

Supplementary information for

**"Nanomedicines reveal how PBOV1 promotes
hepatocellular carcinoma for effective gene
therapy"**

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Supplemental Tables

Supplemental Table 1. Comparison of Clinicopathological Features of Patients with Different PBOV1 Expression Level

Category	Subcategory	Cases	PBOV1		<i>P</i> value
			lower expression	higher expression	
Gender	male	250	79	171	0.256
	female	36	15	21	
Age	<60 years	224	69	155	0.171
	≥60 years	62	25	37	
HBsAg	negative	45	13	32	0.606
	positive	241	81	160	
AFP(ng/ml)	<200	131	43	88	1
	≥200	155	51	104	
Tumor Size	<5cm	72	36	36	0.000(<0.001)
	≥5cm	214	58	156	
Tumor Number	single	220	80	140	0.025
	multiple	66	14	52	
Surgical Margin	<1cm	82	34	48	0.053
	≥1cm	204	60	144	
Liver Cirrhosis	absent	67	19	48	0.458
	present	219	75	144	
Capsulation	capsulated	192	65	127	0.688
	non-capsulated	94	29	65	
Vascular Invasion	absent	211	78	133	0.015
	present	75	16	59	
Edmonson Grading	I-II	223	79	144	0.095
	III-IV	63	15	48	

Supplemental Table 2. Univariate and Multivariate Analyses of Various Prognostic Parameters in Patients with HCC Cox-regression Analysis

1,3,5 year overall survival rate

1,3,5 year disease free survival rate

lower expression 81.9%、56.9%、49.3%

lower expression 58.4%、48.3%、38.2%

higher expression 66.0%、37.7%、29.8%

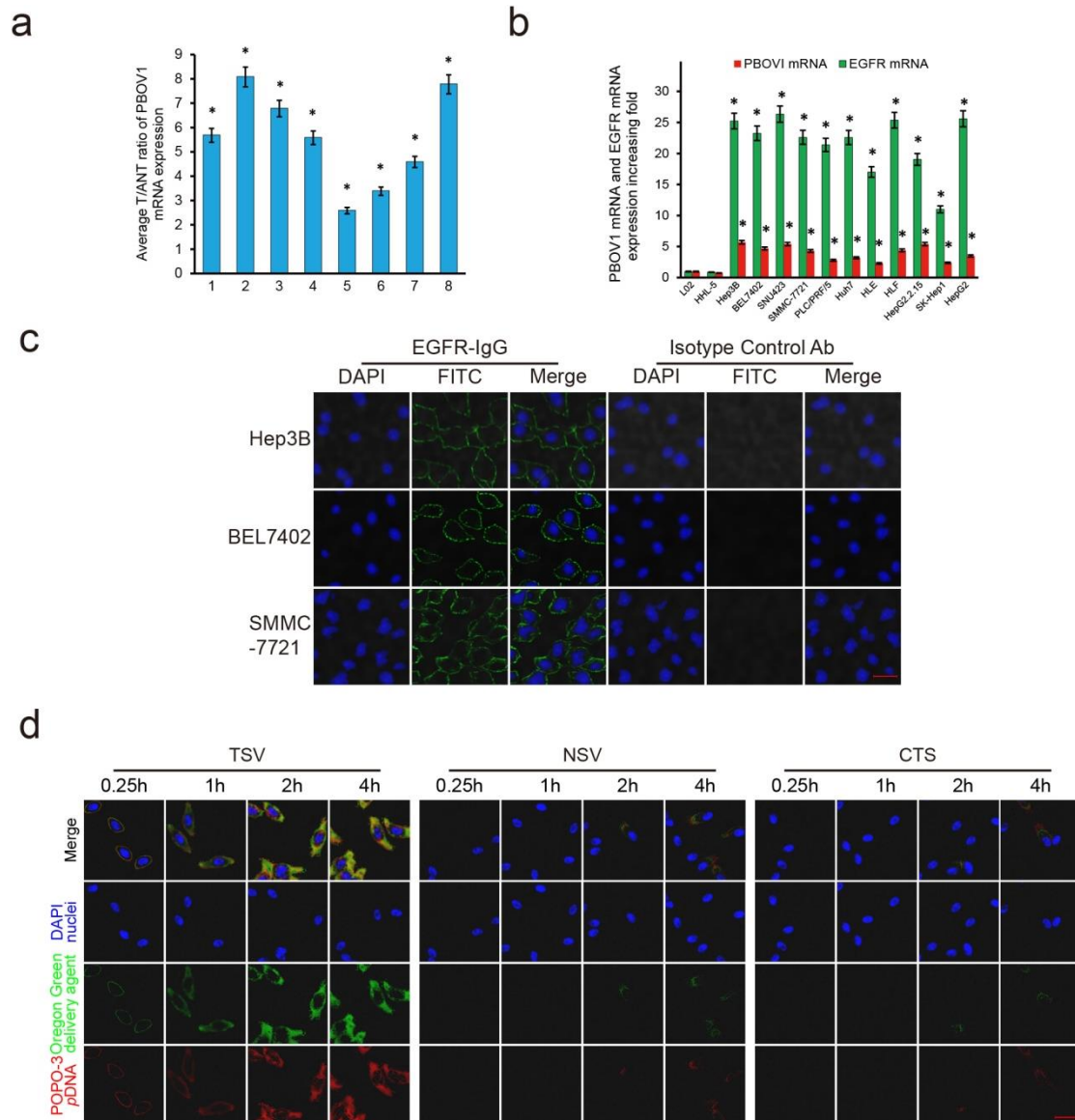
higher expression 39.3%、25.5%、19.2%

Category	Subcategory	Disease-free Survival			Overall Survival		
		Univariate Analysis	Multivariate Analysis	HR (95% CI)	Univariate Analysis	Multivariate Analysis	HR (95% CI)
Gender	male	0.945			0.269		
	female						
Age	<60 years	0.538			0.348		
	≥60 years						
HBs-Ag	negative	0.793			0.361		
	positive						
AFP(ng/ml)	<200	0.066			0.247		
	≥200						
Tumor Size	<5cm	0	0 (<0.001)	2.270	0.000	0 (<0.001)	2.590
	≥5cm			(1.569-3.285)			(1.690-3.969)
Tumor Number	single	0.005	0.005	1.565	0.002	0.002	1.673
	multiple			(1.147-2.135)			(1.208-2.317)
Surgical Margin	<1cm	0.325			0.305		
	≥1cm						
Liver Cirrhosis	absent	0.786			0.852		
	present						
Capsulation	capsulated	0.041	0.035	1.355	0.084		
	non-capsulated			(1.022-1.796)			
Vascular Invasion	absent	0.659			0.569		
	present						
Edmonson Grading	I-II	0.6			0.590		
	III-IV						
PBOV1	Lower Expression	0.003	0.004	1.583	0.020	0.033	1.448
	Higher Expression			(1.155-2.168)			(1.030-2.035)

Supplemental Table 3. Information of Antibodies Used in the Study.

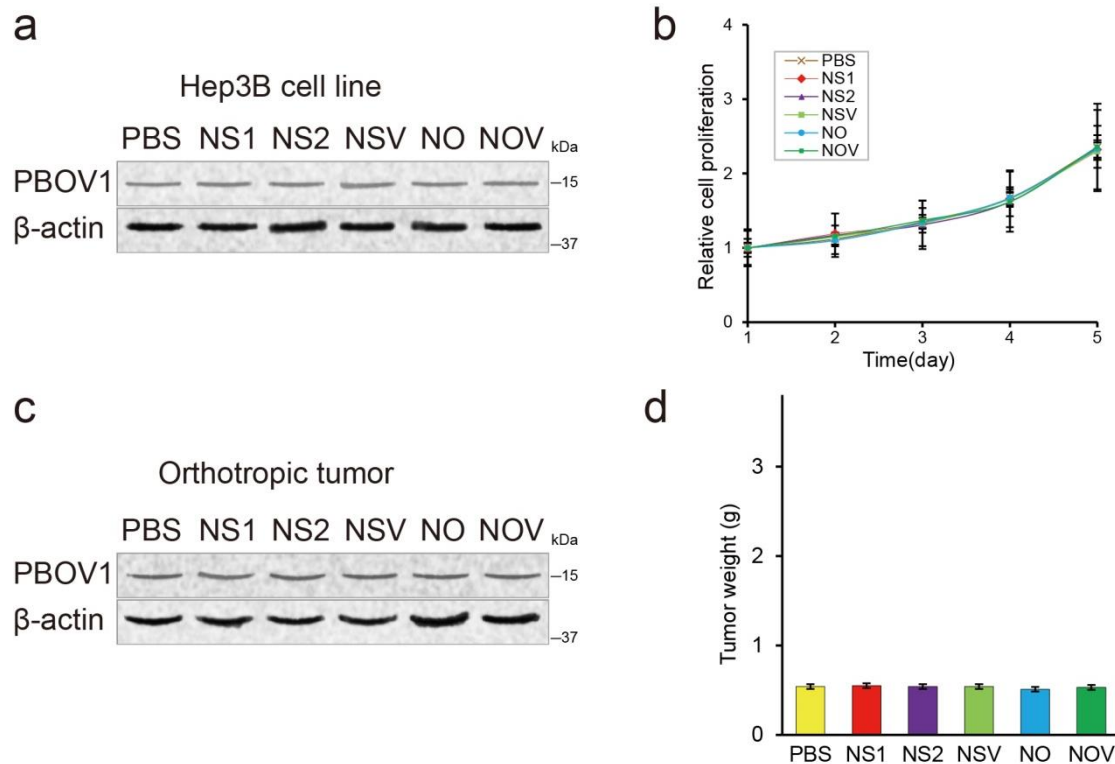
Antibodies	Company	Catalog No.	Application	Dilution fold
Anti-PBOV1	Abcam	ab216045	WB,IHC,IF	200,50,200
Anti-beta Actin	Abcam	ab8226	WB	1000
Anti-Sodium Potassium ATPase	Abcam	ab76020	WB	20000
Anti-EGFR	Abcam	ab52894	WB,IF	1000,250
Anti-Survivin	Abcam	ab76424	WB	5000
Anti-Cyclin D1	Abcam	ab134175	WB	10000
Anti-active Caspase-3	Abcam	ab32042	WB	500
Anti-HIF-1 alpha	Abcam	ab51608	WB	200
Anti-alpha Catenin	Abcam	ab51032	WB,IF	50000,200
Anti-E Cadherin	Abcam	ab15148	WB,IF	500,200
Anti-N Cadherin	Abcam	ab76057	WB,IF	1000,200
Anti-Vimentin	Abcam	ab92547	WB,IF	1000,250
Anti-Ki67	Abcam	ab15580	IF	500
Anti-CD31	Abcam	ab28364	IF	20
Anti-Oct4	Abcam	ab181557	WB	1000
Anti-Nanog	Abcam	ab21624	WB	2000
Anti-c-Myc	Abcam	ab32072	WB	1000
Anti-CD133	Abcam	ab19898	WB,IF	1000,1000
Anti-EpCAM	Abcam	ab32392	WB	2000
Anti-Phospho β -Catenin (Ser33/37/Thr41)	Cell Signaling	#9561	WB	1000
Anti-beta Catenin	Abcam	ab32572	WB,IF	5000,250
Anti-EpCAM PerCP/Cy5.5®	Abcam	ab157319	Flow	200
Anti-CD133 PE	eBioscience	12-1339-41	Flow	200
Anti-BrdU	Abcam	ab15580	IF	1000
Anti-P84	Abcam	ab487	WB	500

Supplemental Figures

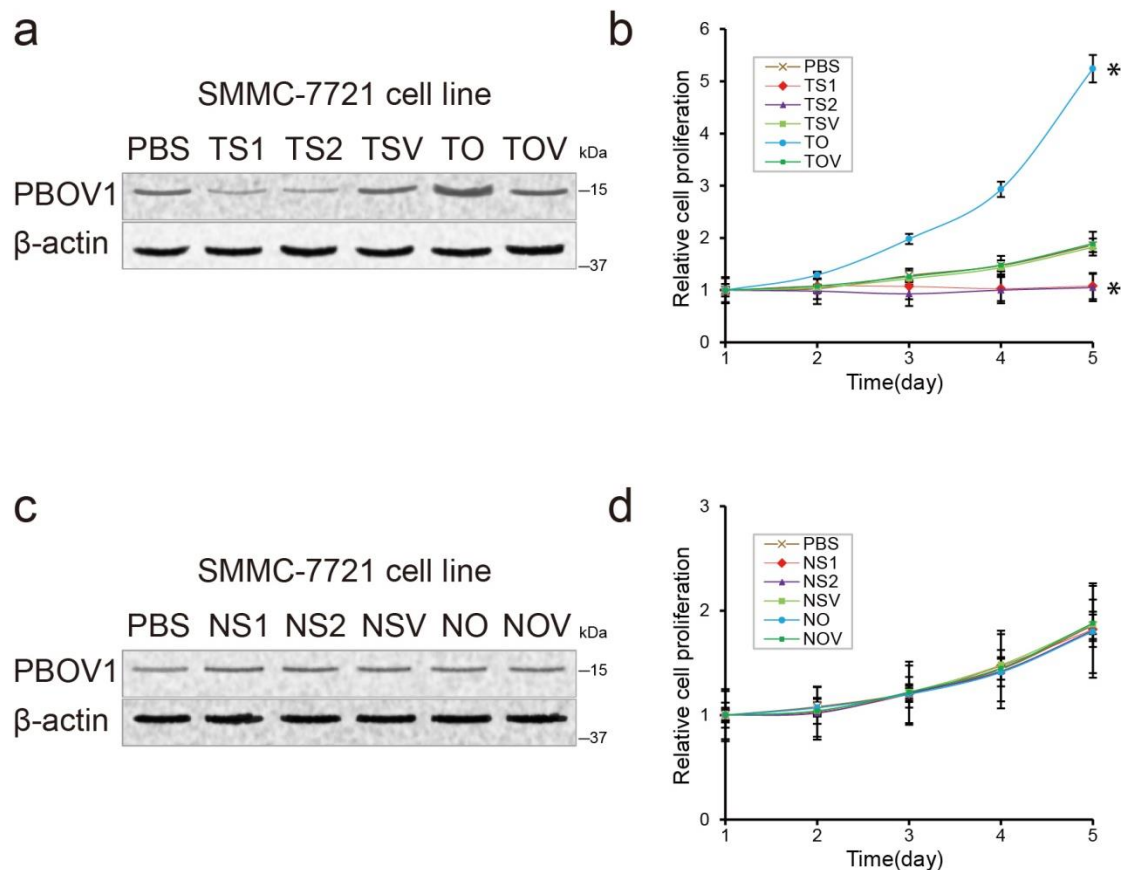


Supplementary Figure 1 Clues of expressions of PBOV1 and EGFR in HCC and cell intracellular uptake of nanomedicines. **a** RT-PCR analysis of PBOV1 mRNA expressions in HCC tissues and paired ANT liver tissues from the same patient. **b** RT-PCR analysis of PBOV1 mRNA expression levels in the human normal hepatocyte cell lines (L02, HHL-5) and multiple HCC cell lines (Hep3B, BEL7402, SNU423, SMMC-7721, PLC/PRF/5, Huh7, HLE, HLF, HepG2.2.1, SK-Hep1 and HepG2). **c** CLSM imaging of Hep3B, BEL7402 and SMMC-7721 cells by EGFR immunofluorescence staining with EGFR-IgG antibody and Isotype control antibody (Blue, DAPI indicating nuclei; Green, FITC indicating EGFR primary antibody aggregation on cell membrane). **d** CLSM imaging of different groups of Hep3B cells incubated for 0.25, 1, 2, 4 h, respectively at Fe concentration of 20 $\mu\text{g/mL}$ (Blue, DAPI indicating nuclei; red, POPO-3

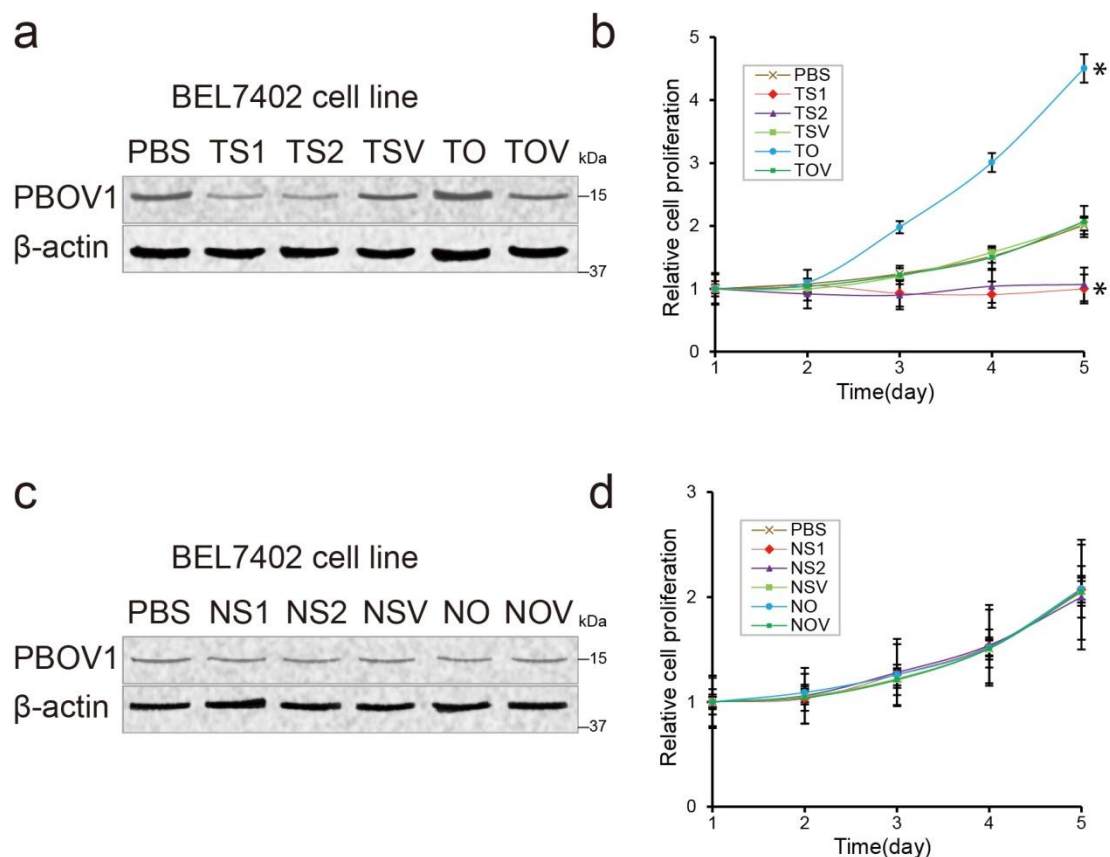
indicating *p*DNA; green, Oregon Green 488 indicating delivery agent; Overlapping of red and green fluorescence to generate a yellow one). Scale bar, 10 μ m. Data were represented as means $\pm$ S.D. Error bars represent the S.D. values (n=3; **P*<0.05. Significant differences were analyzed by Student's *t*-test, compared with PBS control group).



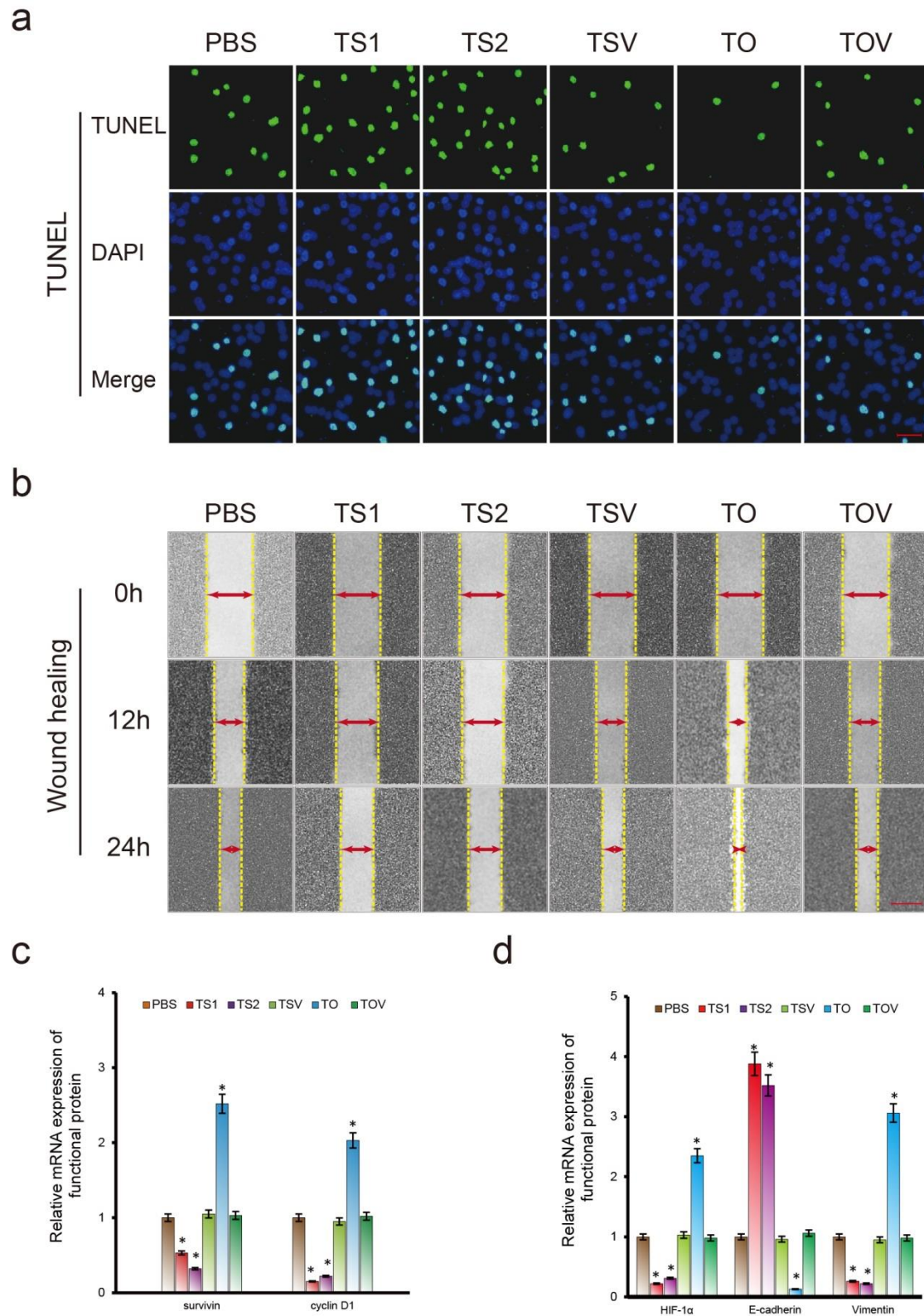
Supplementary Figure 2 PBOV1 expression and cell proliferation were not changed upon treatment of non-targeted nanomedicines. **a** PBOV1 protein expression levels in Hep3B cells determined by western blotting assay. **b** Cell growth viability determined by MTT assay (n=3). **c** Protein expression levels of PBOV1 determined by western blotting assay in tumor tissues at day 20 after treatment began in liver orthotopic model. **d** Tumor weights measured at day 20 after treatment began in liver orthotopic model (n=12). Data were represented as means $\pm$ S.D. Error bars represent the S.D. values (* $P < 0.05$). Significant differences were analyzed by Two-way ANOVA with multiple comparisons for **b**; Student's *t*-test for **d**, compared with PBS control group).



Supplementary Figure 3 Antitumor activities of scAb-EGFR-Targeted and non-targeted nanomedicines in HCC cell line SMMC-7721. **a** PBOV1 protein expression levels in SMMC-7721 cells treated with scAb-EGFR-targeted nanomedicines determined by western blotting assay. **b** Cell growth rates of SMMC-7721 cells treated with scAb-EGFR-targeted nanomedicines determined by MTT assay. **c** PBOV1 protein expression levels in SMMC-7721 cells treated with non-targeted nanomedicines determined by western blotting assay. **d** Cell growth rates of SMMC-7721 cells treated with non-targeted nanomedicines determined by MTT assay. Data were represented as means $\pm$ S.D. Error bars represent the S.D. values ($n=3$, $*P < 0.05$. Significant differences were analyzed by Two-way ANOVA with multiple comparisons for **b** and **d**).

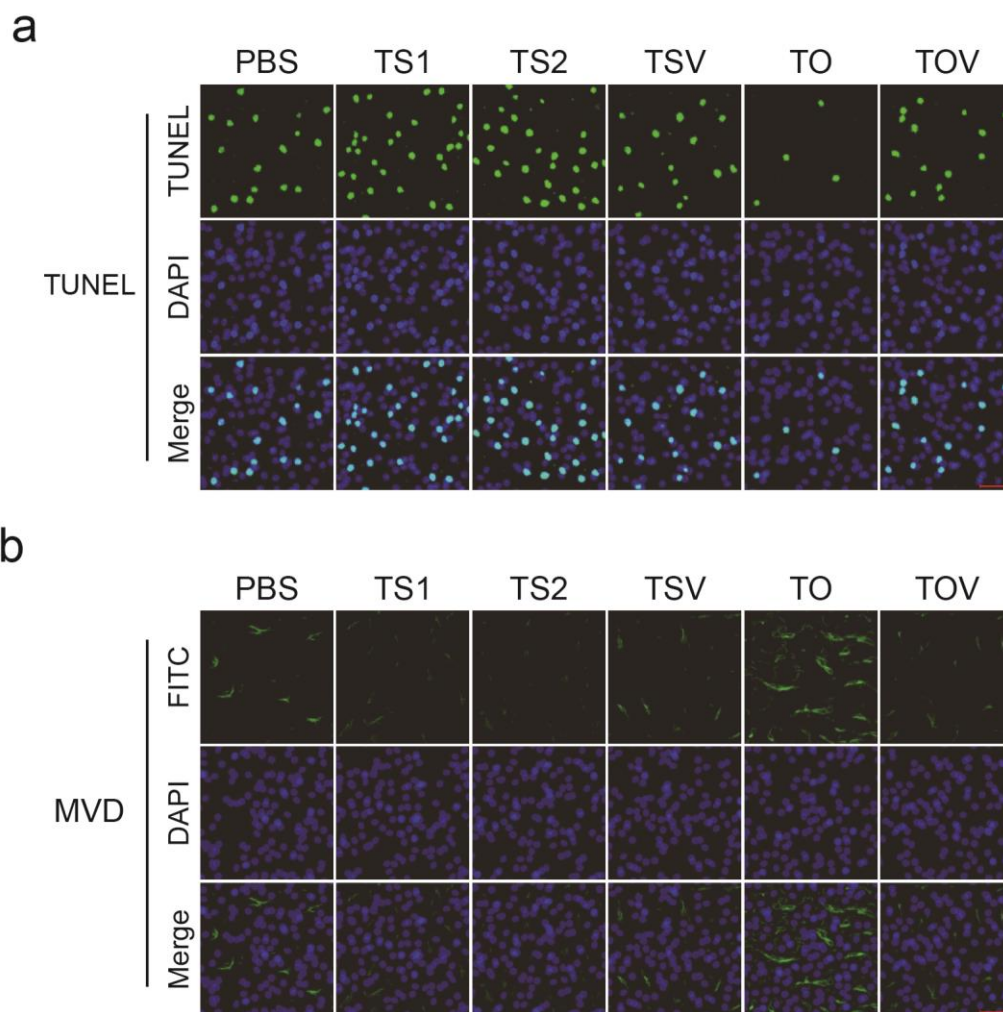


Supplementary Figure 4 Antitumor activities of scAb-EGFR-targeted and non-targeted nanomedicines in HCC cell line BEL7402. **a** PBOV1 protein expression levels determined by western blotting assay in BEL7402 cells treated with scAb-EGFR-targeted nanomedicines. **b** Cell growth rates determined by MTT assay in BEL7402 cells treated with scAb-EGFR-targeted nanomedicines. **c** PBOV1 protein expression levels determined by western blotting assay in BEL7402 cells treated with non-targeted nanomedicines. **d** Cell growth rates determined by MTT assay in BEL7402 cells treated with non-targeted nanomedicines. Data were represented as means $\pm$ S.D. Error bars represent the S.D. values ($n=3$, $*P<0.05$. Significant differences were analyzed by Two-way ANOVA with multiple comparisons for **b** and **d**).



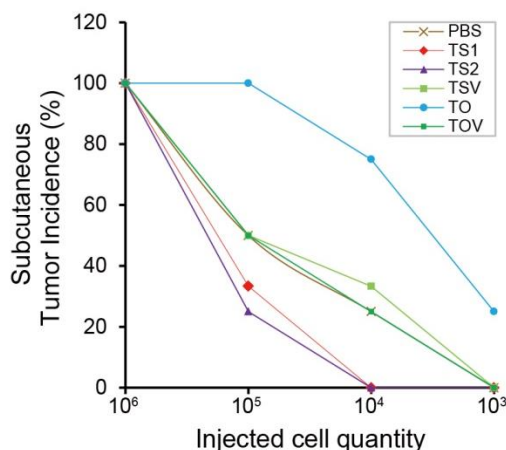
Supplementary Figure 5 The relationship between PBOV1 expression level and Hep3B cell apoptosis / mobility in vitro. **a** Hep3B cell apoptosis examined by TUNEL assay (Scale bar, 20 μ m; Blue, DAPI indicating nuclei; green, FITC indicating apoptotic nuclei). **b** Hep3B cell mobility measured by testing the rate of wound healing at 0, 12 and 24 h after serum-starvation and wound creation (Scale bar, 500 μ m). **c** RT-PCR analysis on mRNA expression levels of

proliferation marker survivin and cell cycle marker cyclin D1. **d** RT-PCR analysis on mRNA expression levels of angiogenesis marker HIF-1 α , epithelial marker E-cadherin and mesenchymal marker Vimentin mRNA expression. Data were represented as means $\pm$ S.D. Error bars represent the S.D. values (n=3; * P <0.05. Significant differences were analyzed by Student's *t*-test, compared with PBS control group).

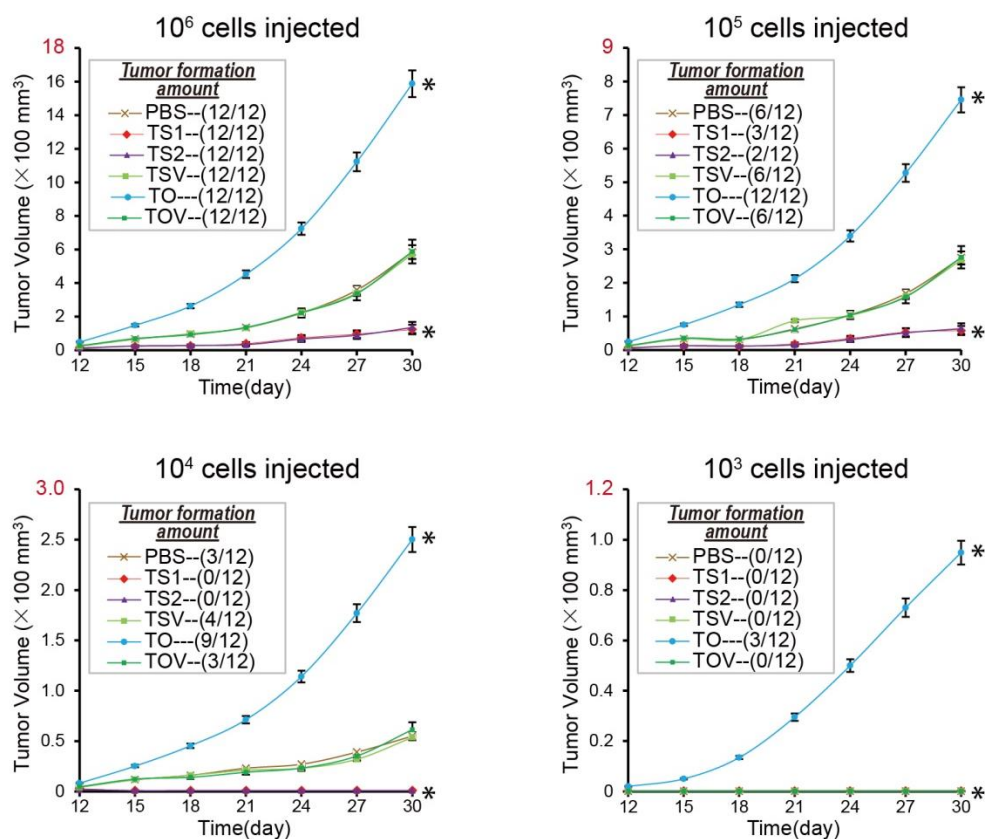


Supplementary Figure 6 In vivo antitumor activities of nanomedicines in liver orthotropic model. **a** Tumor apoptosis detected by TUNEL assay (Blue, DAPI indicating nuclei; green, FITC indicating apoptotic nuclei). **b** Tumor angiogenesis determined by MVD assay (Blue, DAPI indicating nuclei; green, FITC indicating CD31 protein). Scale bar, 10 μ m.

a

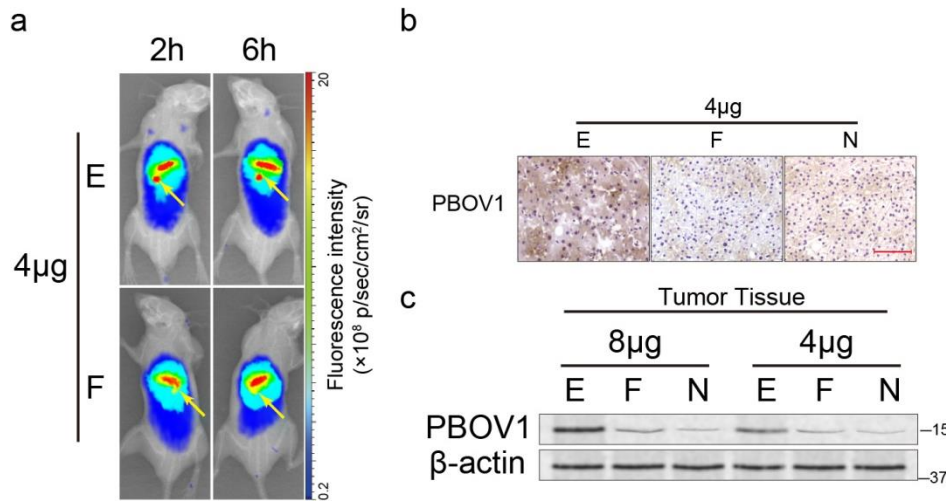


b

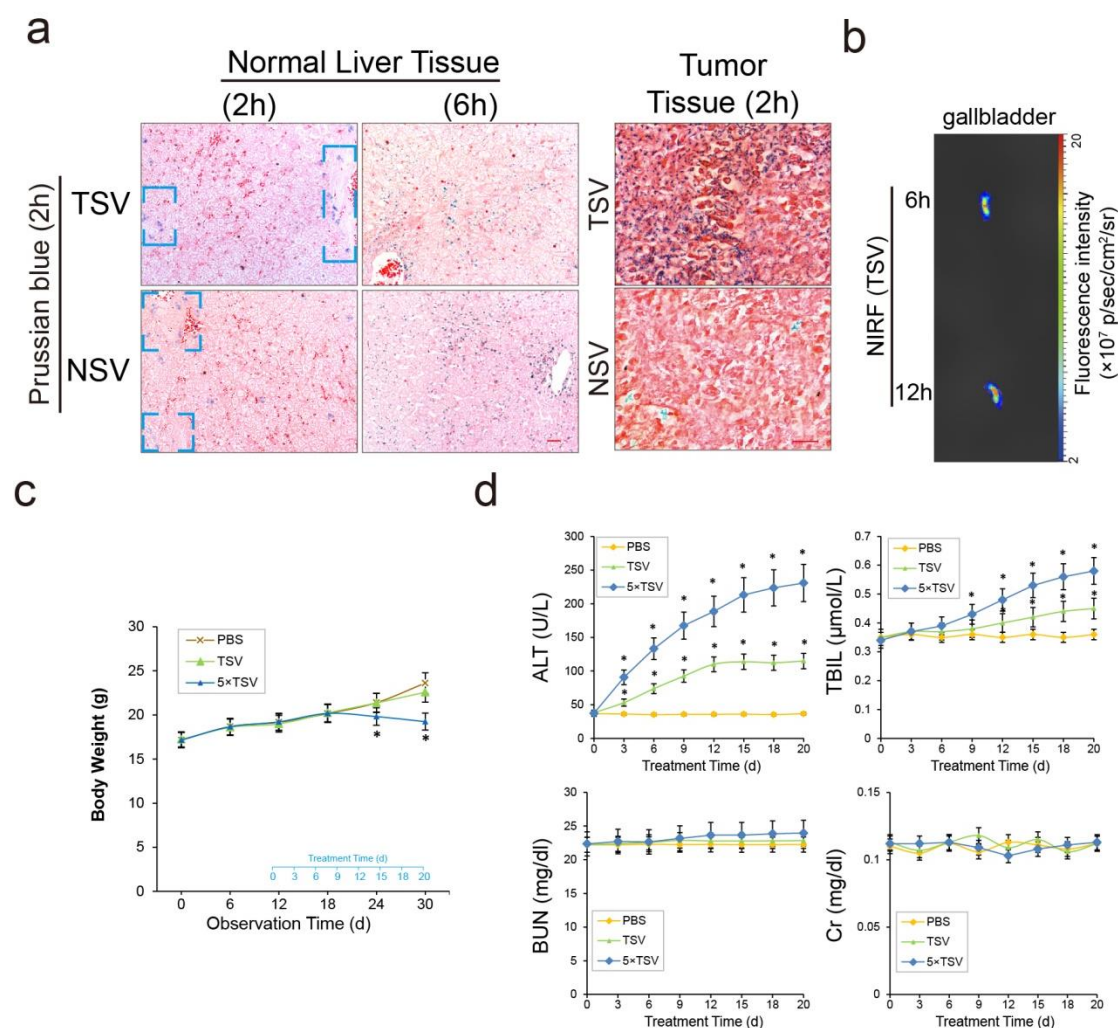


Supplementary Figure 7 Nanomedicines regulated in vivo HCC tumorigenesis activities in subcutaneous tumor model. **a** Oncogenic potential determined by limiting dilution assay to measure tumor incidence; various amounts of Hep3B cells (1×10^3 , 1×10^4 , 1×10^5 or 1×10^6) were injected subcutaneously before nanomedicine treatment. **b** Effect of nanomedicine treatments on tumor incidence rates and growth speeds in subcutaneous tumor model established via subcutaneously injecting Hep3B cells of various numbers (1×10^3 , 1×10^4 , 1×10^5 , 1×10^6). Data were represented as means $\pm$ S.D. Error bars represent the S.D. values (n=12; * $P < 0.05$. Significant

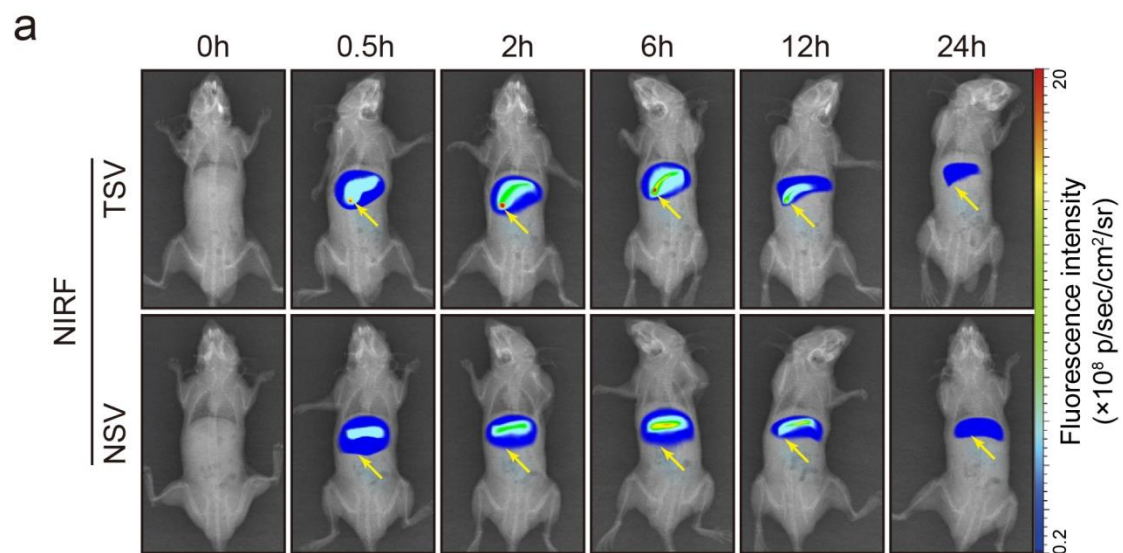
differences were analyzed by Two-way ANOVA with multiple comparisons).



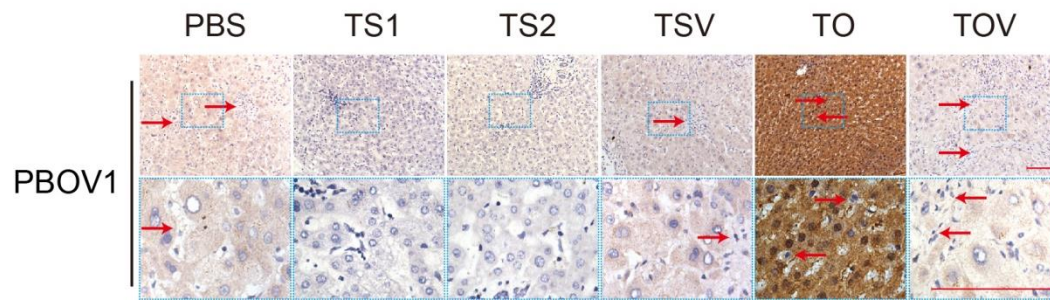
Supplementary Figure 8 In vivo delivery of scAb-EGFR-modified nanomedicine in comparison to that of folate-modified one. **a** In vivo NIRF imaging of mice at 2, 6 h after single injection of scAb-EGFR (E) of folate (F) targeted nanomedicine at dose of 4 µg PBOV1 plasmid; carrier labeled with Alexa Fluor 750 for imaging; yellow arrows indicated tumor location. **b** Protein expression levels of PBOV1 determined by IHC in liver orthotopic tumors of mice receiving scAb-EGFR-targeted (E), folate-targeted (F), or non-targeted (N) nanomedicine injection once a day for 20 d at 4 µg PBOV1 plasmid per injection. Scale bar, 100 µm. **c** Protein expression levels of PBOV1 determined by western blotting assay in liver orthotopic tumors of mice receiving scAb-EGFR-targeted (E), folate-targeted (F), or non-targeted (N) nanomedicine injection once a day for 20 d at 4 µg or 8 µg PBOV1 plasmid per injection.



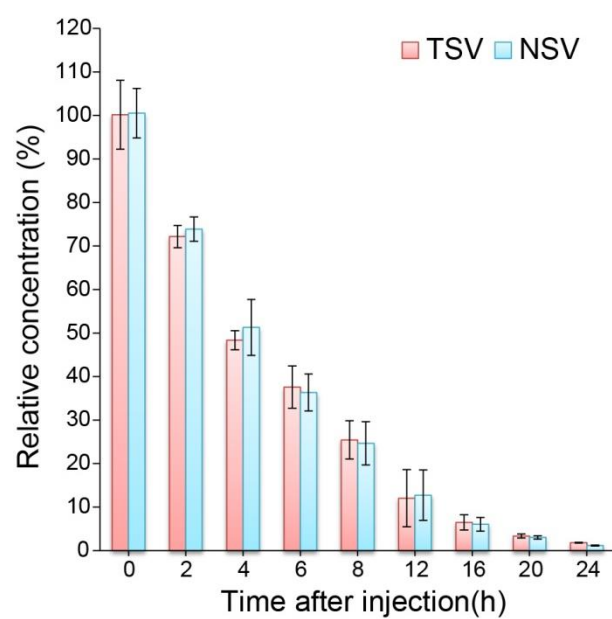
Supplementary Figure 9 Liver metabolism and toxicity of nanomedicines in Hep3B liver orthotopic model. **a** Prussian blue staining assays performed on histological sections of liver and tumor tissues after single injection. Scale bar, 20 μm . **b** Ex vivo NIRF images of mice gallbladders excised at 2 h after single injection of TSV group nanomedicine. Before testing, Cholecystectomy was performed, including ligation of cystic duct, retention of bile, and eventual removal of gallbladder. **c** Body weights of mice receiving normal dose or overdose of nanomedicine in comparison with mice receiving PBS injection. **d** Serum liver function markers alanine transaminase (ALT) / total bilirubin (TBIL) and renal function markers blood urea nitrogen (BUN) / creatinine (Cr) assessed during the period of 20 d treatment with nanomedicine at normal dose or overdose; injection performed once a day at 8 μg plasmid in TSV group (normal dose) and 40 μg plasmid in 5 $\times$ TSV group (overdose). Data were represented as means $\pm$ S.D. Error bars represent the S.D. values (n=12; $*P < 0.05$. Significant differences were analyzed by Student's *t*-test, compared with PBS control group).



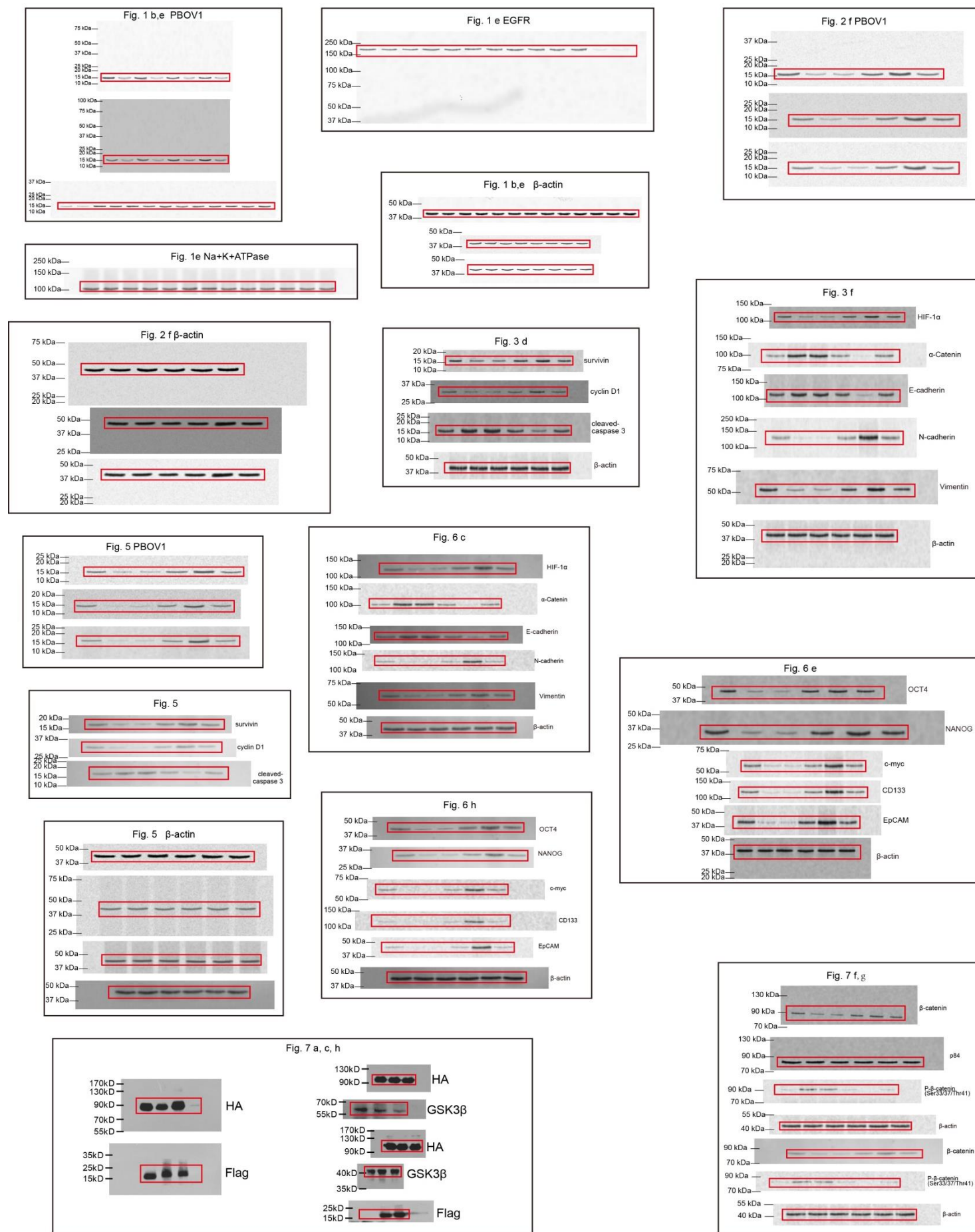
Supplementary Figure 10 In vivo biodistribution of nanomedicines in Hep3B liver orthotropic model. Near-infrared fluorescence imaging (NIRF) imaging of mice determined at various time points after single injection of Alexa Fluor 750-labeled nanomedicines via tail vein (Yellow arrows indicate the location of tumor).



Supplementary Figure 11 In vivo effects of nanomedicines. Protein expression levels of PBOV1 determined by IHC in liver orthotopic tumors in various treatment groups (upper panel), and magnified view (bottom panel) showing cancer cell specificity of PBOV1 expression in tumor tissue. Stromal cells including immune cells and fibrocytes without PBOV1 immunohistochemical staining were indicated by red arrows. Scale bar, 50 μm .



Supplementary Figure 12 In vivo concentrations of the two siRNA nanomedicines measured by the fluorescence intensity of the blood collected after tail-vein injection. Data were represented as means $\pm$ S.D. Error bars represent the S.D. values (n=6).



Supplementary Figure 13 Scanned images of key western blots.