Supplementary Data

Supplementary materials and methods

FACS analysis

To confirm BiTE binding for, 2×10^6 cells were collected by centrifugation and incubated with 20μg/mL of BiTE in phosphate-buffered saline (PBS) containing 1% newborn calf serum for 45 min at 4°C. After being washed with cold PBS, cells were incubated for another 45 min at 4°C with mouse anti-His antibody (Kang-Chen Bio-tech, Shanghai, China). Cells were again washed with cold PBS before being incubated for an additional 45 min at 4 °C with a FITC-conjugated goat anti-mouse antibody (Kang-Chen Bio-tech, Shanghai, China) in the dark. For each sample, at least 10,000 cells were analyzed by FACS cytometry (Beckman Coulter Epics Altra, Miami, FL) and MultiCycle AV for Windows (version 5.0; Phoenix Flow Systems, San Diego, CA). FACS analysis was also performed using anti-EpCAM monoclonal antibodies 1H8 and 2F2 as reference.[38]

Lentivirus Production and Transduction of Target Cells

Encoding sequences for human Gal-1, EpCAM-myc tag, mouse EpCAM-myc-tag, and Human and mouse chimeric EpCAM-myc tag were amplified by PCR and then inserted into a pWPT vector. To produce virus particles, 20 μg of pWPT-EpCAM was transfected with 10 μg of packaging plasmid pAX2 and 6 μg of the vesicular stomatitis virus (VSV-G) envelope plasmid pMD2G (generous gifts from Dr T. Didier) into 293T cells using a calcium phosphate transfection system. Huh-7 or CHO-K1 cells were infected with recombinant lentiviral particles to produce polyclonal cells

with stable expression of Gal-1 or EpCAM and confirmed by western blot.

The human Gal-1 shRNA sequences were inserted into a pshRNA-copGFP vector. To produce virus particles, 1 μg of pshRNA-copGFP was transfected with 1.25 μg of pREV, 1.25 μg of PVSV-G and 2.5 μg of pGag-pol into 293T cells using a Lipofectamine™ 2000 transfection system. SMMC-7721 cells were transduced with recombinant lentiviral particles to produce polyclonal cells with stable expression of Gal-1 shRNA or negative control (NC) shRNA and the expression of the target gene was confirmed by western blot.

Supplementary table

Supplementary table 1. Gal-1 shRNA sequences

Gal-1 shRNA sequences	Target sequences
CCTGAATCTCAAACCTGGACTTCCTGTCA GATCCAGGTTTGAGATTCAGGTTTTT	CCTGAATCTCAAACCTGGA
CACCATCGTGTGCAACAGCCTTCCTGTCA GAGCTGTTGCACACGATGGTGTTTTT	CACCATCGTGTGCAACAGC
CCTGTGCCTGCACTTCAACCTTCCTGTCA GAGTTGAAGTGCAGGCACAGGTTTTT	CCTGTGCCTGCACTTCAAC

Supplementary table 2. Real-time PCR and RT-PCR primer sequences

primers	sequence
CD133 forward primer	TGGATGCAGAACTTGACAACGT
CD133 reverse primer	ATACCTGCTACGACAGTCGTGGT
β-actin forward primer	CATCCTCACCCTGAAGTACCC
β-actin reverse primer	AGCCTGGATAGCAACGTACATG
PD-L1 forward primer	TTGGGAAATGGAGGATAAGA
PD-L1 forward primer	GGATGTGCCAGAGGTAGTTCT
c-FLIP forward primer	ACAGAGTGAGGCGATTTGAC
c-FLIP forward primer	GAACAGACTGCTTGTACTTCT
PI-9 forward primer	TCTGCCCTGGCCATGGTTCTCCTA
PI-9 forward primer	CTGGCCTTTGCTCCTCCTGGTTTA

Supplementary table 3. IC_{50} concentration of DOX or 5-FU on $CD133^{+}EpCAM^{+}$ and $CD133^{-}EpCAM^{-}$ Huh-7 cells

	$CD133^{+}EpCAM^{+}$	$CD133^{-}EpCAM^{-}$	p
Doxorubicin	0.70 ± 0.15 μg/mL	0.33 ± 0.05 μg/mL	$p<0.05$
5-FU	14.38±7.42 μg/mL	5.17±0.91 μg/mL	$p<0.05$

Supplementary table 4. IC_{50} concentration of DOX or 5-FU on $CD133^{+}EpCAM^{+}$ and $CD133^{-}EpCAM^{-}$ Hep3B cells

	$CD133^{+}EpCAM^{+}$	$CD133^{-}EpCAM^{-}$	p
Doxorubicin	80.77 ± 15.23 ng/mL	18.97 ± 3.26 ng/mL	$p<0.05$
5-FU	4.31±0.58 μg/mL	2.27±0.82 μg/mL	$p<0.01$

Supplementary figure legends

Supplementary Figure S1. FACS and ELISA analysis of the binding of 1H8 and 2F2 to EpCAM. (A) FACS analysis of the binding of 1H8 and 2F2 to hepatocellular carcinoma cell lines. (B) ELISA analysis of the binding of 1H8 and 2F2 to recombinant EpCAM. 1H8 and 2F2 are effectively bound to recombinant EpCAM protein (Sino Biological Inc.) as C-10 (C-10 is a mouse IgG_1 monoclonal antibody raised against amino acids 24-93 of EpCAM of human origin, SANTA CRUZ BIOTECHNOLOGY, INC. SC-25308). IgG1 is isotype control. mean±SD, n=3. (**p<0.01; ***p<0.001).

Supplementary Figure S2. Mapping of the binding domains of mAbs 1H8 and 2F2 on EpCAM. (A) Structure and exon boundaries of EpCAM. (B) The binding domain of mAbs 1H8 and 2F2 were determined using CHO-K1 cells expressing chimeric EpCAM by FACS assay.

Supplementary Figure S3. The binding capacity of 1H8/CD3 or CD3scFv to PBMCs and hepatocellular carcinoma cell lines was determined by FACS assay. (A) The binding capacity of 1H8/CD3 to PBMCs and hepatocellular carcinoma cell lines was determined by FACS assay. (B) The binding capacity of CD3scFv to PBMCs was determined by FACS assay.

Supplementary Figure S4. Mapping of the binding domains of 1H8/CD3 using CHO-K1 cells expressing human, mouse and mouse/human chimeras of EpCAM by

FACS assay.

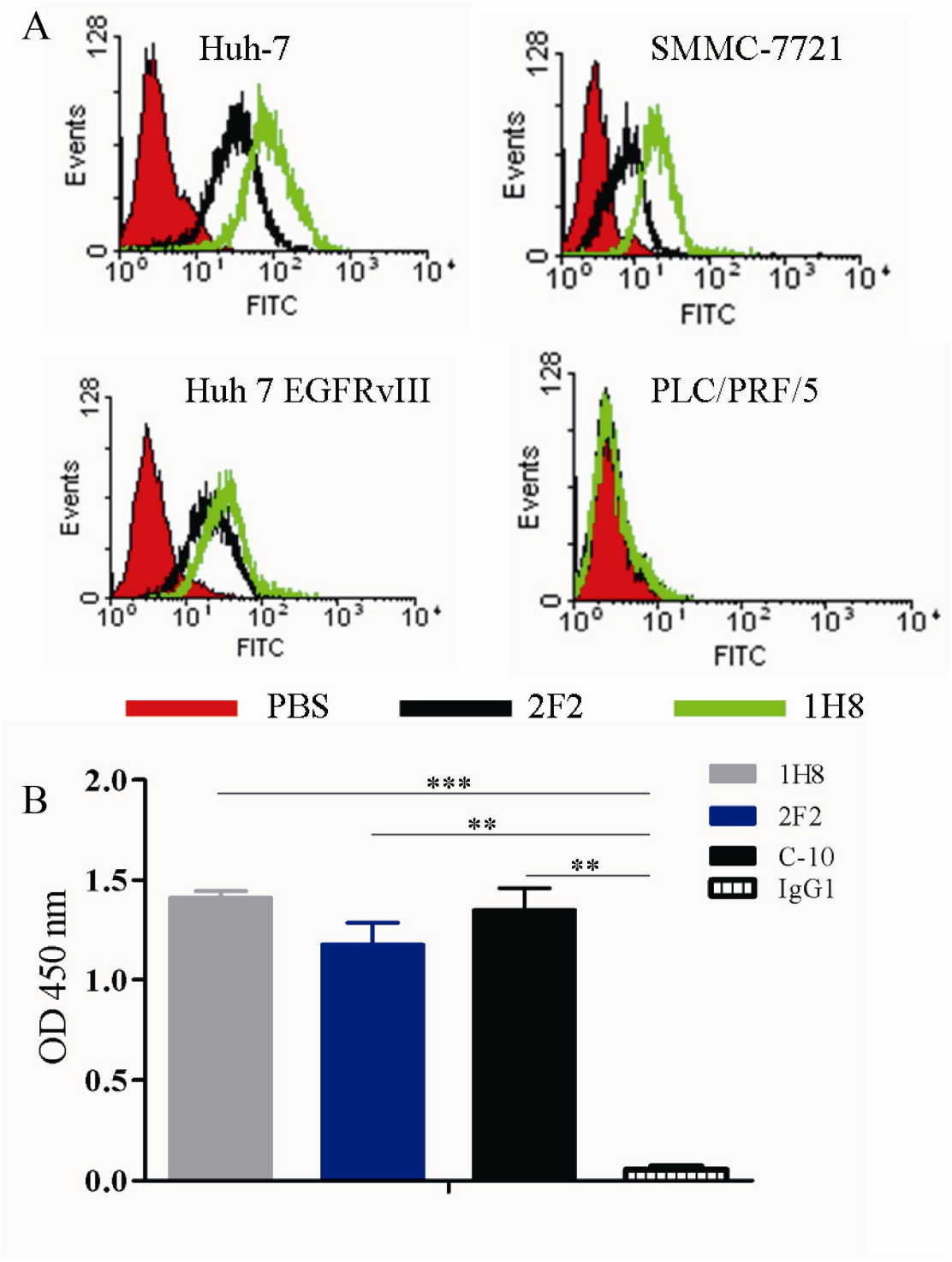
Supplementary Figure S5. $CD133^{+}EpCAM^{+}$ Huh-7 and Hep3B cells possess characteristics of CSC. (A) Colony formation ability was increased in $CD133^{+}EpCAM^{+}$ Hep3B cells compared with $CD133^{-}EpCAM^{-}$ Hep3B cells (**p<0.01). (B) $CD133^{+}EpCAM^{+}$ or $CD133^{-}EpCAM^{-}$ Huh-7 and Hep3B cells were treated with doxorubicin or 5-FU for 72 h, and then cell proliferation was detected by CCK-8 assay. Cell inhibition assay showed that IC_{50} concentrations of Doxorubicin and 5-FU were higher in cells coexpressing CD133 and EpCAM (mean±SD, n=3).

Supplementary Figure S6. PD-L1, PI-9 c-FLIP and Gal-1 expression in HCC cells. (A) PD-L1, PI-9 and cFLIP mRNA expression in HCC cells was detected by RT-PCR. HCC cells were incubated with IFN-γ (10 ng/mL) for 48 h. (B) c-FLIP mRNA expression in HCC cells was detected by Real-time PCR. (C) Cell extracts from Huh-7, mock or Gal-1-transfected Huh-7 cells were subjected to western blot analysis. (D) Cell extracts from SMMC-7721, NC shRNA- or Gal-1 shRNA-transfected SMMC-7721 cells were subjected to western blot analysis. mean±SD, n=3. (*p<0.05; **p<0.01; p<0.001).

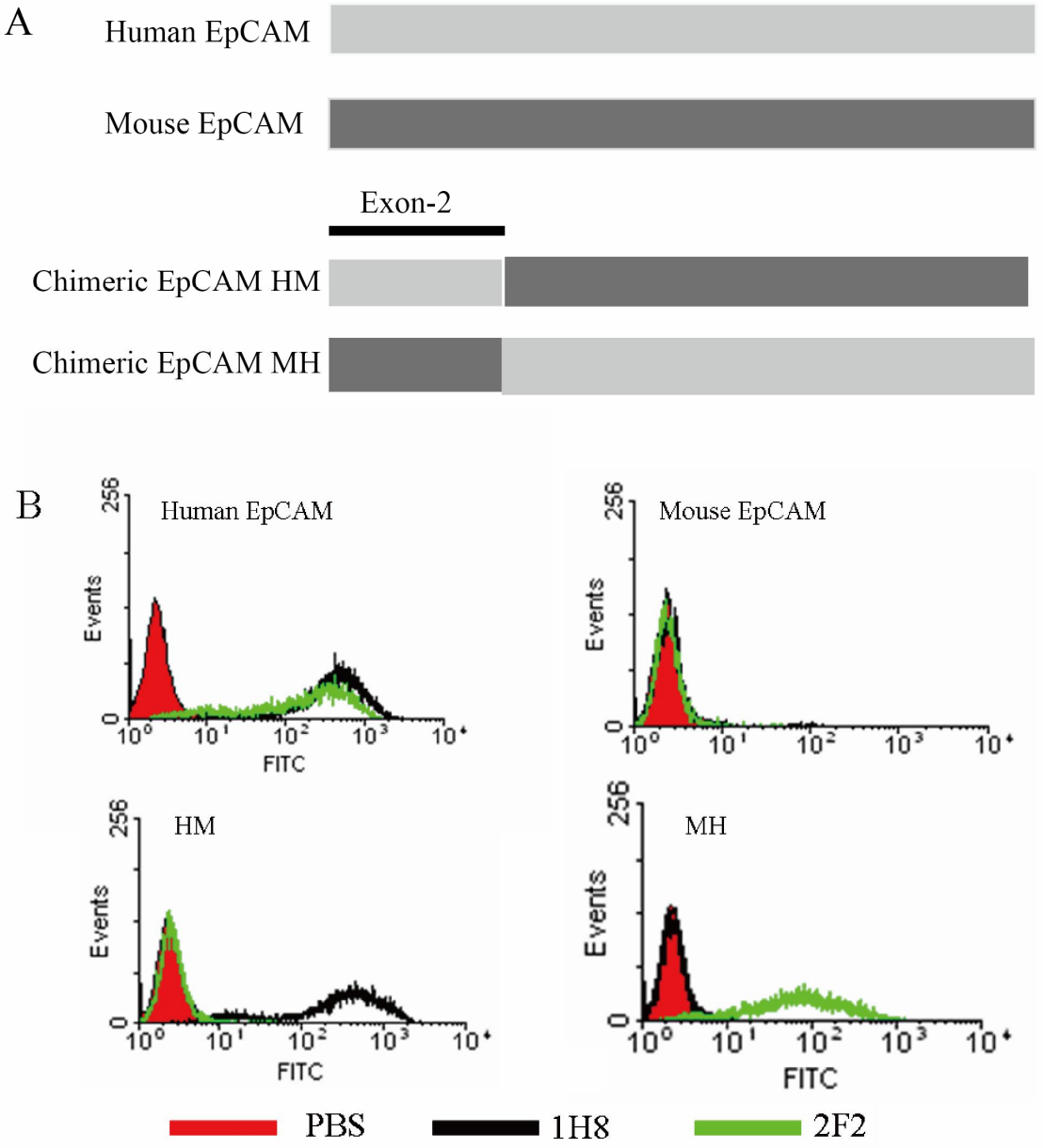
Supplementary Figure S7. CSC content after in vivo treatment. Real-time PCR analysis for EpCAM, CD133, Nanog, Sox-2, Oct-3/4, Notch and Klf-4 as CSC markers in tumor tissues after 30 days of in vivo treatment with 1H8/CD3, 1H8 or

PBS. mean±SD, n=3. (*p<0.05; **p<0.01; ***p<0.001).

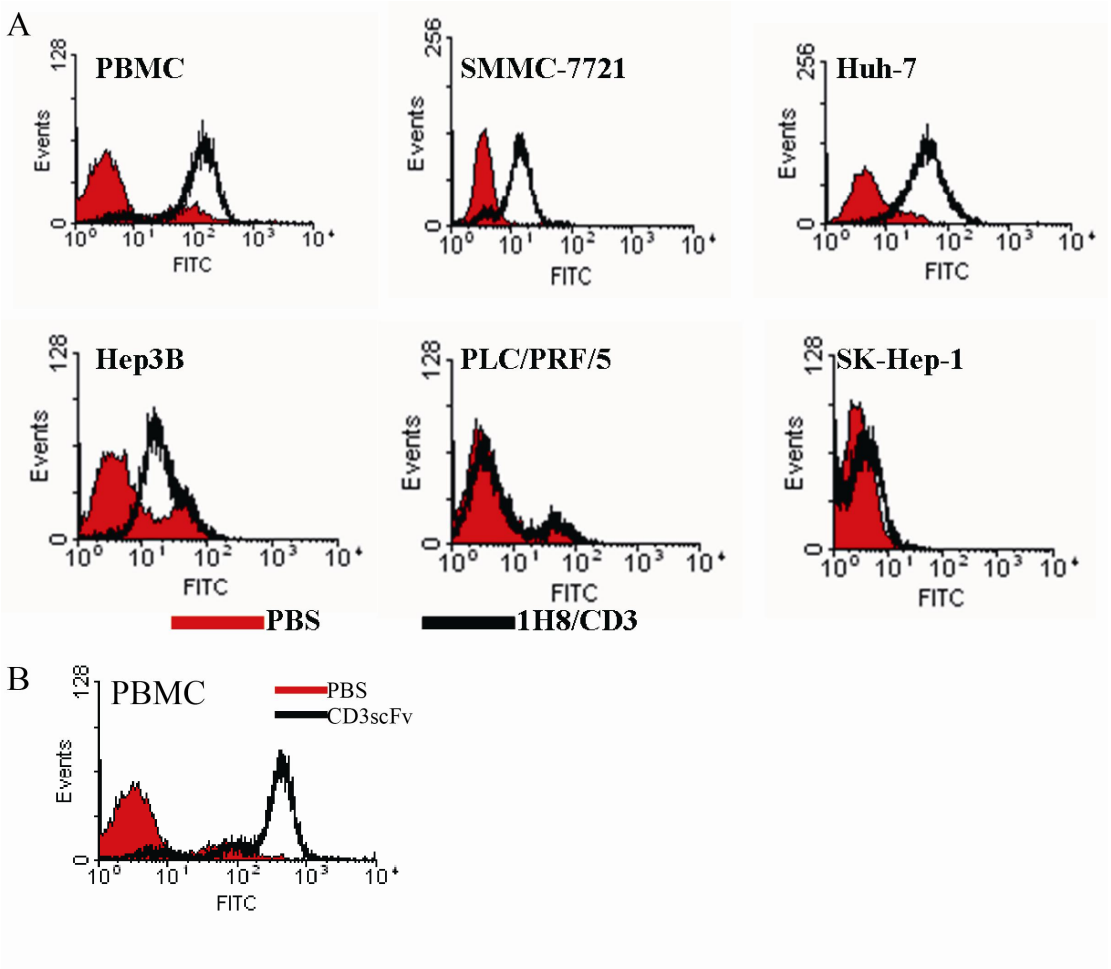
Supplementary Figure S1



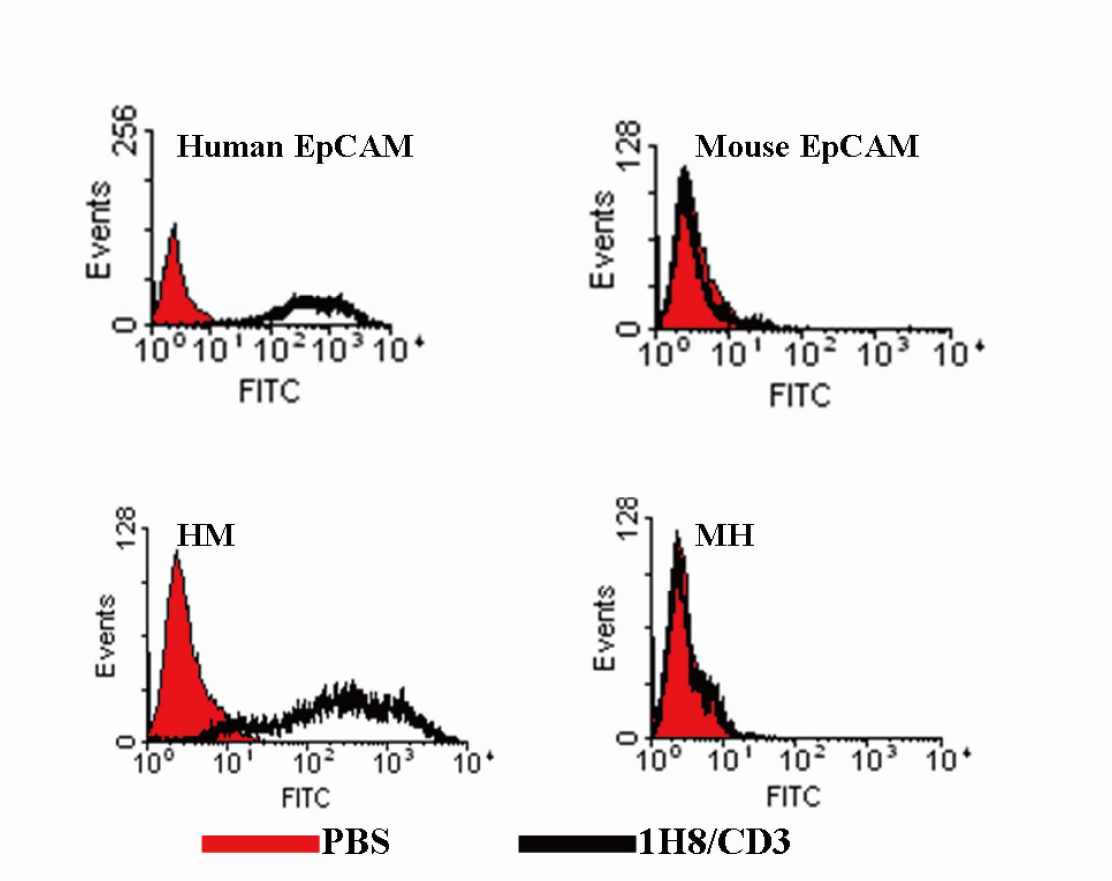
Supplementary Figure S2



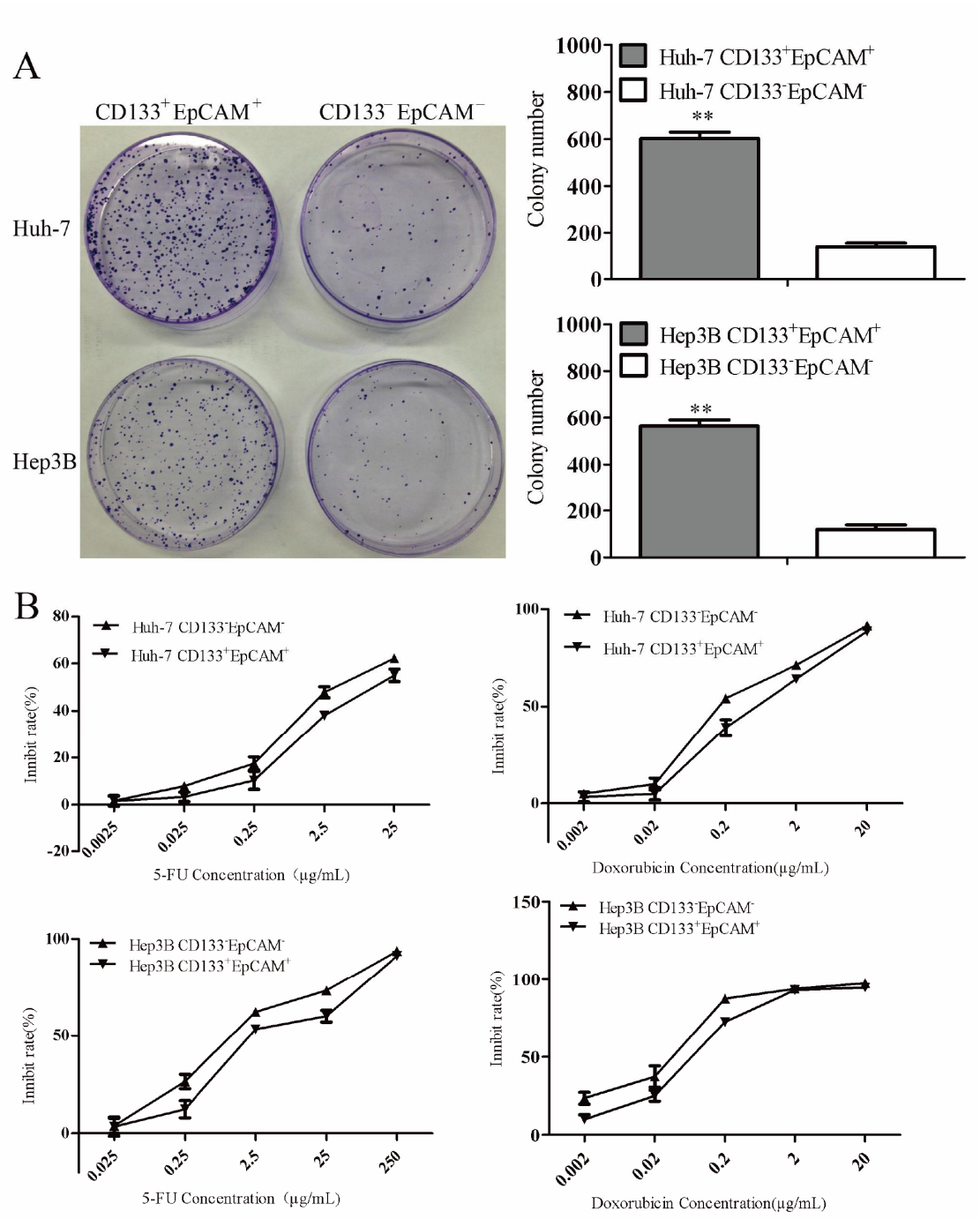
Supplementary Figure S3



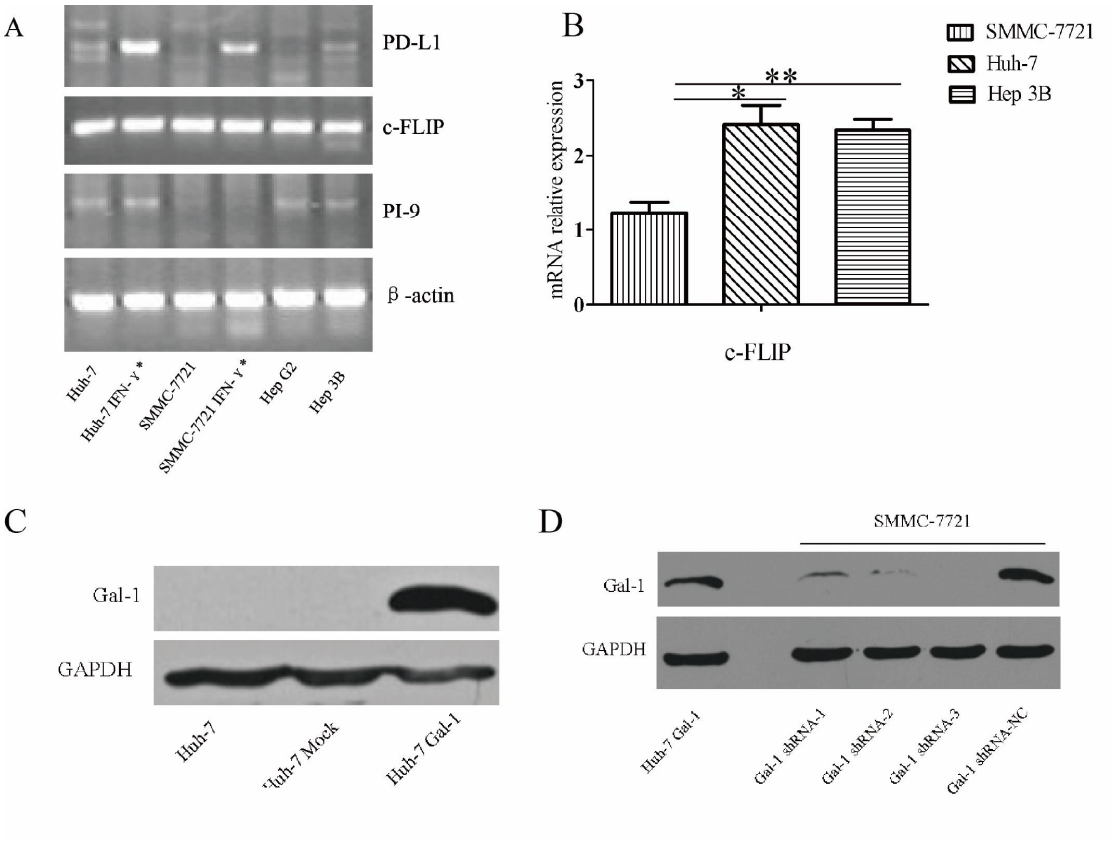
Supplementary Figure S4



Supplementary Figure S5



Supplementary Figure S6



Supplementary Figure S7