Supplementary Material: Index of Cancer-Associated Fibroblasts Is Superior to the Epithelial–Mesenchymal Transition Score in Prognosis Prediction

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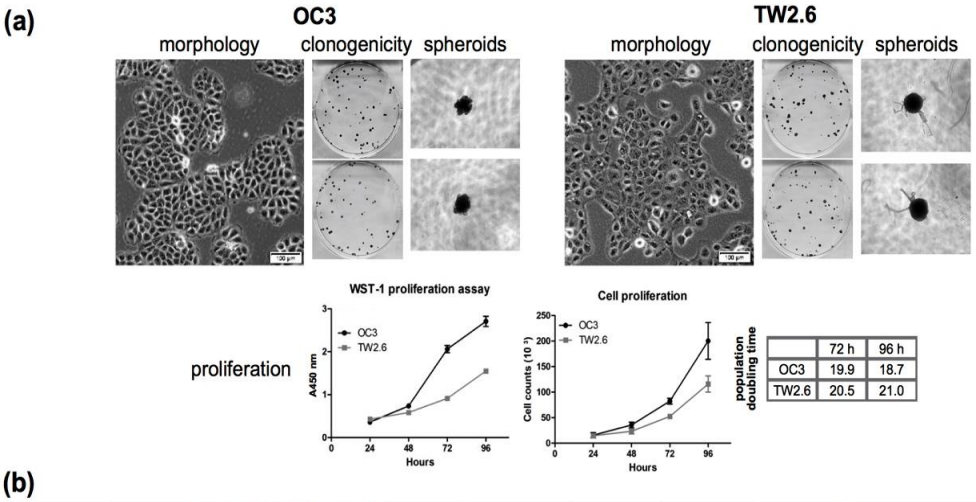
1. Supplementary Figures

(a)



	72 h	96 h
OC3	19.9	18.7
TW2.6	20.5	21.0

(b)

	PartekFlow_HISAT2_XenoFilteR					Xenome					
Sample ID	total	human	mouse	Others	Sample ID	total	human	mouse	both	ambiguous	neither
OC3-NOG01	41,471,895	28,460,414	12,190,173	821,308	OC3-NOG01	41,517,383	28,029,353	12,276,003	134,681	975,944	101,402
OC3-NOG03	42,313,589	31,209,536	10,385,464	718,589	OC3-NOG03	42,353,148	30,734,427	10,447,230	71,811	1,035,256	64,424
OC3-NOG04	39,963,604	26,740,316	12,424,179	799,109	OC3-NOG04	39,997,897	26,336,510	12,494,910	72,330	1,023,592	70,555
OC3-NOG05	44,982,542	30,080,198	14,064,061	838,283	OC3-NOG05	45,013,746	29,627,916	14,157,829	74,616	1,057,009	96,376
OC3-NOG06	44,498,707	20,495,914	22,898,066	1,104,727	OC3-NOG06	44,579,995	20,100,426	23,149,086	74,264	1,174,916	81,303
OC3-NOG08	18,991,855	13,476,414	5,163,847	351,594	OC3-NOG08	18,992,084	13,260,608	5,154,072	26,044	548,212	3,148
TW2.6-NOG07	45,739,782	40,595,127	4,437,510	707,145	TW2.6-NOG07	45,767,962	39,890,968	4,460,034	135,377	1,133,734	147,849
TW2.6-NOG08	44,509,967	37,819,003	6,059,922	631,042	TW2.6-NOG08	44,535,081	37,137,979	6,090,260	90,010	1,121,545	95,287
TW2.6-NOG09	46,540,933	37,969,664	7,838,264	733,005	TW2.6-NOG09	46,600,991	37,252,699	7,885,405	110,807	1,265,000	87,080
TW2.6-NOG10	39,921,892	34,221,142	4,654,397	1,046,353	TW2.6-NOG10	40,159,093	33,644,305	4,824,968	539,679	1,014,588	135,553
TW2.6-NOG14	17,375,551	15,151,224	1,998,324	226,003	TW2.6-NOG14	17,375,735	14,776,655	2,022,112	27,608	546,205	3,155

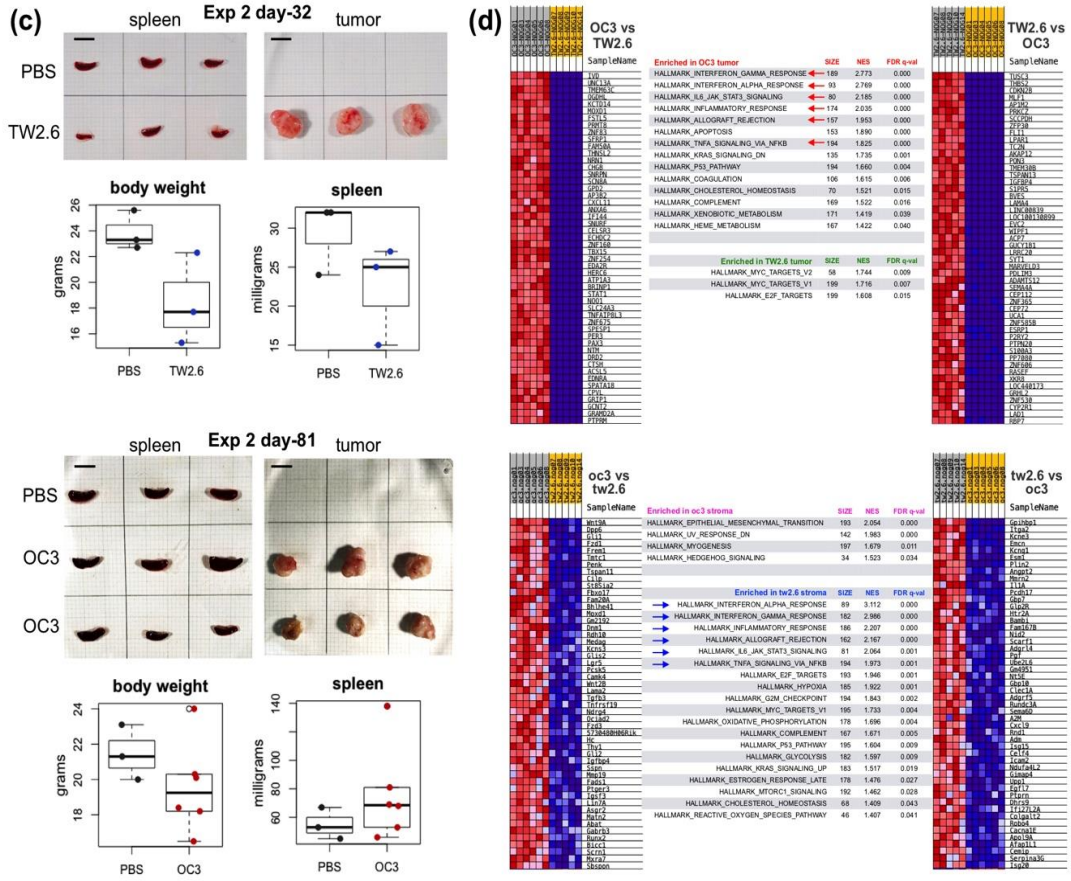


Figure S1. Supplementary information related to Figure 1. (**a**) Comparative analysis of morphology (scale bar = 100 μm), clonogenicity, spheroid formation, and proliferation between OC3 and TW2.6 cells grown in vitro; (**b**) Breakdown of RNA-seq reads generated from pipeline 1 (XenoFilteR) and pipeline 2 (Xenome); (**c**) Comparisons of spleens and tumors from control mice (PBS) with that from OC3-NOG (n = 6) and TW2.6-NOG (n = 3) in Exp 2. To reach ~500 mm^3 in tumor size, OC3-NOG were euthanized on day 81; TW2.6-NOG were euthanized on day 32. Scale bar, 1 cm. (**d**) Gene set enrichment analysis (GSEA) of tumor cell reads (upper) and murine stroma reads (lower). Heat maps show top 50 genes differentially expressed in OC3 tumor (upper left), TW2.6 tumor (upper right), OC3 stroma (lower left) and TW2.6 stroma (lower right). Expression values of high, moderate, low, and lowest are represented as red, pink, light blue, and dark blue, respectively [1]. Reports of enriched pathways for each analysis are listed in the middle. Pathways with FDR q-value > 0.05 were removed.

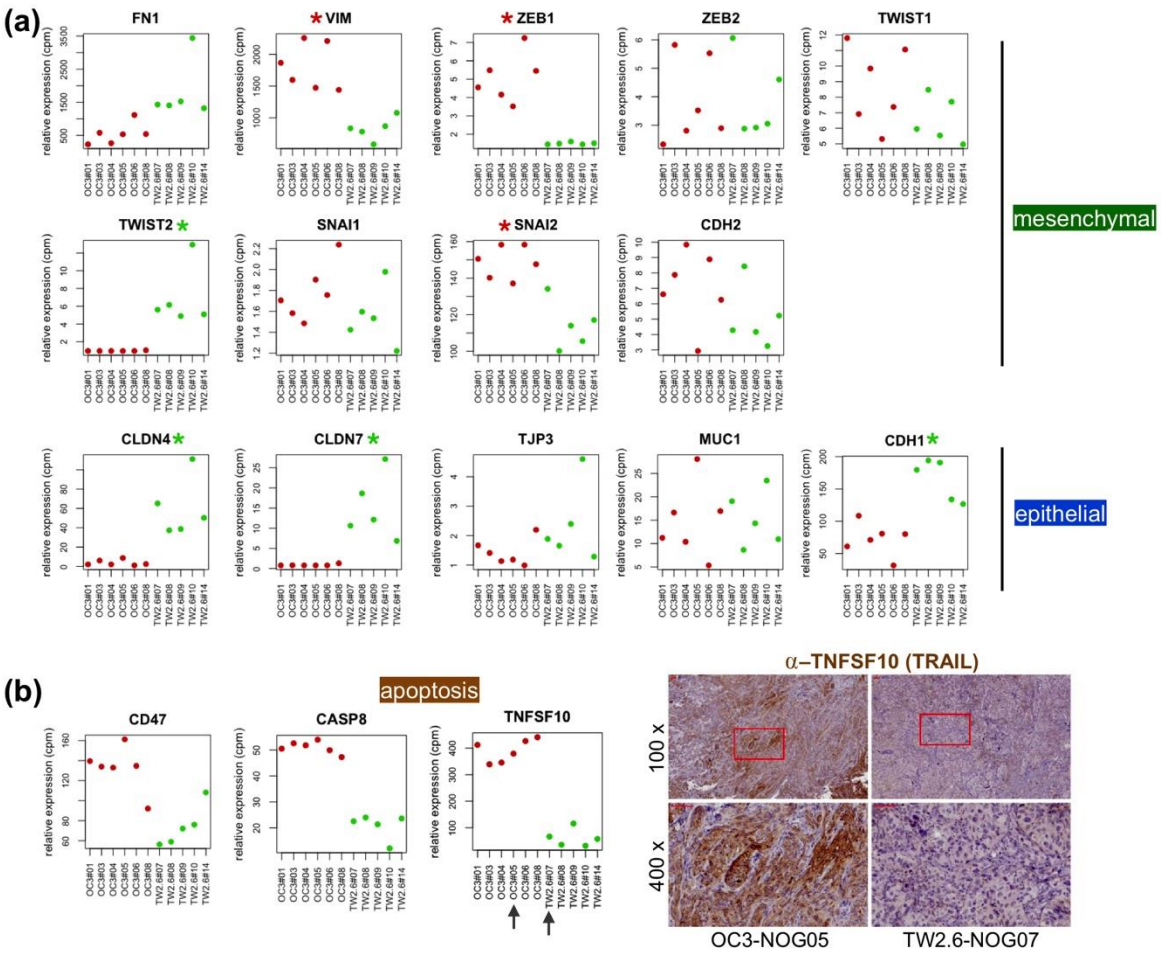
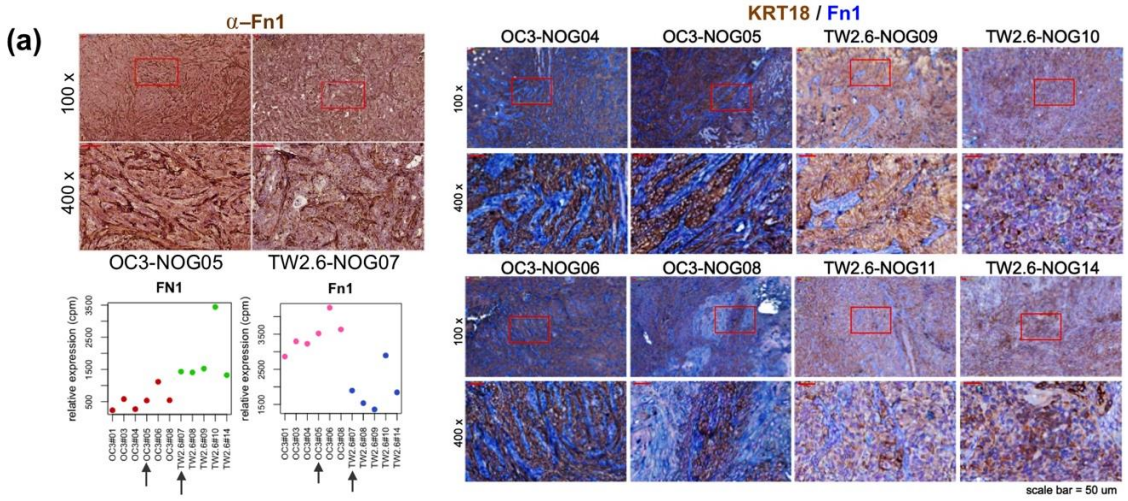
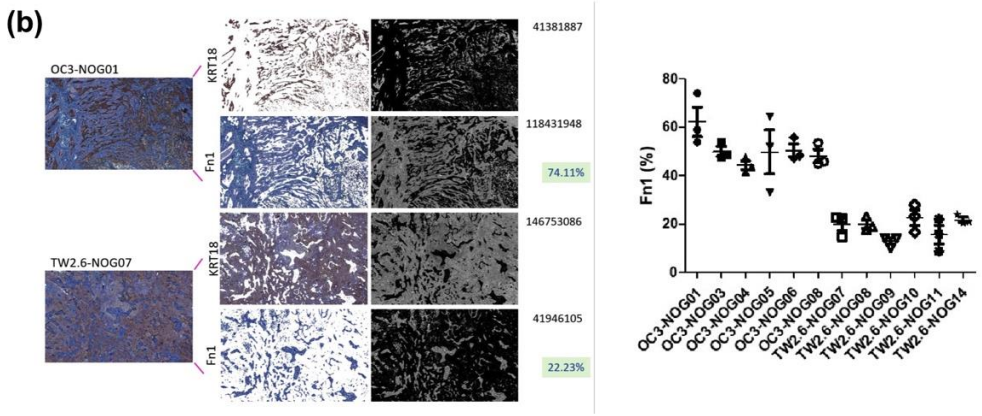
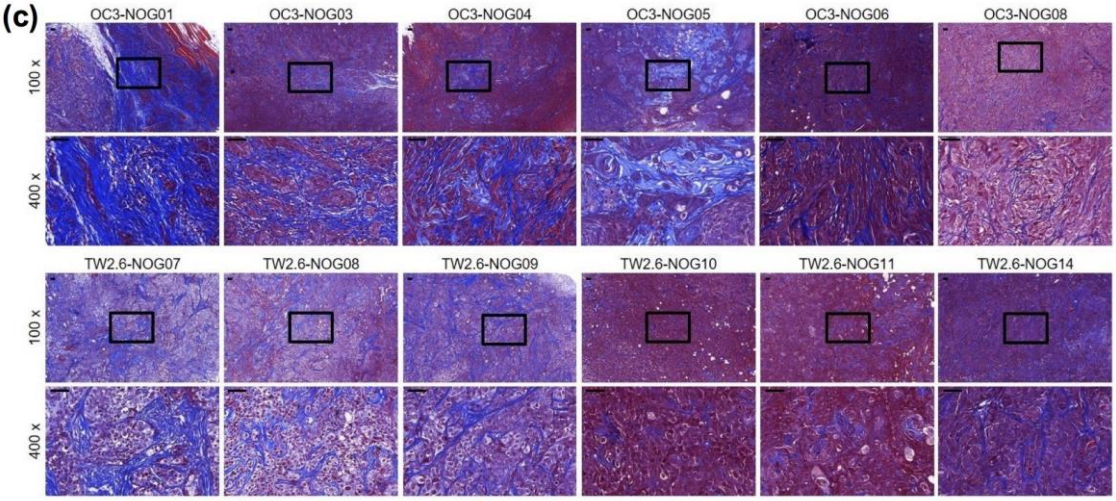
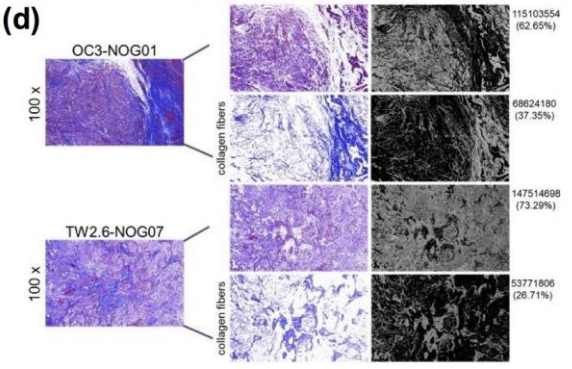


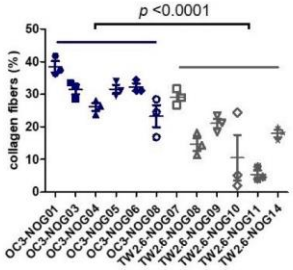
Figure S2. Supplementary information related to Figure 2. (**a**) Plots of 14 EMT related differentially expressed genes in OC3 and TW2.6 tumor cells, including mesenchymal genes (FN1, VIM, ZEB1, ZEB2, TWIST1, TWIST2, SNAI1, SNAI2, CDH2), and epithelial genes (CLDN4, CLDN7, TJP3, MUC1, CDH1); (**b**) Plots of apoptosis related DEGs (CD47, CASP8, TNFSF10) in tumor cells. Representative immunohistochemistry images of indicated xenograft sections stained for human TNFSF10 (brown). Scale bar, 50 μm.


(a)
α–Fn1
100 x
400 x
OC3-NOG05
TW2.6-NOG07
FN1
Fn1
relative expression (cpm)
KRT18 / Fn1
OC3-NOG04
OC3-NOG05
TW2.6-NOG09
TW2.6-NOG10
OC3-NOG06
OC3-NOG08
TW2.6-NOG11
TW2.6-NOG14
scale bar = 50 um


(b)
OC3-NOG01
TW2.6-NOG07
KRT18
Fn1
41381887
118431948
74.11%
146753086
41946105
22.23%
Fn1 (%)
OC3-NOG01
OC3-NOG03
OC3-NOG04
OC3-NOG05
OC3-NOG06
OC3-NOG08
TW2.6-NOG07
TW2.6-NOG08
TW2.6-NOG09
TW2.6-NOG10
TW2.6-NOG11
TW2.6-NOG14


(c)
OC3-NOG01
OC3-NOG03
OC3-NOG04
OC3-NOG05
OC3-NOG06
OC3-NOG08
TW2.6-NOG07
TW2.6-NOG08
TW2.6-NOG09
TW2.6-NOG10
TW2.6-NOG11
TW2.6-NOG14
100 x
400 x


(d)
OC3-NOG01
TW2.6-NOG07
collagen fibers
115103554
(62.65%)
68624180
(37.35%)
147514698
(73.29%)
53771806
(26.71%)


p <0.0001
collagen fibers (%)
OC3-NOG01
OC3-NOG03
OC3-NOG04
OC3-NOG05
OC3-NOG06
OC3-NOG08
TW2.6-NOG07
TW2.6-NOG08
TW2.6-NOG09
TW2.6-NOG10
TW2.6-NOG11
TW2.6-NOG14

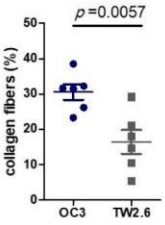
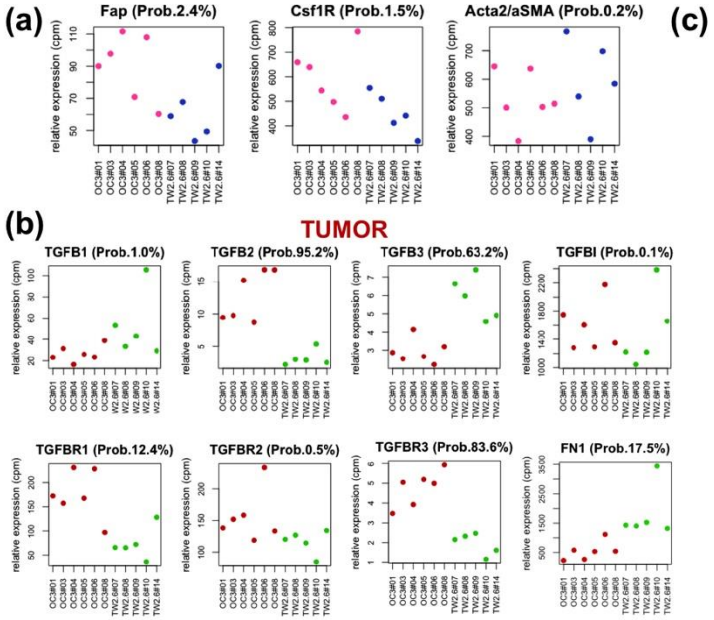

p =0.0057
collagen fibers (%)
OC3
TW2.6

Figure S3. Supplementary information related to Figure 3. (**a**) (left) Representative immunohistochemistry images of indicated tissue sections stained for murine Fn1 (brown). Scale bars, 50 μm. Plots denote expression levels (cpm) of human FN1 and murine Fn1 in OC3 and TW2.6 CDXs. Arrows indicate samples used for IHC staining experiments. (right) Representative IHC images of indicative sections co-stained with human KRT18 (brown) and murine Fn1 (blue). (**b**) Quantitative analysis of dual IHC staining depicted in (**a**). Note that TW2.6-NOG11 was not included in RNA-seq analysis. (**c**) Representative Masson's trichrome stain of OC3 and TW2.6 sections (collagen: blue; cytoplasm: pink; nuclei: dark brown). Scale bar, 50 μm. (**d**) Quantification of Masson's trichrome stain depicted in (**c**). Results show mean ± SEM of three 100x magnification fields from each section.



(c)

Covariate	HR (95% CI for HR)	p.value
LNmeta	1.8 (0.65-5)	0.26
AngioLymphatic invasion (ALI)	1.9 (0.67-5.3)	0.23
EMT_Score	**4.2 (1.3-14)**	**0.015***
TGFB1	0.65 (0.23-1.8)	0.41
TGFB2	**4.9 (1.4-17)**	**0.015***
TGFB3	0.89 (0.32-2.5)	0.83
TGFBI	**11 (2.4-50)**	**0.0021****
TGFBR1	1.4 (0.49-3.8)	0.55
TGFBR2	1.3 (0.49-3.7)	0.57
TGFBR3	0.56 (0.2-1.6)	0.27
FN1	1.8 (0.63-5)	0.28

NCKU-OrCA-40T (GSE37991)

Figure S4. Supplementary information related to Figure 4. (**a**) Plots denote expression of genes conventionally attributed to activated fibroblasts (Fap, Csf1R) and myoblasts (Acta2) in the stroma of OC3 and TW2.6 CDXs. (**b**) Plots denote RNA-seq reads of human TGF-β related genes and FN1 in OC3 and TW2.6 CDXs. (**c**) Cox proportional hazard ratios of two clinical features (LN meta and angiolymphatic invasion), EMT score, indicated TGF beta-axis genes, and FN1 for the 40 OSCC patients in the NCKU-OrCA-40TN cohort (GSE37991).

2. Supplementary Materials and Methods

Text S1: Clonogenic Survival Assay

Cells were cultured in six-well plates (100 cells per well) for 10 to 14 d. Cells were fixed with 70% ethanol for 30 min at 4 °C, stained with 1% crystal violet for 30 min, and rinsed with water thoroughly. The stained colonies (>50 cells) were counted under a microscope. Colony count for each treatment was presented as mean ± SD derived from quadruple wells.

Text S2: Spheroid Invasion Assays

The Cultrex 96 Well 3D Spheroid BME Cell Invasion Assay kit (#3500-096-K; Trevigen, Gaithersburg, MD, USA) was used according to the manufacturer's protocol. In each assay, 1,500 OC3 or TW2.6 cells resuspended in spheroid formation solution were seeded in a 96-well round bottom plate to form aggregated spheroids (72 h), followed by adding invasion matrix (72 h). The bright-field spheroids images were captured by using an Olympus IX73 microscope.

Text S3: Cell Proliferation Assays

Cell proliferation was determined by using a WST-1 assay (Roche, Indianapolis, IN, USA) or trypan blue exclusion method (Figure S1a). In WST-1 assay, 2000 cells per well were seeded in a 96-well plate and WST-1 proliferation assay was performed on the following 4 consecutive days. 10 μL of WST-1 reagent was added to each well containing 100 μL of medium. Four hours after incubation at 37 °C, the microplate was gently mixed for 1 min and the absorbance was measured by microplate reader Spectramax 250 (Molecular Devices, Hampton, NH, USA) at 450 nm with a reference wavelength at 630 nm. In parallel, for each sample at each time point, 5000 cells per well were seeded in a 48-well plate, followed by trypan blue exclusion assay to determine cell viability.

Reference

1. Subramanian, A.; Tamayo, P.; Mootha, V.K.; Mukherjee, S.; Ebert, B.L.; Gillette, M.A.; Paulovich, A.; Pomeroy, S.L.; Golub, T.R.; Lander, E.S.; et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci.* **2005**, *102*, 15545–15550, doi:10.1073/pnas.0506580102.