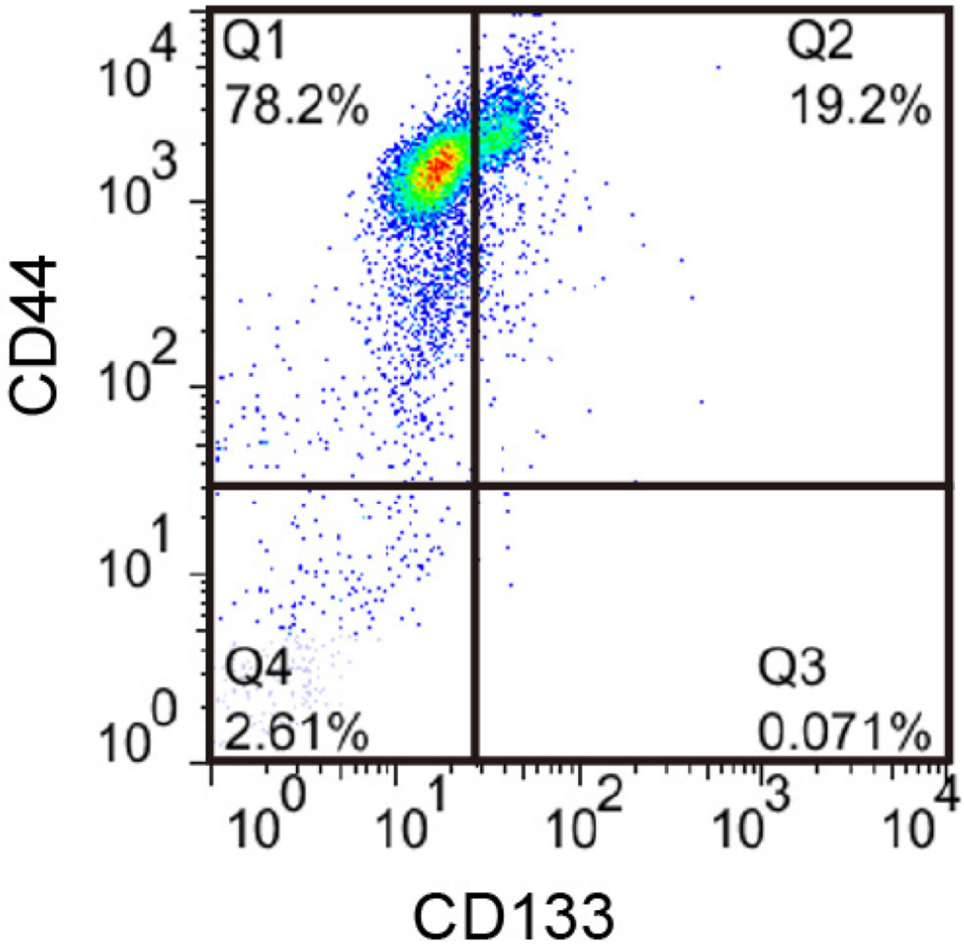
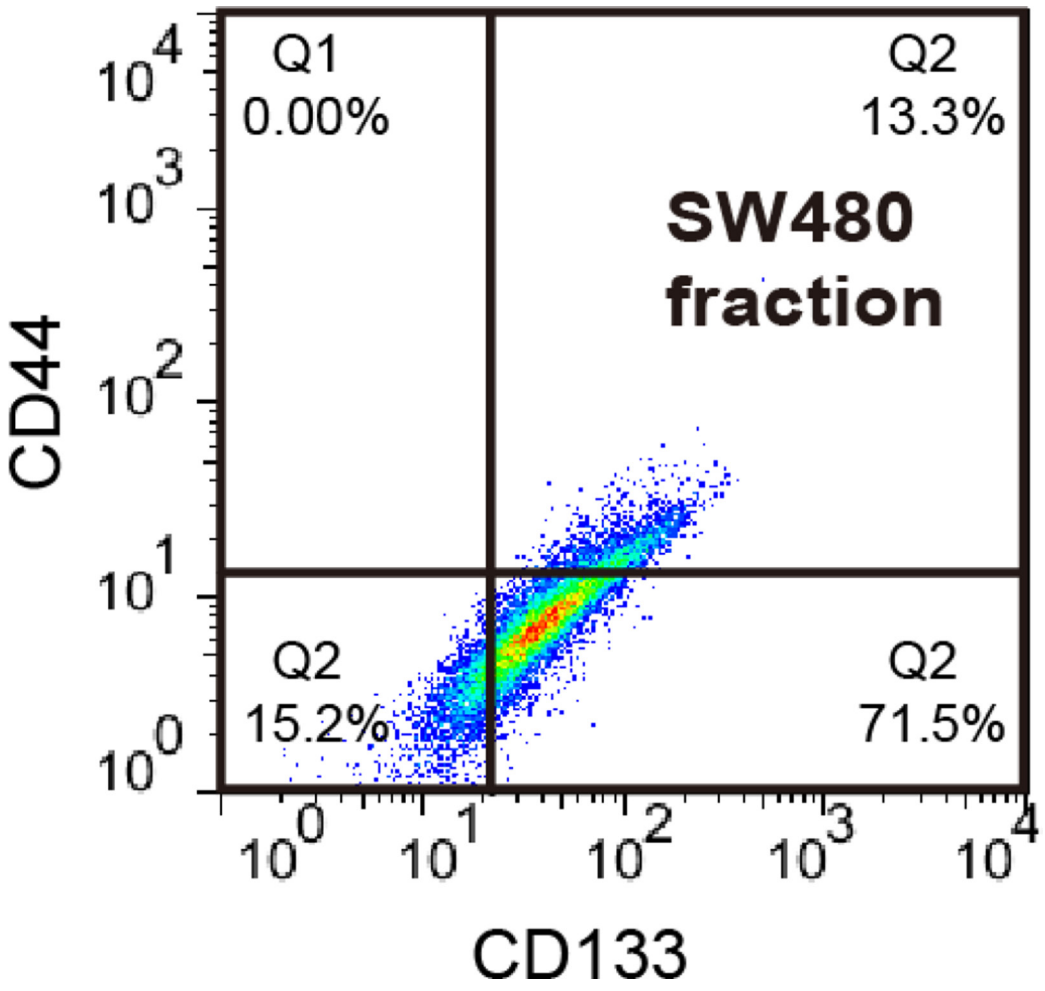
Reduced CD146 expression promotes tumorigenesis and cancer stemness in colorectal cancer through activating Wnt/β-catenin signaling

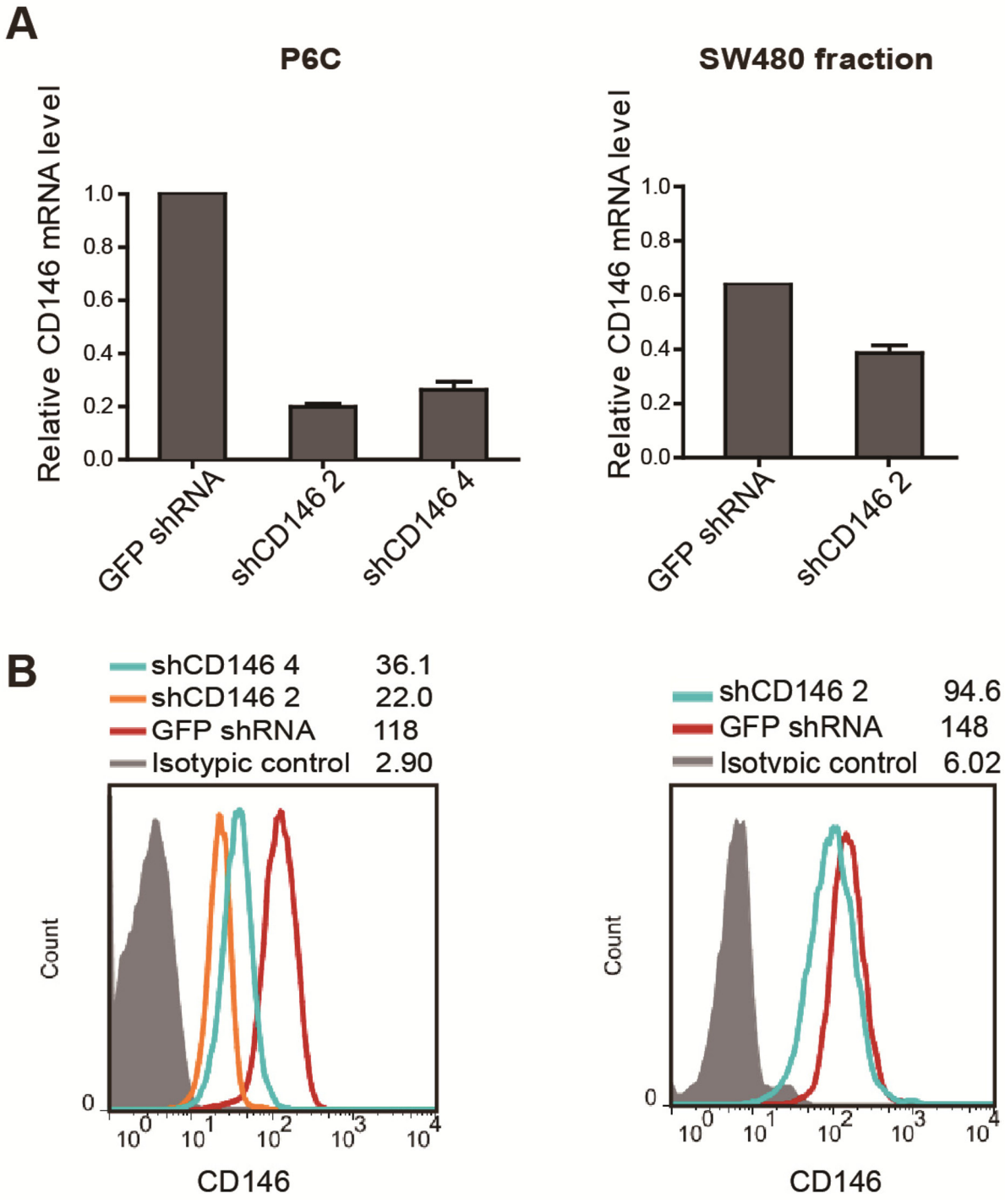
SUPPLEMENTARY FIGURES AND TABLES



Supplementary Figure S1: Phenotypic identification of primary CRC cell line P6C. FACS analysis of CD44 and CD133 expression on P6C cells.



Supplementary Figure S2: FACS sorting of SW480 fraction based on the expression of CD44 and CD133. SW480 fraction denotes the $CD44^+CD133^+$ subset in established CRC cell line SW480.

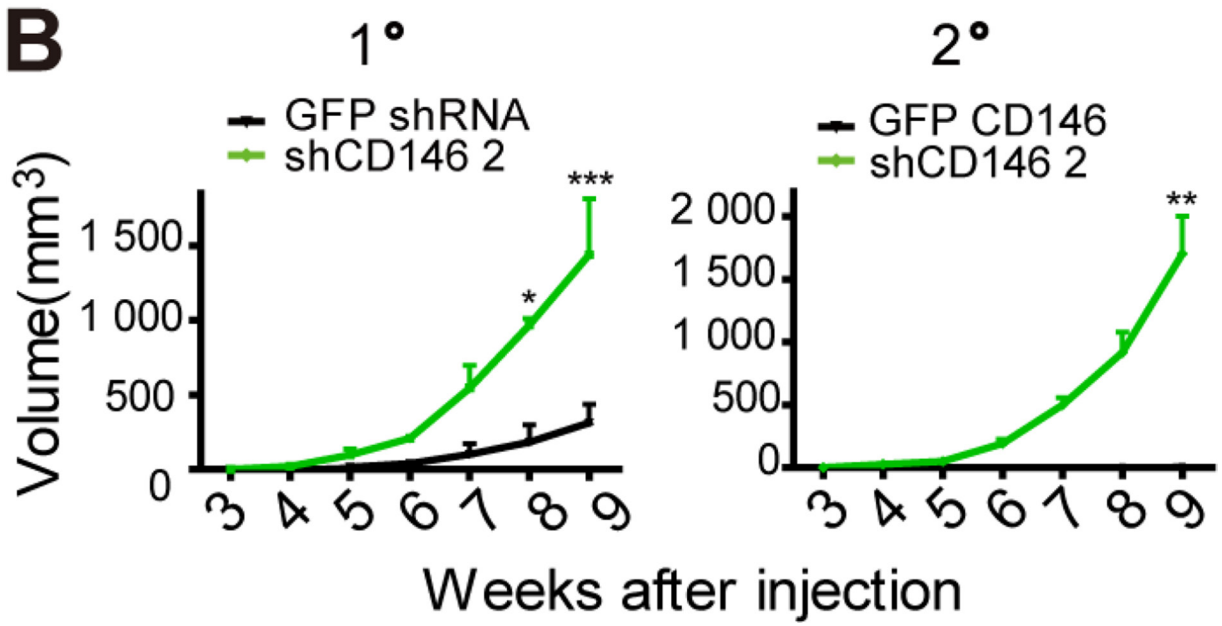


Supplementary Figure S3: Knockdown efficiency of CD146 in P6C and SW480 fraction with shRNAs transfection. **A.** The mRNA expression levels of CD146 in P6C cells and SW480 fraction, as assessed by qRT-PCR analysis. GFP shRNA was used as a negative control. The relative mRNA level of CD146 was normalized to human GAPDH mRNA expression. Error bars, mean ± s.d. **B.** The protein levels of CD146 in P6C cells (left) and SW480 fraction (right) were analyzed by FACS. Histograms of one representative experiment are shown.

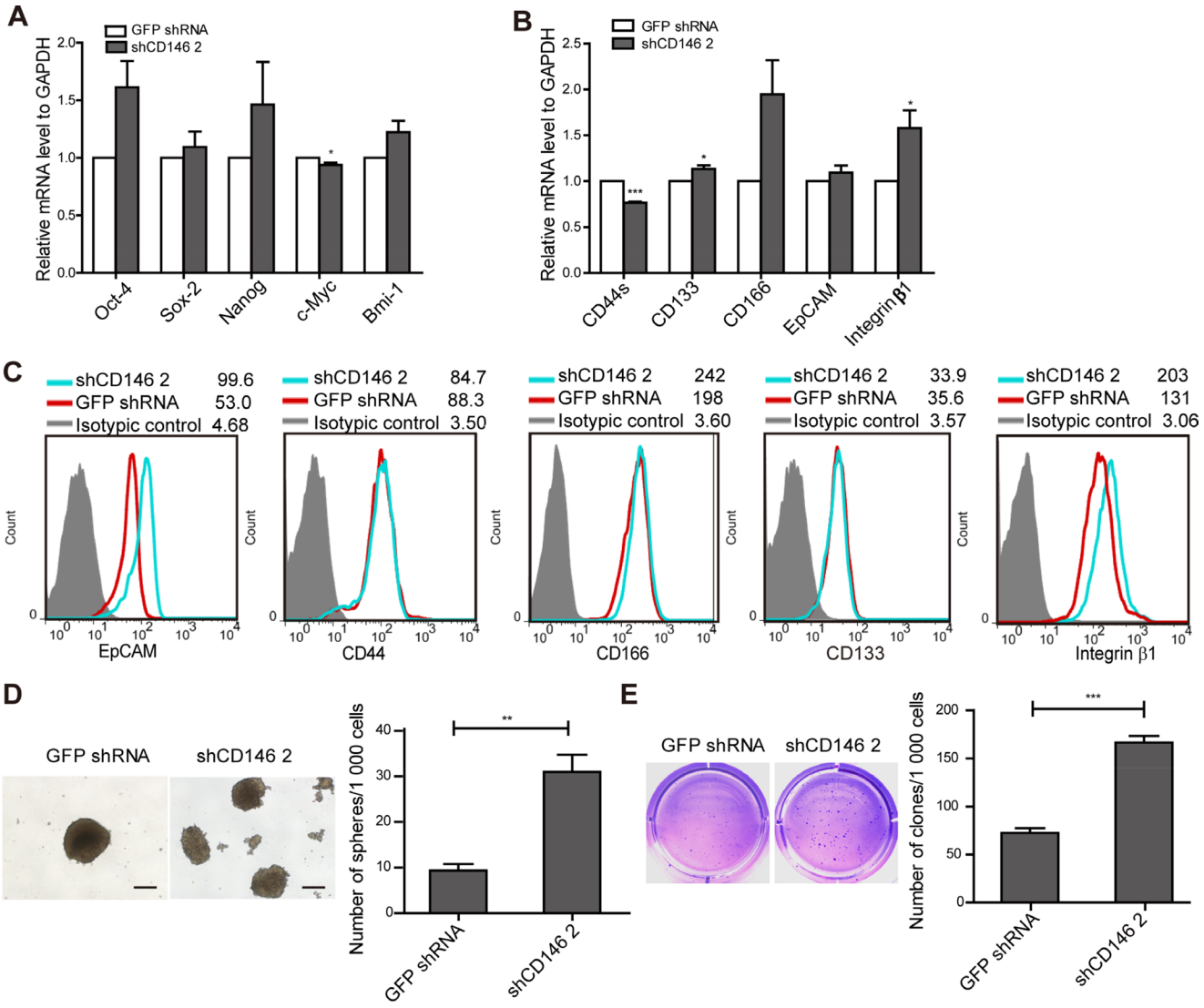
A

	Cell subset	Incidence	Latency
1°	GFP shRNA	2/5	32
	shCD146 2	4/5	19
2°	GFP shRNA	1/5	45
	shCD146 2	2/5	23

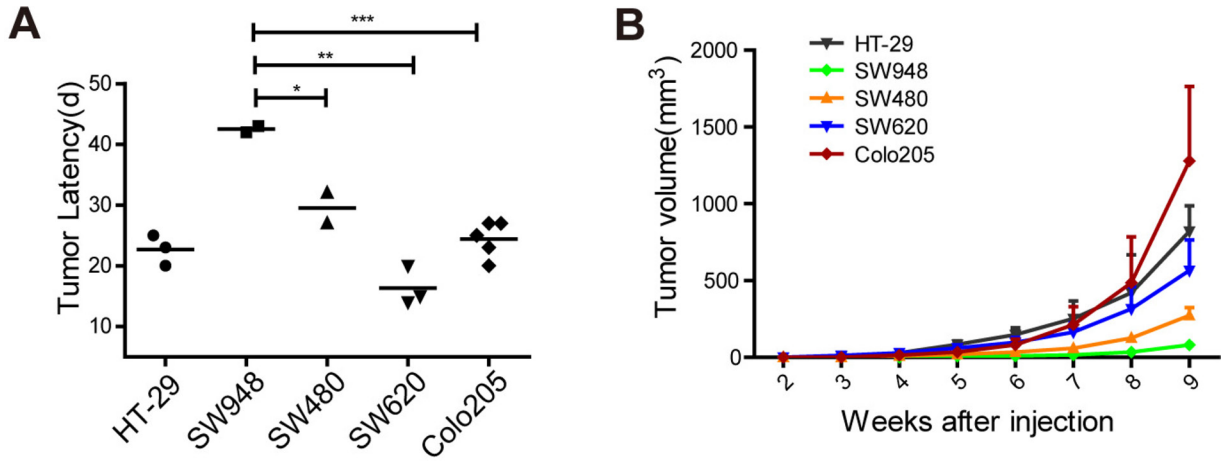
Notes: 1 000 single cells were injected .



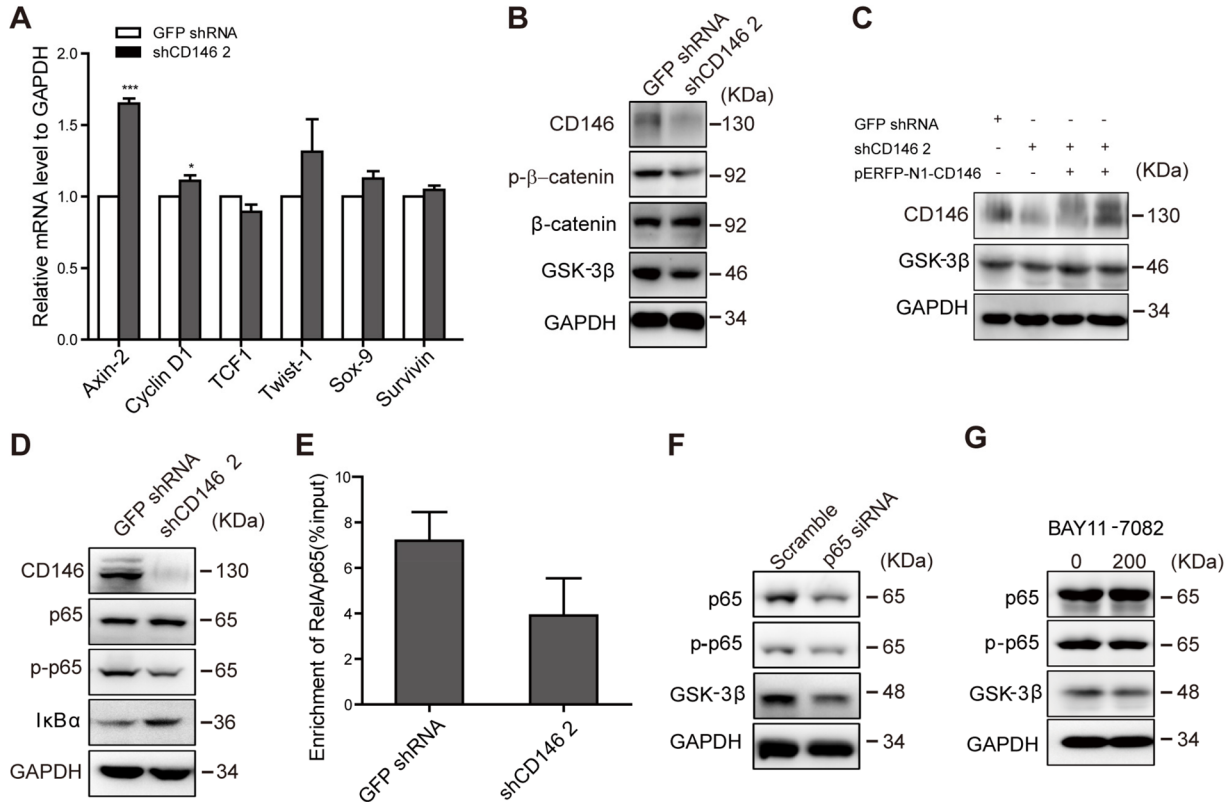
Supplementary Figure S4: Reduced CD146 expression promotes colorectal tumorigenesis *in vivo*. A. Reduced expression of CD146 facilitates tumor formation in serial transplantations. 1000 SW480 fraction cells were used for each injection (n=5). **B.** Tumor volumes and growth curves were monitored in serial recipients. Significance of differences at indicate time point were determined by two-way ANOVA analysis, **P<0.05, **P<0.01, ***P<0.001.* Error bars, mean ± s.d.



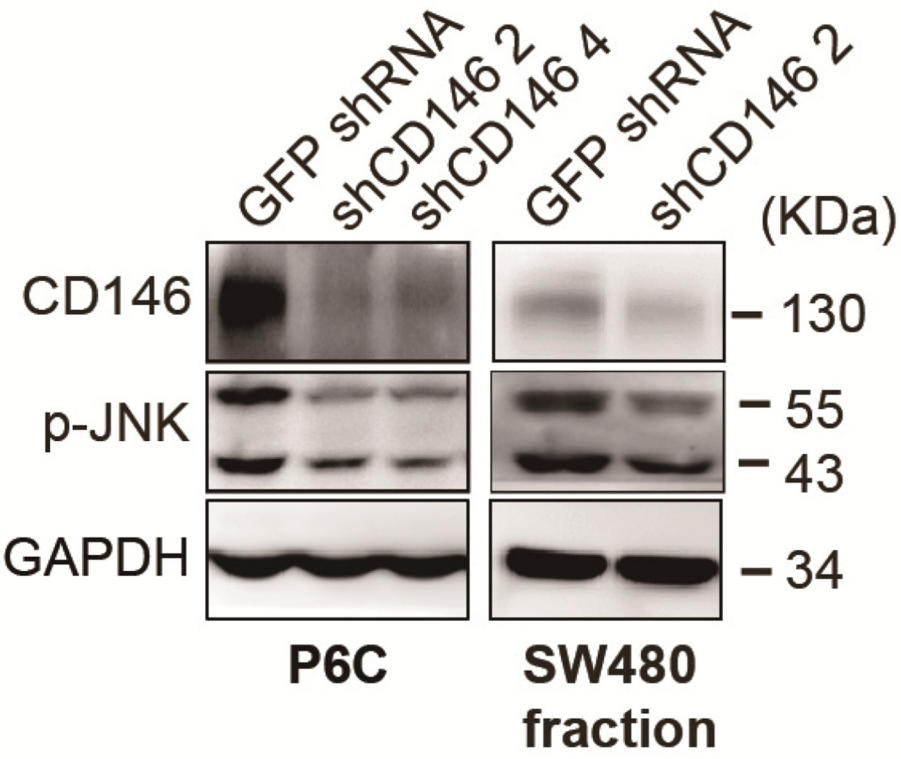
Supplementary Figure S5: Knockdown of CD146 in SW480 fraction restores a stem cell phenotype. A. The mRNA expression levels of stemness-related transcription factors in SW480 fraction, as determined by qRT-PCR. Data were expressed as mean value ± s.d. of three independent experiments. **B.** The mRNA expression levels of colorectal CSC markers in SW480 fraction. **C.** FACS analysis of CSC surface markers. **D.** CD146 knockdown promotes sphere formation. 1000 SW480 fraction cells were cultured in ultra low adherent plates in triplicates under SFM condition. **E.** CD146 knockdown increases clone formation efficiency. **P<0.05, **P<0.01, ***P<0.001.* Error bars, mean ± s.d.



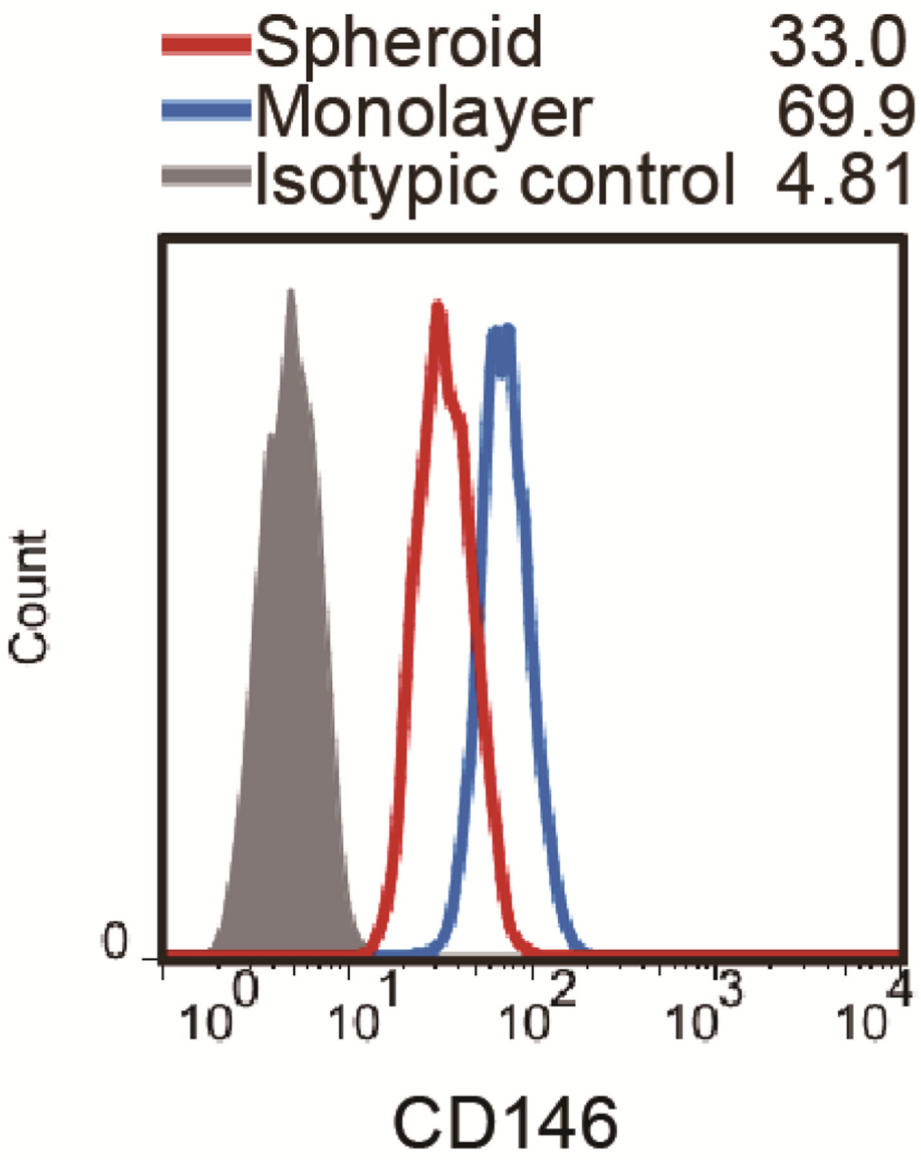
Supplementary Figure S6: Tumorigenicity of five established human CRC cell lines *in vivo*. A. Tumor incidence and latency in xenotransplantation. 1000 cells were used for each subcutaneous injection to NOD/SCID mice (n=5). **B.** Tumor volumes and growth curves were monitored and measured weekly. **P<0.05,*P<0.01, ***P<0.001*. Error bars, mean ± s.d.



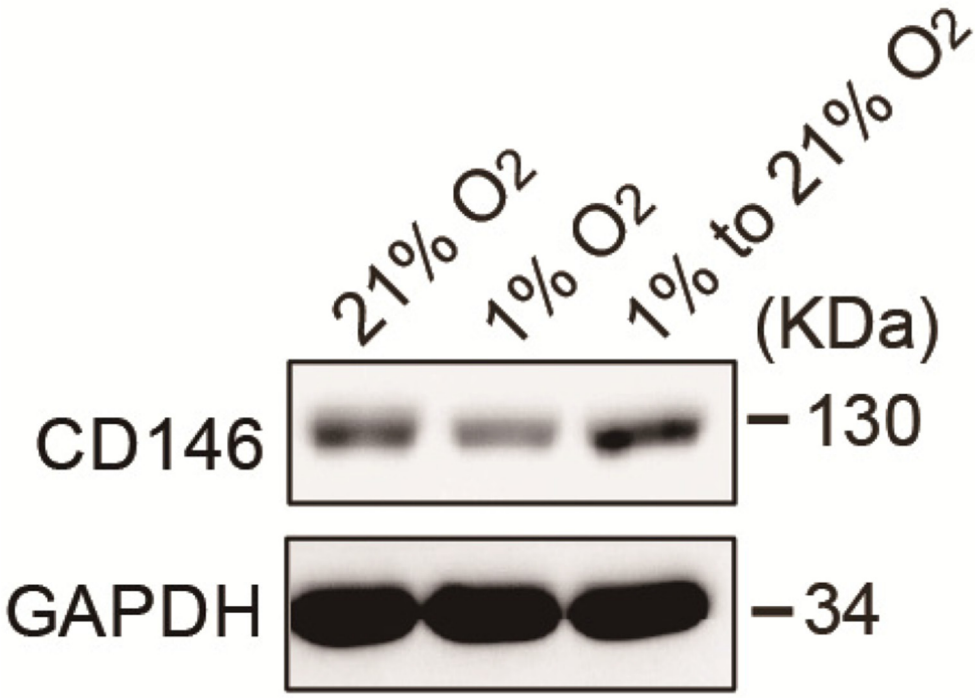
Supplementary Figure S7: Knockdown of CD146 activates canonical Wnt signaling in SW480 fraction cells. A. The mRNA expression levels of Wnt target genes upon CD146 knockdown. Data were expressed as mean value ± s.d. of three independent experiments. **B.** Knockdown of CD146 represses β-catenin phosphorylation and GSK-3β expression. GAPDH were used as a loading control. **C.** Rescued CD146 expression up-regulates the protein level of GSK-3β. **D.** Knockdown of CD146 inhibits NF-κB/p65 signaling pathway. **E.** Binding of NF-κB/p65 to the predicted site in GSK-3β gene promoter was validated by ChIP assays. NF-κB/p65 enrichment was normalized to input control. **F-G**. GSK-3β expression was inhibited by both transfection of p65-specific siRNA and treatment with NF-κB inhibitor BAY11-7082 (200 ng/ml). **P<0.05, ***P<0.001*. Error bars, mean ± s.d.



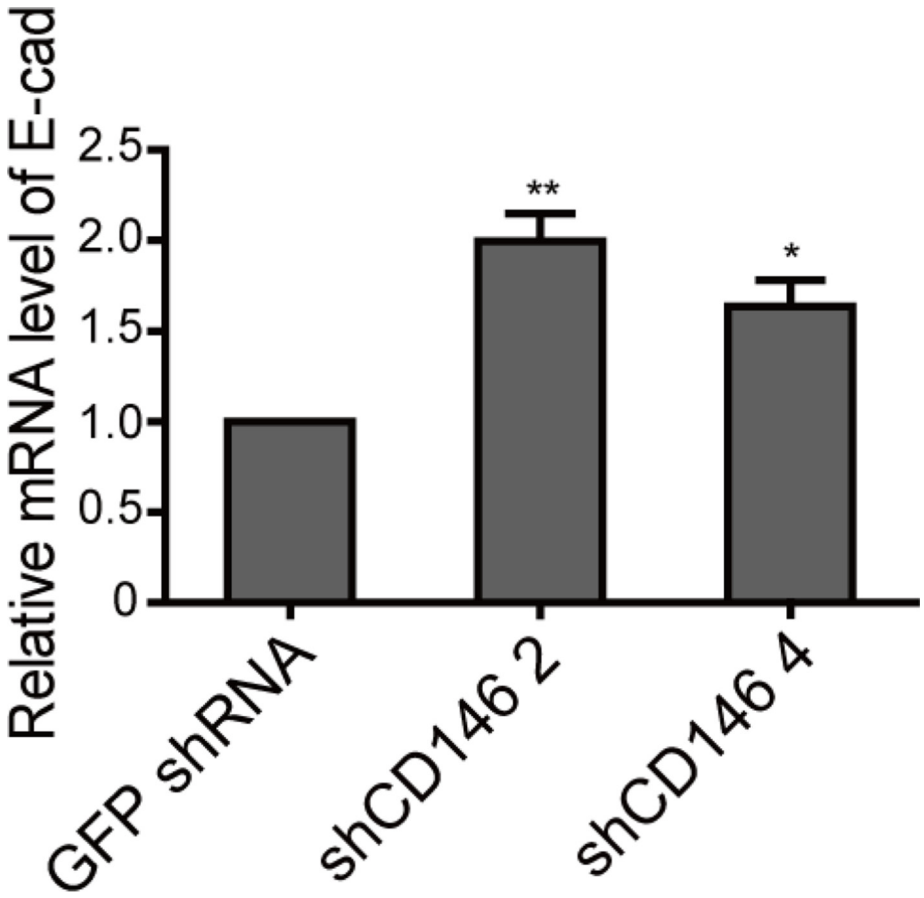
Supplementary Figure S8: Knockdown of CD146 inactivates non-canonical Wnt/ PCP pathway. JNK kinase activity was determined by western blotting in P6C cells and SW480 fraction.



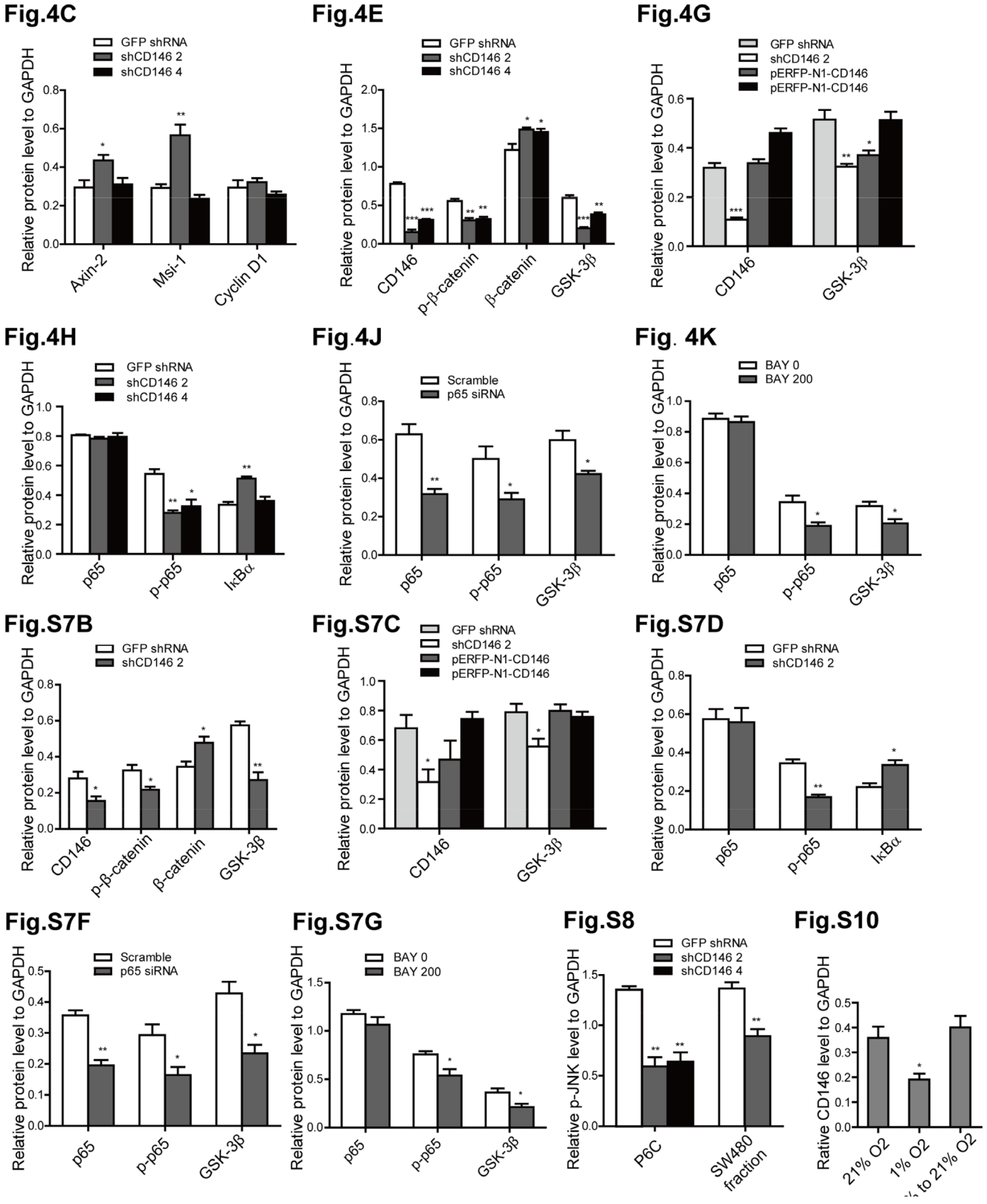
Supplementary Figure S9: CD146 is downregulated in spheres compared to the monolayer counterparts. P6C cells were cultured as adherent monolayer in medium containing 10% FBS. Spheroid cultures of P6C were maintained in ultra low adherent plates under SFM condition. Histograms of one representative experiment are shown.



Supplementary Figure S10: CD146 is downregulated under hypoxic condition. P6C cells maintained in normoxic condition (21% O_2) were sub-cultured in hypoxia (1% O_2) for 24h and were then switched back to the normal condition.



Supplementary Figure S11: Knockdown of CD146 induces the upregulation of E-cadherin in P6C cells. Relative mRNA level of E-cadherin was determined by qRT-PCR. **P<0.05, **P<0.01.* Error bars, mean ± s.d.



Supplementary Figure S12: Quantitative analysis of Western blot. Relative protein expression was determined by measuring the intensities of the bands and then normalizing to the internal control (GAPDH). **P<0.05,*P<0.01, ***P<0.001.* Error bars, mean ± s.d.

Supplementary Table S1: Pathway analysis based on the KEGG database

Pathway	Hits	Total	Percent	Enrichment test p-value	q-value
Wnt	20	151	13.25%	0.1627	0.7036
Notch	8	47	17.02%	0.1315	0.7036
Hedgehog	7	56	12.50%	0.3631	0.7055
ABC transporters	6	44	13.64%	0.314	0.7055

Hit threshold: fold change of gene expression≥2

Supplementary Table S2: Differentially expressed genes in P6C cells upon CD146 knockdown

Gene Symbol	Accession Number	Probe ID	Fold Change	
			shCD146 2 vs GFP shRNA	shCD146 4 vs GFP shRNA
LEF1	NM_001130714	221558_s_at	1.31	1.43
HNF1A	NM_002127	210515_at	1.63	1.51
CCND1	NM_053056	208712_at	1.35	0.81
AXIN2	NM_004655	222696_at	2.52	2.2
CD44	NM_000610	212063_at	1.14	1.36
BIRC5	NM_001012270	202094_at	0.93	0.66
ASCL2	NM_005170	207607_at	0.72	0.79
MSI1	NM_002442	206333_at	7.81	5.41
SOX9	NM_000346	202935_s_at	0.74	3.25
SOX2	NM_003106	213722_at	2.3	3.24
NANOG	NM_024865	220184_at	19.84	3.24
POU5F1	NM_002701	208286_x_at	1.7	0.78
WNT9A	NM_003395	230643_at	2.3	2.36
TWIST1	NM_000474	213943_at	5.66	1.35
EGFR	NM_005228	224999_at	0.97	0.89
FZD7	NM_003507	203705_s_at	3.01	1
EPCAM	NM_002354	201839_s_at	2.07	2.54
MYC	NM_002467	244089_at	1.05	1.98
NMYC	NM_005378	209757_s_at	23.09	47.22
JUN	NM_002228	201464_x_at	0.66	0.65
FGF9	NM_002010	206404_at	2.89	0.7
JAG1	NM_000214	209097_s_at	0.74	2.28
CDH1	NM_004360	201130_s_at	1.69	0.42
ABCB1	NM_000927	243951_at	2.63	1.77
RUNX2	NM_001015051	232231_at	1.32	4.78
CTNNB1	NM_001098209	201533_at	1.39	1.05
GSK3B	NM_001146156	209945_s_at	0.45	0.89
GSK3A	NM_019884	632_at	0.97	1.06
CREBBP	NM_001079846	228177_at	3.4	1.45
FZD1	NM_003505	204451_at	6.02	1.9
FZD3	NM_017412	227524_at	2.62	6.56
FZD8	NM_031866	224325_at	1.14	2.26
LRP5	NM_002335	209468_at	1.86	2.39
SFRP1	NM_003012	202035_s_at	0.48	0.32
DKK1	NM_012242	204602_at	0.45	0.98
MCAM	NM_006500	209087_x_at	0.14	0.23

Supplementary Table S3: shRNA used in CD146 knockdown

Name	Sequences	Respective cDNA locations
GFP shRNA	non-effective shRNA	nonsense sequence
shCD146 1	ATTCCTCAAGTCATCTGGT	506~524bp (190~196aa)
shCD146 2	GTTGAATCTGTCTTGTGAA	1299~1318bp (454~460aa)
shCD146 3	TGGCATTCAAGGAGAGGAA	1342~1360bp (468~474aa)
shCD146 4	GCTGGTTAAAGAAGACAAA	634~652bp(231~237aa)

Supplementary Table S4: Primers used in qRT-PCR

Gene	Sequences(5'-3')
MCAM (CD146)	Forward: TCAACGGCACGGCAAGTG Reverse: AGGCCGTGCATTCAACACC
CD44s	TCATAGAAGGGCACGTGGTG TGGGAGGTGTTGGATGTGAG
CD133	CTATTCAGGATATACTCTCAGCATT TTTCTGTGGATGTAACTTTCAGTG
CD166	CGT CTG CTC TTC TGC CTC TT TAG GTG CCT CAA ACA CGT TG
EpCAM	CGCAGCTCAGGAAGAATGTG TGAAGTACACTGGCATTGACG
ITGB1 (Integrin β1)	TTCAGTGAATGGCAACAATG AGCAACCACGCCTGCTAC
POU5F1 (Oct-4)	TTTAATCCCACATCATGTATCACT CTATCTACTGTGTCCCAGG
SOX2	GTATCAGGAGTTGTCAAGGCAGAG TCCTAGTCTTAAAGAGGCAGCAAAC
NANOG	CGATCTCCTGACCTTGT CACGCCTGTAAATCCCA
MYC (c-Myc)	CGGAACTCTTGTGCGTAAGG CTCAGCCAAGGTTGTGAGGT
BMI1	TTCGACCTTTGCAGATACCCATAAC TGCCAATTGCTTCTAATGGAACAG
AXIN2	AGTGTGAGGTCCACGGAAAC CTGGTGCAAAGACATAGCCA
MSI1	GGTTTCCAAGCCACAACCTA TCGGGGAACTGGTAGGTGTA
CCND1 (Cyclin D1)	CTGGAGGTCTGCGAGGAACA CCTTCATCTTAGAGGCCACGAA
HNF1A (TCF1)	AGGAGTGCAATAGGGCGGAATG CCGGTTGGCAAACCAGTTGTAG
LEF1	CGAAGAGGAAGGCGATTTAG CTGAGAGGTTTGTGCTTGTC
TWIST1	TGCCAATCAGCCACTGAAAG TTTGCAGGCCAGTTTGATCCC
SOX9	TGGGCAAGCTCTGGAGACTTC ATCCGGGTGGTCCTTCTTGTG
BIRC5 (Survivin)	AGAACTGGCCCTTCTTGGAGG CTTTTTATGTTCCTCTATGGGGTC
WNT9A	GCAAGATGCTGGATGGGTC GAGGATGGTCAGGGGCTC
E-cadherin	TGCCCAGAAAATGAAAAAGG GTGTATGTGGCAATGCGTTC
GAPDH	CAGCCTCAAGATCATCAGCA GTCTTCTGGTGGCAGTGAT

Supplementary Table S5: Primers used in ChIP assays

primers	Sequences(5'-3')
Pair 1	TTCGCTTTCTTCTCCCCACC GATTGGCTGGAAAACTCCGC
Pair 2	CGCTTTCTTCTCCCCACCTT ATTGGCTGGAAAACTCCGCT
Pair 3	TCGCTTTCTTCTCCCCACCT TTGGCTGGAAAACTCCGCT