Artemin is hypoxia responsive and promotes oncogenicity and increased tumor initiating capacity in hepatocellular carcinoma

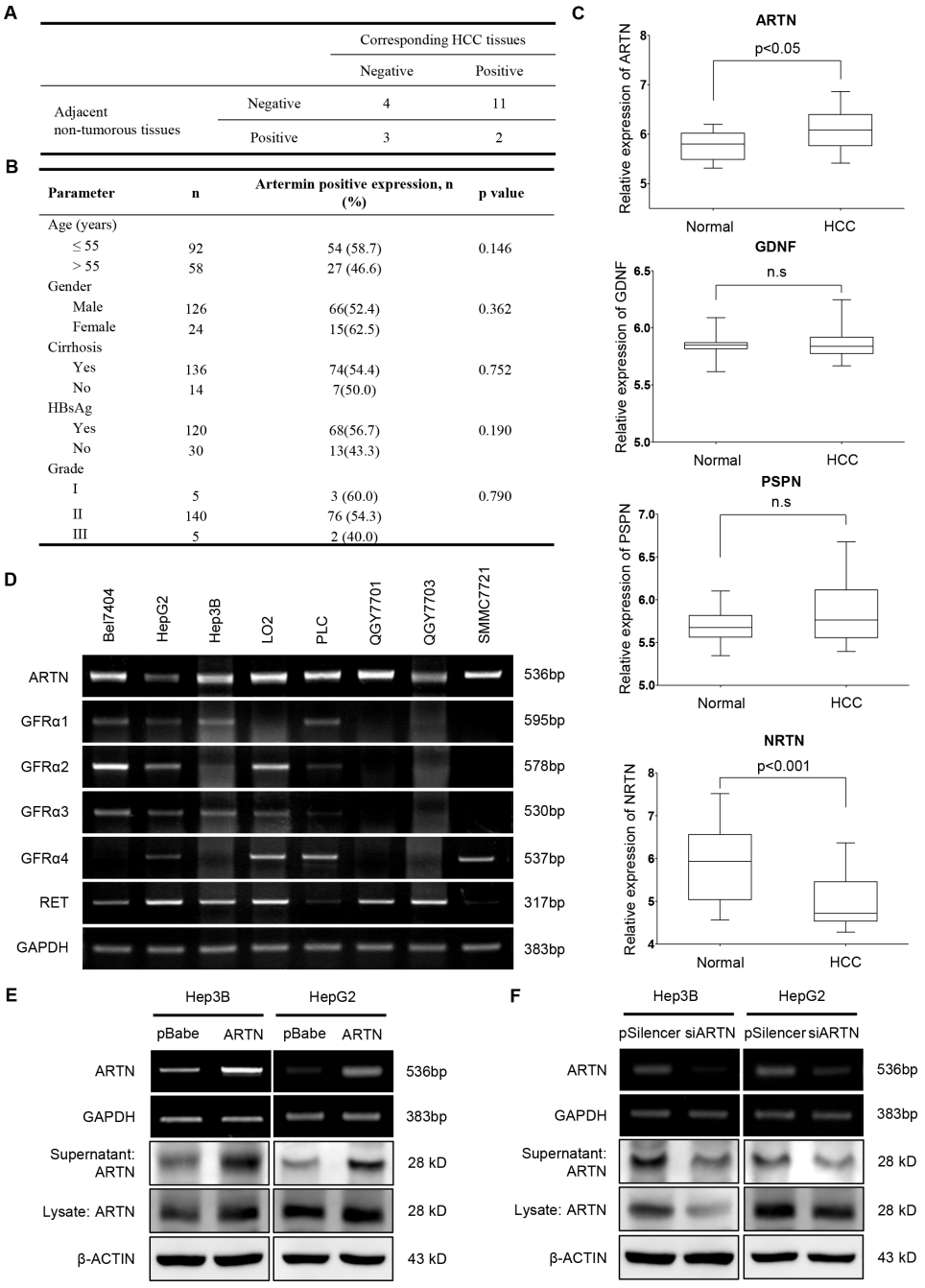
Supplementary Materials

A

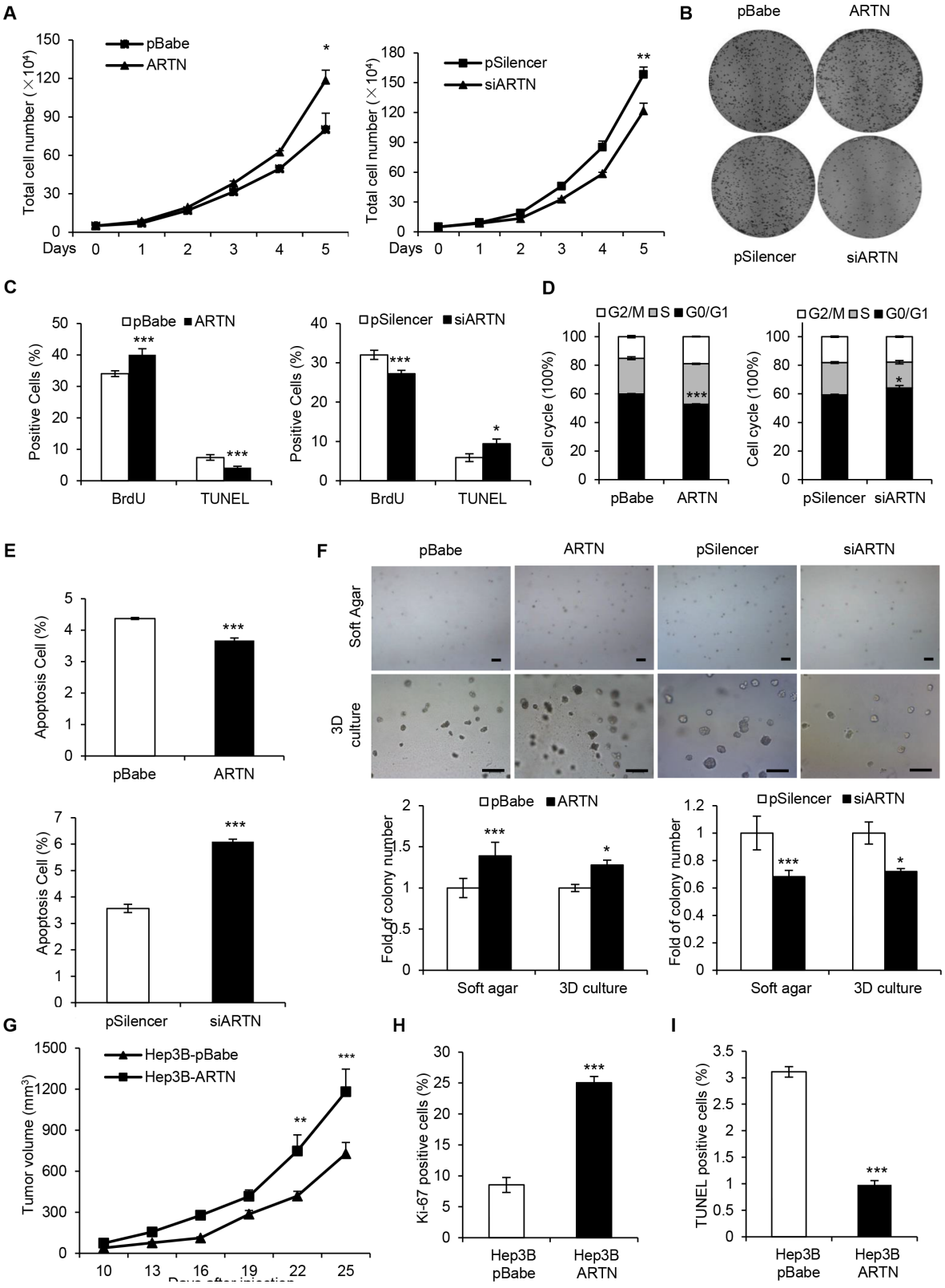
		Corresponding HCC tissues	
		Negative	Positive
Adjacent non-tumorous tissues	Negative	4	11
	Positive	3	2

B

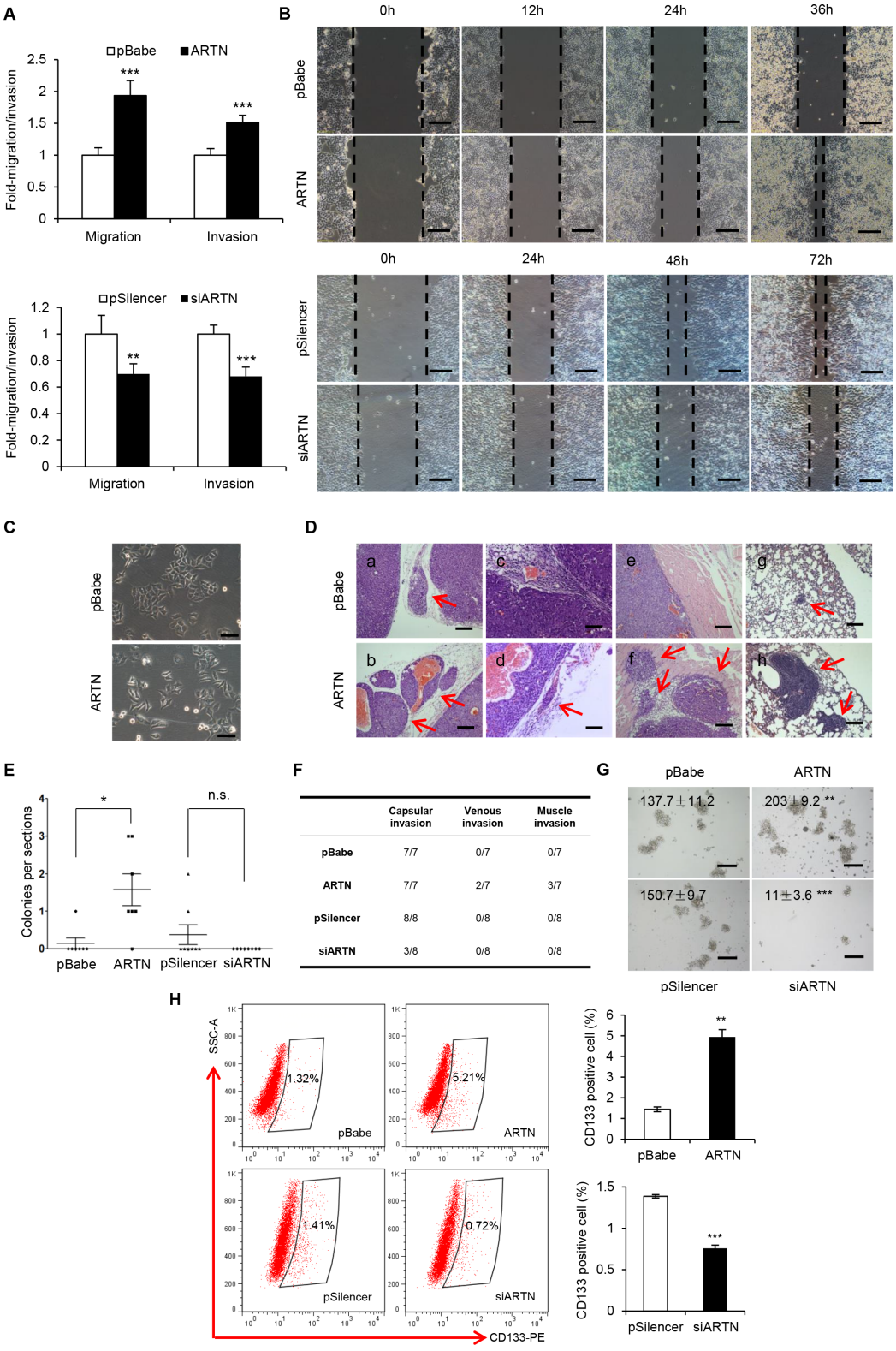
Parameter	n	Artermin positive expression, n (%)	p value
Age (years)			
≤ 55	92	54 (58.7)	0.146
> 55	58	27 (46.6)	
Gender			
Male	126	66(52.4)	0.362
Female	24	15(62.5)	
Cirrhosis			
Yes	136	74(54.4)	0.752
No	14	7(50.0)	
HBsAg			
Yes	120	68(56.7)	0.190
No	30	13(43.3)	
Grade			
I	5	3 (60.0)	0.790
II	140	76 (54.3)	
III	5	2 (40.0)	



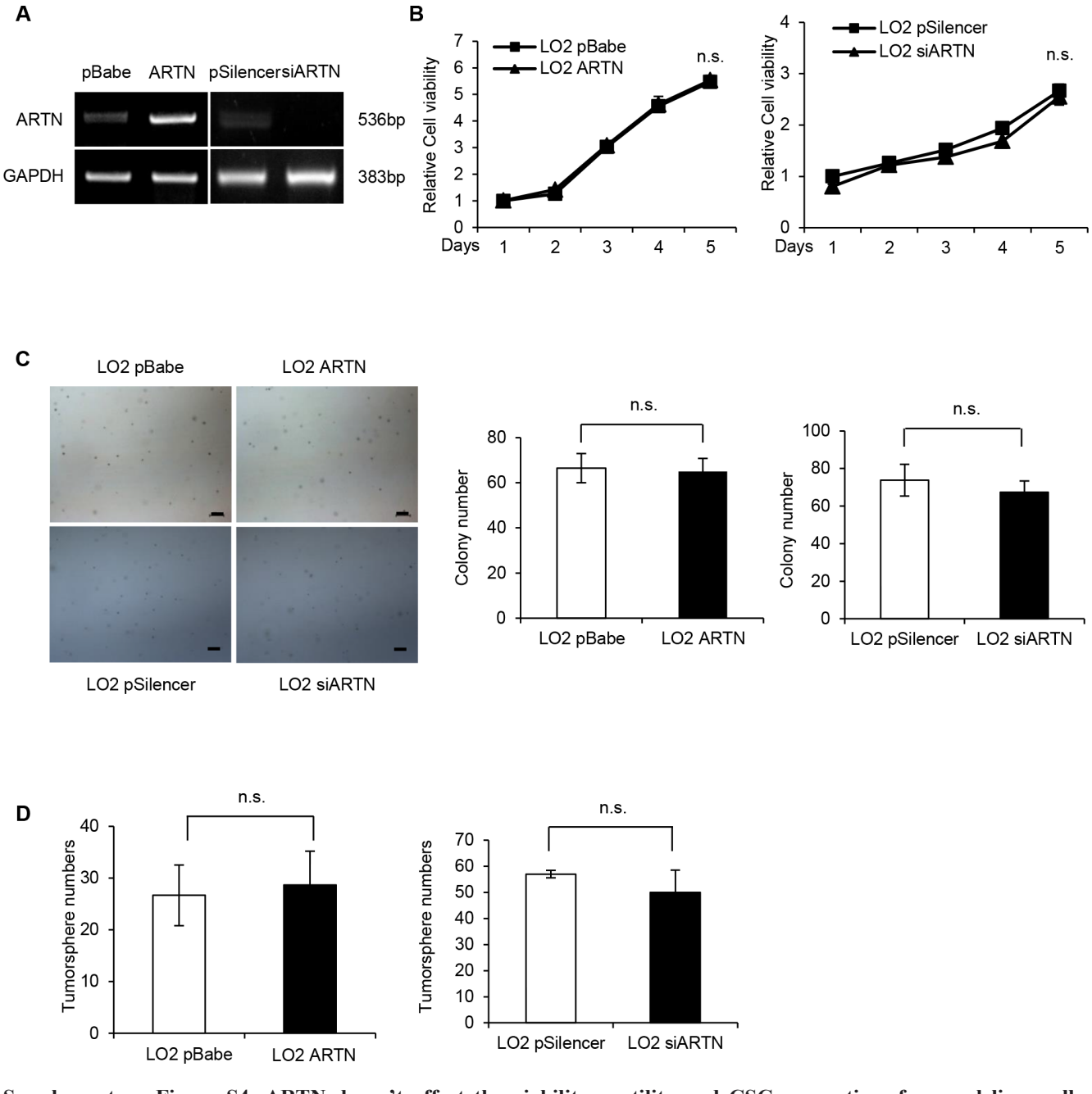
Supplementary Figure S1: ARTN expression patterns in HCC specimens, HCC cell lines and LO2 cells. (**A**) IHC analysis of ARTN expression in human HCC specimens and paired adjacent non-tumorous liver tissues (paired Student *t* test). (**B**) The relationship between ARTN and other clinicopathological characteristics in HCC patients. (**C**) The mRNA expressions of all GDNF family members in HCC samples compared with normal liver tissues based on a published HCC mRNA array dataset. (**D**) The expression of ARTN, GFRα1-4 and RET mRNA in HCC cell lines, determined by RT-PCR. (**E–F**) Verification of forced and knockdown expression of ARTN in Hep3B and HepG2 cell lines by RT-PCR and Western blot ($\chi 2$ test).



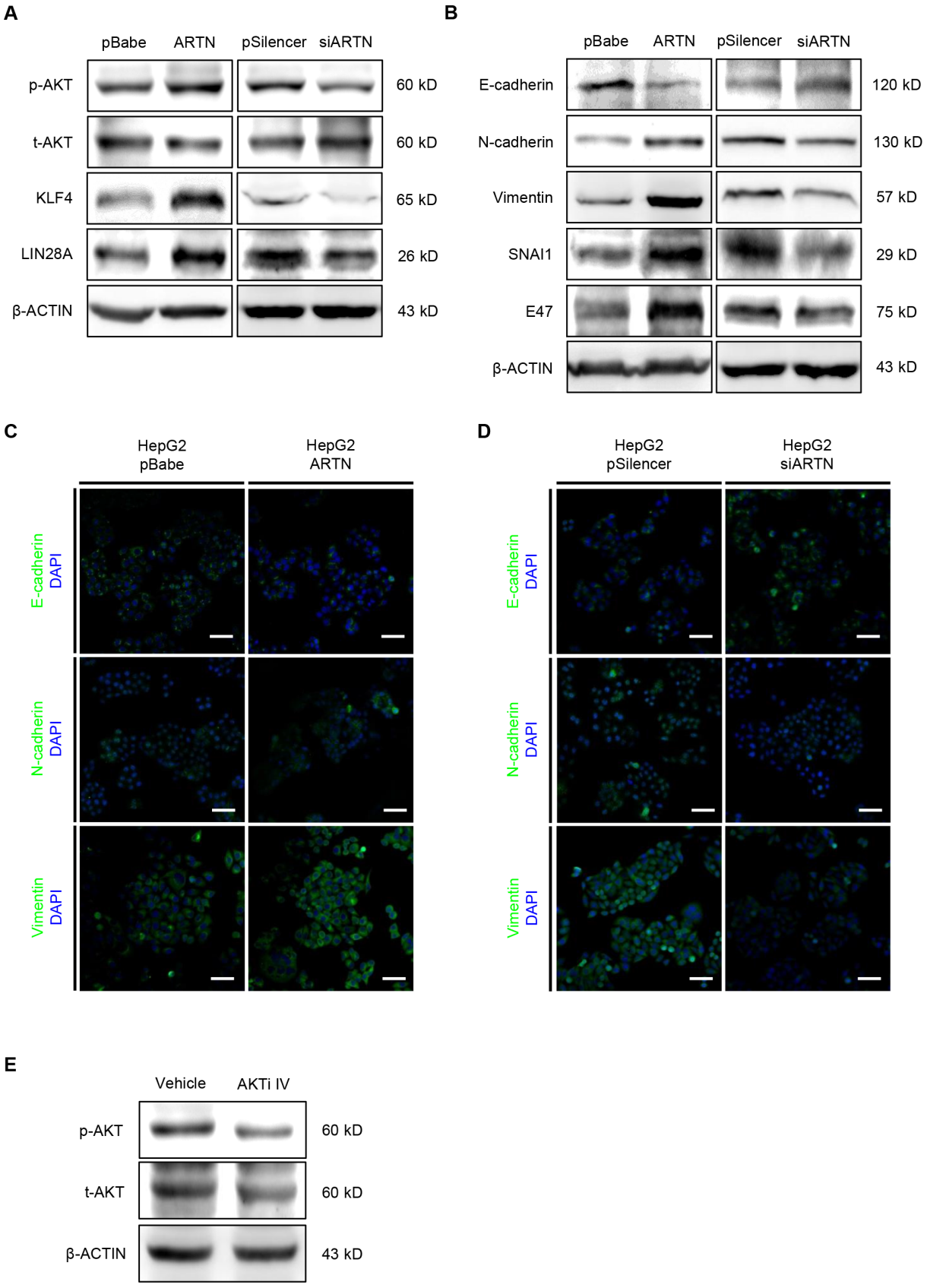
Supplementary Figure S2: ARTN promotes oncogenicity of HepG2 cells *in vitro*. (**A**) Proliferation of HepG2 cells was determined by total cell number counting. (**B**) Foci formation assay of HepG2 cells in monolayer culture. (**C**) The percentages of BrdU and TUNEL positive HepG2 cells. (**D**) Cell cycle analysis. The HepG2 cells were stained with PI and then detected by flow cytometry. The percentages of cells in three phases are shown in histogram. (**E**) Apoptosis cells were detected by Annexin V and PI staining with flow cytometry. (**F**) Soft agar (Upper panels, 40×) and 3D Matrigel (Lower panels, 100×) colony formation of HepG2 cells. Colony numbers were counted and are shown in histogram. (**G**) Tumor growth curve of Hep3B-pBabe and Hep3B-ARTN cells injected into the flank of male nude mice. (**H–I**) Tumor cell proliferation and apoptosis were examined by Ki-67 and TUNEL staining, respectively. Mean ± SD, $n = 3$, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (Student t test).



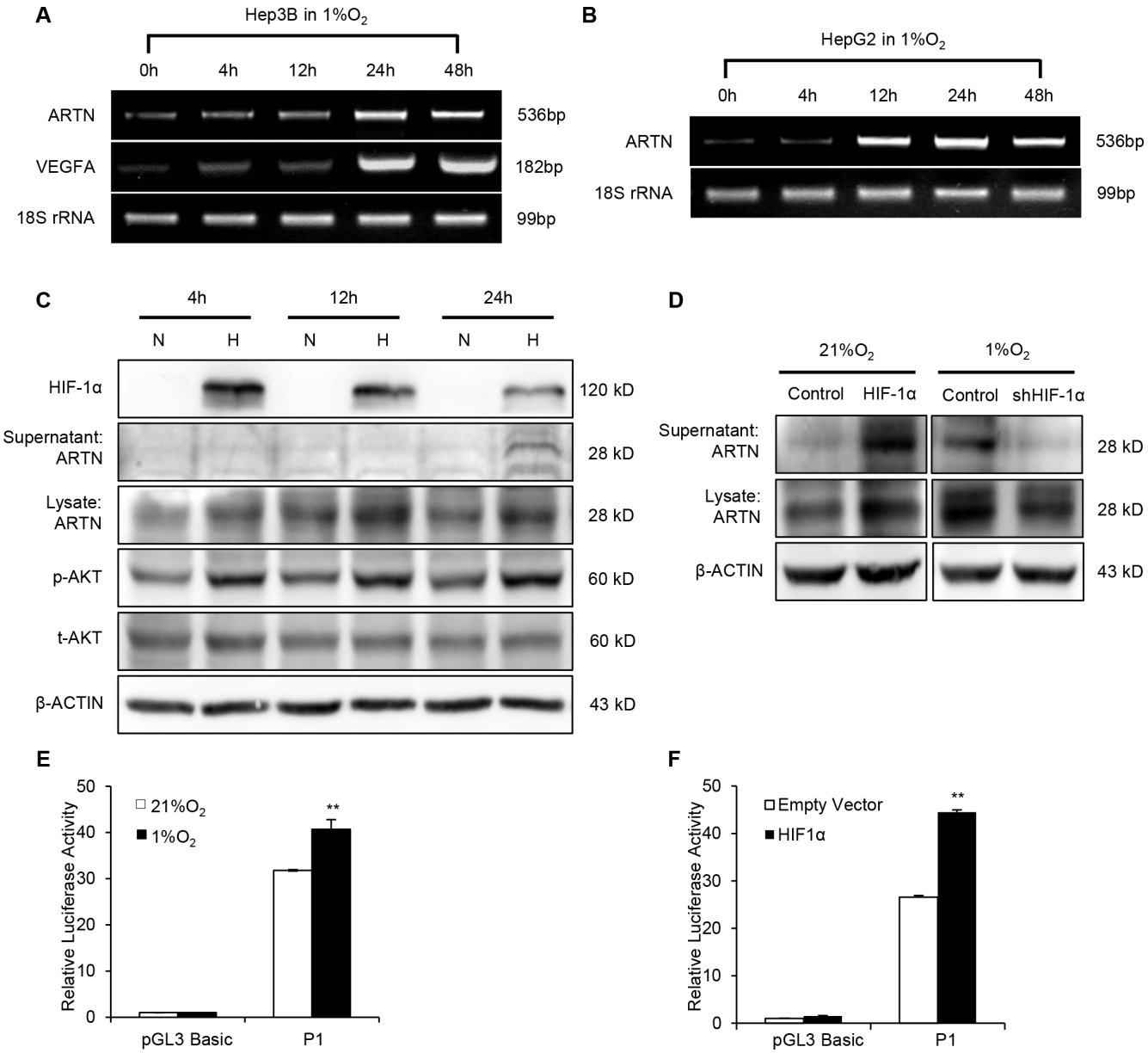
Supplementary Figure S3: ARTN regulates the metastatic capacity and CSC properties of HepG2 cells *in vitro*. (**A**) Transwell migration and invasion assay of HepG2 cells. (**B**) Wound healing assay of HepG2-ARTN and HepG2-siARTN cells compared with their respective control cells. Magnification, 100×. (**C**) Morphology of HepG2-pBabe and HepG2-ARTN cells. Representative pictures were taken using phase-contrast microscopy at 200× magnification. (**D**) H & E staining of tumors and lungs from mice xenograft model. Arrows indicated invasion of capsular (a, b), venous (c, d), muscles (f) and lung metastasis foci (g, h). (**E**) Numbers of lung micrometastases per section in individual mice (each data point represented a different mouse). (**F**) The quantification of the mice showed invasive phenotypes in each group. (**G**) Tumorsphere formation assays of HepG2 cells. Representative images of tumorspheres generated by HepG2 cells were captured by microscope at 100× magnification. (**H**) CD133 positive population in HepG2 cells. Results were shown in histogram. Mean ± SD, $n = 3$, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (Student t test).



Supplementary Figure S4: ARTN doesn't affect the viability, motility and CSC properties of normal liver cells. (**A**) Establishment of ARTN overexpression and knockdown in LO2 cells was verified by RT-PCR. (**B**) Relative cell viability of LO2-ARTN and LO2-siARTN cells compared with their respective control cells. Cells were incubated with CCK8 and then detected at the wavelength of 450 nm. (**C**) Soft agar assay of LO2 cells. Representative images of colonies formed by LO2 cells were taken at 40× magnification. The results were shown in histogram. (**D**) Tumorsphere formation of LO2 cells. Mean ± SD, $n = 3$, n.s., not significant (Student t test).



Supplementary Figure S5: ARTN modulates CSC and EMT via regulation of several important factors. (**A**) Western blot analysis of p-AKT and important transcription factors (KLF4, LIN28A) of CSC pathway in HepG2 cells. (**B**) Western blot analysis of EMT markers and inducers in HepG2 cells. (**C–D**) Immunofluorescene staining of E-cadherin, N-cadherin and Vimentin in HepG2 cells. Representative pictures were taken using confocal microscopy at 200 × magnification. (**E**) The p-AKT level was detected after treatment of AKT inhibitor IV (AKTi IV) for 24 hours.



Supplementary Figure S6: ARTN is induced by hypoxic condition in HCC cells. (**A**) Time-course analysis of ARTN mRNA under hypoxia condition in Hep3B cells. The levels of ARTN mRNA were determined by RT-PCR. VEGFA mRNA was used as a positive control. (**B**) Time-course analysis of ARTN mRNA under hypoxia condition in HepG2 cells. (**C**) Time-course analysis of HIF-1α, ARTN and p-AKT protein in HepG2 cells. Cells were incubated under normoxia or hypoxia for every indicated time. The protein levels were determined by Western blot. N, 21% O_2; H, 1% O_2. (**D**) The expression of ARTN was related with HIF-1α level in HepG2 cells, determined by Western blot. In normoxia, HepG2 cells were transiently transfected with HIF-1α cDNA or the control construct, or HIF-1α was knockdown by shRNA plasmid under hypoxia for 48 hours. (**E**) Luciferase activity of promoter (P1). HepG2 cells were transfected with the promoter (P1) constructs and then incubated in normoxic (21% O_2) or hypoxic (1% O_2) conditions. (**F**) Analysis of promoter (P1) activity in HepG2 cells after transfection of HIF-1α cDNA or the control construct under normoxia. Mean ± SD, $n = 3$, $^{**}P < 0.05$ (Student t test).

Supplementary Table S1: Sequences of the oligonucleotides for RT-PCR analysis, shRNA, plasmid construction and ChIP assays

Primers for RT-PCR	**Sense Strand (5′-3′)**	**Antisense Strand (5′-3′)**
ARTN	TGAGCAGCGTCGCAGAGG	GAGGCGGTCCACGGTTCT
GFRα1	TTGCAGGACTCCTGCAAGACG	GACCACAGCTTGGAGGAGCAG
GFRα2	CAGCTGCTCCTATGAGGACAAG	CTGGGATGATATTTGTCGTGAGC
GFRα3	CTGCTCACTTTCTTCGAGAAGG	CAGGGTTTTCATTCTGGTGTGC
GFRα4	ATGGTGCCATTCAGGCCTTTGC	ATGGTCTCTGACCTGCTCTAGG
RET	CGTGAAGAGGAGCCAGGGTC	TAACCATCATCTTCTCCAGGTCT
GAPDH	TCCCATCACCATCTTCCAGG	CCATCACGCCACAGTTTCC
VEGFA	ACGGACAGACAGACAGACACC	GAACAGCCCAGAAGTTGGAC
18S rRNA	CGGCGACGACCCATTCGAAC	GAATCGAACCCTGATTCCCCGTC
Primers for plasmid construction	**Sense Strand (5′-3′)**	**Antisense Strand (5′-3′)**
ARTN cloning primer	CGGATCCATGGAACTTGGAC TTGGAGGCC	AGAATTCTCAGCCCAGGCAGCC GCAGG
siARTN cloning primer	AACAGCACCTGGAGAACCG	
ARTN promoter P1 cloning primer	ATAAGCTAGCAACCCTCCCATG GTCCCCAGTG	CGTAAGCTTGGCAAGTGGGCTGGC TTACCTC
ARTN promoter P2 cloning primer	CCGAGCTAGCTTTAGCCTGGAC CAAAGGGAAG	CGTAAGCTTGGGAGGGTTTTGAGC AGAATGG
ARTN promoter P1 HIF-1α Mut A primer	GGACCCCCAAATCTCAGCAGTC AGCCGCCC	GGGCGGCTGACTGCTGAGATTTGG GGGTCC
ARTN promoter P1 HIF-1α Mut B primer	CAGCAGTCAGCCGCCGGACCG GCTTACCCC	GGGGTAAGCCGGTCCGGCGGCTG ACTGCTG
sh Scr	CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG	
sh HIF-1α	CCGGCCAGTTATGATTGTGAAGTTACTCGAGTAACTTCACAATCATAAC TGGTTTTT	
Primers for ChIP assay	**Sense Strand (5′-3′)**	**Antisense Strand (5′-3′)**
HIF-1α Site A and B Primers	GAAGAATCGGGTGGAGCA	GGAGCGAGGGGTAAGCC
NC site Primer	ATGGTTGCCACTGGGGATCT	TGCCAAAGCCTAGGGGAAGA

Supplementary Table S2: List of proteins tested by antibodies and characteristics of the corresponding antibodies used

Protein	Assay	Antibody	Origin	Dilution	Incubation period
ARTN	WB	gpab	#sc-9330, Santa Cruz	1:1000	overnight
ARTN	IHC	gpab	#sc-9330, Santa Cruz	1:200	overnight
CD133	FACS	mmab	#130080801, Miltenyi Biotec	0.5 µg	10 min
ACTIN	WB	mmab	#M20010, Abmart	1:5000	overnight
Phospho-AKT	WB	rpab	#CST9271, Cell Signaling	1:1000	overnight
Total-AKT	WB	mmab	#sc-5298, Santa Cruz	1:1000	overnight
KLF4	WB	mmab	#AM2725a, Abgent	1:1000	overnight
LIN28A	WB	rpab	#3978, Cell Signaling	1:1000	overnight
E-Cadherin	WB	mmab	#610181, BD	1:5000	overnight
E-Cadherin	IF	mmab	#610181, BD	1:400	overnight
N-Cadherin	WB	mmab	#610920, BD	1:5000	overnight
N-Cadherin	IF	mmab	#610920, BD	1:400	overnight
VIMENTIN	WB	mmab	#550513, BD	1:5000	overnight
VIMENTIN	IF	mmab	#550513, BD	1:400	overnight
SNAIl	WB	rpab	#sc-28199, Santa Cruz	1:1000	overnight
E47	WB	mmab	#554199, BD	1:5000	overnight
HIF-1α	WB	mmab	#610958, BD	1:5000	overnight
HIF-1α	IHC	mmab	#610958, BD	1:400	2 hour
HIF-1α	CHIP	rpab	# NB100-134, Novus	10µg	overnight

Abbreviations: WB, Western blot; FACS, flow cytometry; ChIP, chromatin immunoprecipitation; IHC, immunohistochemistry; IF, immunofluorescence; mmab, mouse monoclonal antibody; rpab, rabbit polyclonal antibody; gpab, goat polyclonal antibody.