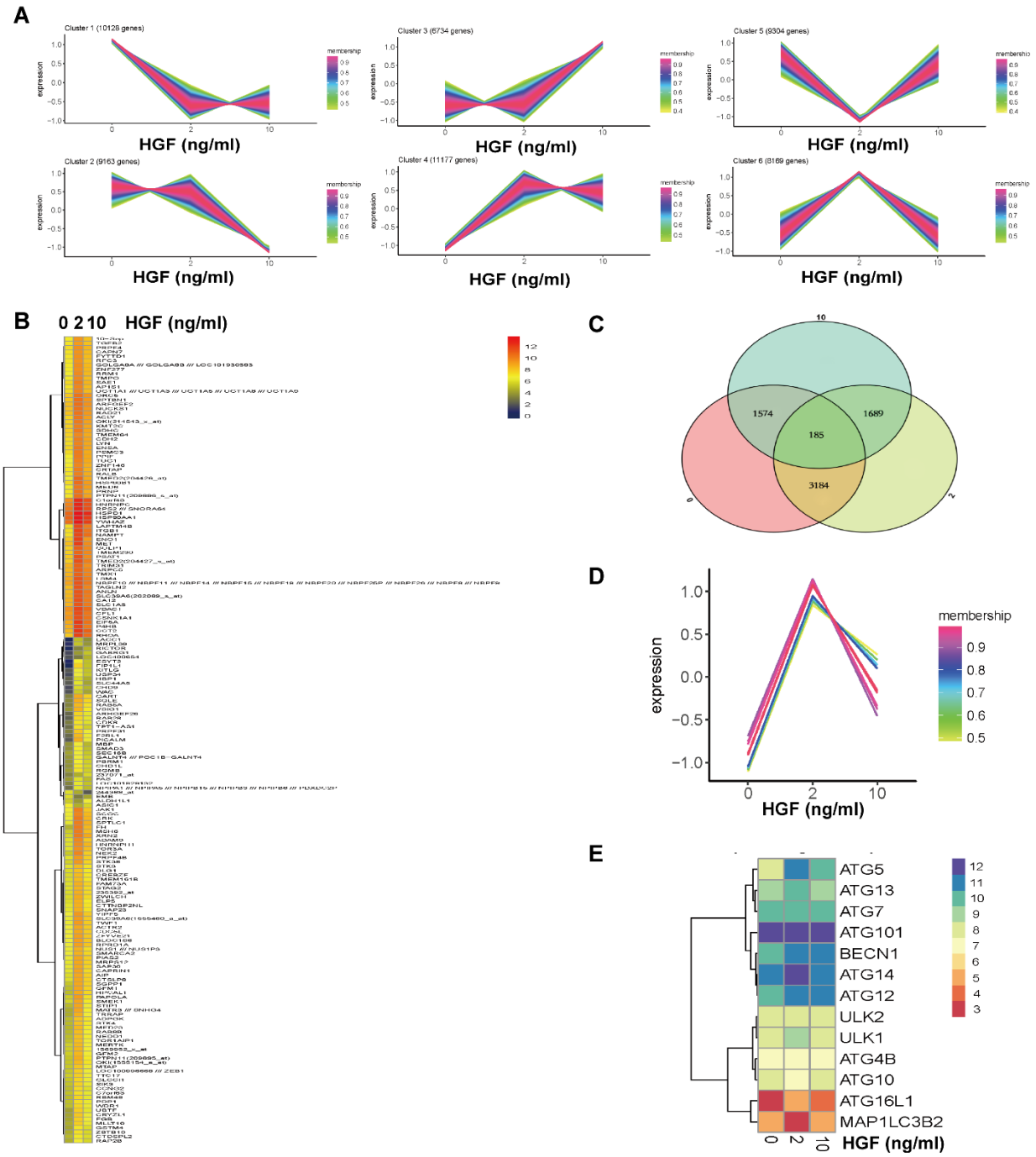

Figure S1. Autophagic proteins in heat-treated residual HCC cells in response to HGF were identified using gene microarrays.