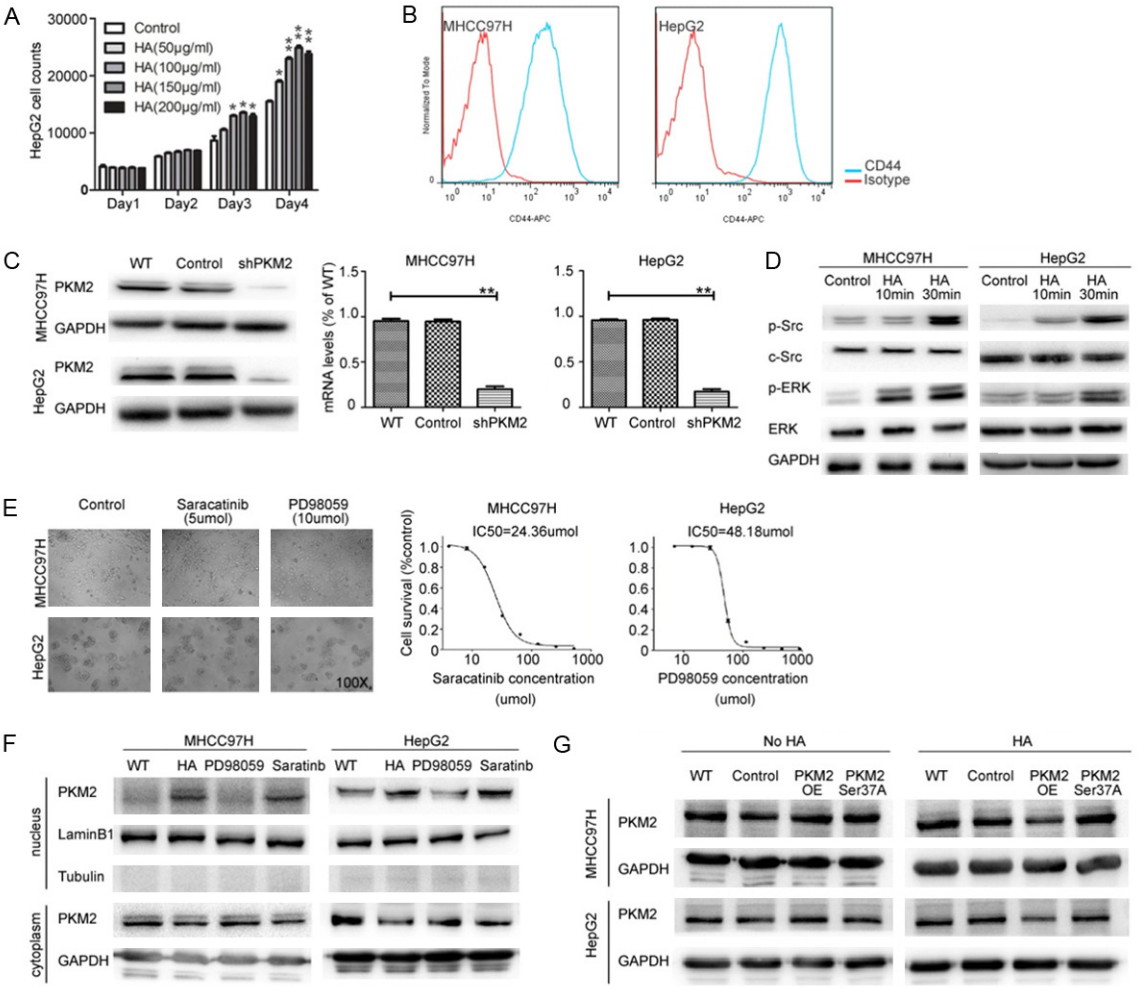

Supplementary Figure 1. A. Different concentrations of HA (50, 100, 150, 200 μg/ml) were included in the HepG2 cell proliferation assay. Compared with the control group, all four HA concentrations promoted the proliferation of HepG2 cells. B. A high percentage of cells were positive for CD44 in both MHCC97H and HepG2 cells. C. MHCC97H and HepG2 cells were transfected with lentivirus vectors, resulting in the stable knockdown of PKM2. D. c-Src and ERK were significantly activated at 30 minutes following HA stimulation. E. HCC cells were incubated for 6h with Saracatinib (4-500 μmol/ml) or PD98059 (7-900 μmol/ml) and the IC50 were calculated, which were much more than the kinase inhibiting concentration of those two drugs that had been reported previously. Treated HCC cells with Saracatinib or PD98059 at reported concentration did not affecting the morphology of the HCCs cells. F. Blocking ERK activation could inhibit HA-induced PKM2 nuclear translocation. G. Cytoplasm protein was extracted. PKM2 Ser37 mutation inhibited the nuclear translocation of PKM2.

Supplementary materials and methods

Lentivirus and tranfection

Four lentiviruses were established by Hanyin Biotechnology, Shanghai, China: an empty vector control (Control), stable knockdown of PKM2 (shPKM2), overexpressed PKM2 (PKM2 OE), and a PKM2 Ser37A mutation (PKM2 Ser37A). All transfections were performed according to the manufacturer's protocols.

Stable knockdown of PKM2 (shPKM2) was accomplished using shRNA (5'-CATCTACCACTTGCAATTA-3') targeting the PKM2 exon 10 or a non-target shRNA control in pHY-LV-KD5.1 vector. Lentivirus targeting PKM2 was constructed with the full-length PKM2 cDNA cloned in pHY-LV-KD5.1 vector to overexpress PKM2 (PKM2 OE) or with an empty vector control.

PKM2 mutation (Ser37A) plasmid (vector pHY-LV-OE1.6) was amplified by over-lapping PCR products using the following primers: F: 5'-CCGGAATTCGCCACCATGGACTACAAGGACGATGACGACAAGTCGAAGCCCCATAGTGAAG-3'; R: 5'-CGGGATCCTCACGGCACAGGAACAACAC-3'; Mutant F: TGCCGCCTGGACATTGATGCACCACCCATCACAGCCC-3'; Mutant R: 5'-GGGCTGTGATGGGTGGTGCATCAATGTCCAGGCGGCAGGGCTGTGATGGGTGGTG-3'. Primers F/mutant R and mutant F/R were used for the first round of PCR independently, then the primer F/R with first round products as template were used for a second round of PCR to generate a full length mutation sequence [1].

Preparation of whole cell lysates, subcellular fractionation and Western blot analysis

The protocol was described in detail in previously by our lab [2]. Briefly, the whole-cell lysates were extracted using RIPA lysis buffer (Beyotime, China) containing PMSF and phosphatase inhibitor (Roche) at 4°C; The cytosol and nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology). Protein samples were separated by 10% SDS-PAGE gel and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% bovine serum albumin (BSA, Beyotime, China) for 2 hours, washed, and then incubated with primary antibodies overnight at 4°C at concentrations, which the manufacturer's protocol recommended. After washing, the membrane was incubated with appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) and detected using enhanced chemiluminescence method (Pierce, IL). The amounts of proteins were quantified by densitometry with ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized to relative internal standards. All of the experiments were performed in triplicate.

Quantitative real-time PCR

Total RNA was extracted using Trizol reagent. cDNA was synthesized from 500 ng RNA with PrimeScript RT Master Mix (Takara, Japan). Quantitative real-time PCR was performed using 2 × SYBR Green PCR Master Mix under the following conditions. 95°C for 30 seconds, followed by 40 cycles at 95°C for 5 seconds, 64°C for 34 seconds, and a melt cure step using a Step One Plus Real-Time PCR System (Applied Biosystems). The target gene expression levels for each experiment were normalized to GAPDH. The relative gene expression levels were detected and calculated by the Ct comparative method.

The RT-PCR primers used were as following. PKM2, 5'-GCTGCCATCTACCACTTGC-3' (forward) and 5'-CCAGACTTGGTGAGGACGATT-3' (reverse); GAPDH, 5'-GCACCGTCAAGGCTGAGAAC-3' (forward) and 5'-TGGTGAAGAACGCCAGTGGA-3'.

Immunofluorescence

PKM2 expression in HCC cell lines was detected using an immunofluorescence assay. Cells were treated with 4% formaldehyde and then permeabilized with 0.5% Triton X-100. Non-specific block binding was carried out with 5% BSA before incubation with the primary antibody (Anti-PKM2, ab137791 at 1:500 dilutions, Abcam, UK) overnight at 4°C. Secondary antibody (Alexa Fluor 594, Invitrogen, CA) was incubated at 37°C for 30 minutes. Nuclei were stained with DAPI (Roche) at 37°C for 5 minutes. Mouse

IgG was used as a negative control. Fluorescence microscopy (Leica Microsystems Imaging Solutions, UK) was used to observe the results.

Cell proliferation and colony formation

Cell proliferation was done as described [2]. In brief, 97H cells and G2 cells (4000 cells/well) were seeded into a 96-well plate. At the indicated time points, 100 μl Cell Kit-8 (Dojindo) was added to the cells for 2 hours, and then the plate was read using an enzyme-linked immunosorbent assay plate reader at 450 nm.

Cells were plated in 6-well plates at a density of 1000 cells/well and incubated for 14 days. The cells were fixed with 2% formalin for 10 minutes and stained with 0.5% crystal violet for 5 minutes prior to be photographed. Cell colonies with a diameter larger than 50 μm were counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell migration and invasion

Cell migration was evaluated using the scratch wound assay. Cells were cultured for 2 days to form a tight cell monolayer, which was then wounded with a 200 μl plastic pipette tip. Cultured by serum-free culture medium for 48 hours, migration cells at the wound front were photographed. Cell migration was calculated as percentages of cell coverage to the initial cell-free zone using ImageJ software. The values are the means of three independent experiments.

Transwell Permeable Supports (Corning, Lowell, MA, USA) with an 8-μm polycarbonate filter membrane was used, with 150 μg Matrigel (BD Biosciences, San Diego, CA, USA) coated on the top surface of the filter. Cells (2×10^4 cells/well) with 150 μl serum-free medium were seeded into upper chamber of each well of a 24-well plate. And lower reservoir was filled medium containing 10% serum. After incubating 24 hours in normal conditions, the cells on the top surface were cleared physically, while the cells translocated the filter were stained and quantitated by visual inspection of the filter membranes.

Measurements of aerobic glycolysis and pyruvate kinase activity

Cells (2×10^5) were seeded in 24-well plates, and the medium was changed after 6 h with serum free DMEM. Then, cells were incubated with the serum free DMEM with or without IM7 (20 μg/ml) or IgG (20 μg/ml) at 4°C for 30 minutes. Next, the medium was changed to 1 ml normal-serum DMEM with or without HA (100 μg/ml, Ultra Low Mw, GLR003, R&D systems) for 12 h, and then the medium was collected for measurement of glucose and lactate concentrations. A lucose assay kit and Lactate assay kit (MAK013 and MAK064, Sigma-Aldrich, USA) were used to determine the levels of glucose and lactate respectively. Glucose consumption was the difference in glucose concentration when compared with DMEM. The activity of PK was measured with a Pyruvate kinase assay (MAK072, Sigma-Aldrich, USA) according to the manufacturer's instruction.

Evaluation of tumor growth in vivo

The animal experiments were carried out in accordance with the guideline of the Shanghai Medical Experimental Animal Care Commission and all animals received human care. Male BALB/c nude mice (5 weeks old, Shanghai Institute of Materia Medica, Chinese Academy of Science) were housed under specific pathogen-free conditions. Twenty-four mice were randomized into four groups and cells (5×10^6 per 200 μl Matrigel (1:8 diluted, 354263, BD Biosciences, CA) with 100 μg/ml HA (GLR003, R&D systems)) were subcutaneously injected. The tumor volumes were measured by vernier caliper every week, and the mice were euthanized after 4 weeks. The tumor volume was calculated by (large diameter) × (small diameter)^2/2.

Flow cytometry

Cells (2×10^5) were stained with directly labeled mouse monoclonal antibodies directed against CD44 (APC-conjugated) in 0.1 ml PBS containing 0.1% bovine serum albumin for 30 minutes at 4°C. Non-

specific IgG was used as a negative control. FACScaliber Flow cytometer (BD Biosciences) and FlowJo software (Tree Star Inc, Ashland, Ore) were used to analyze the stained cells.

Tissue microarray construction

Tissue microarrays (TMAs) were generated in our previous reports [3]. Briefly, representative areas of tumor and adjacent normal tissue were circled away from the necrotic, hemorrhagic and major fibrotic areas. Triplicates of 1-mm diameter cylinders were cored from the areas of interest in the donor blocks and inserted into a recipient paraffin block in a grid pattern using a tissue arrayer (Beecher Instruments, Silver Spring, MD, USA). Sections (4 μm) were then cut from each recipient paraffin block.

References

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