Supplementary Material

Low levels of Sox2 are required for melanoma rumor-repopulating cell dormancy

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Supplementary Material

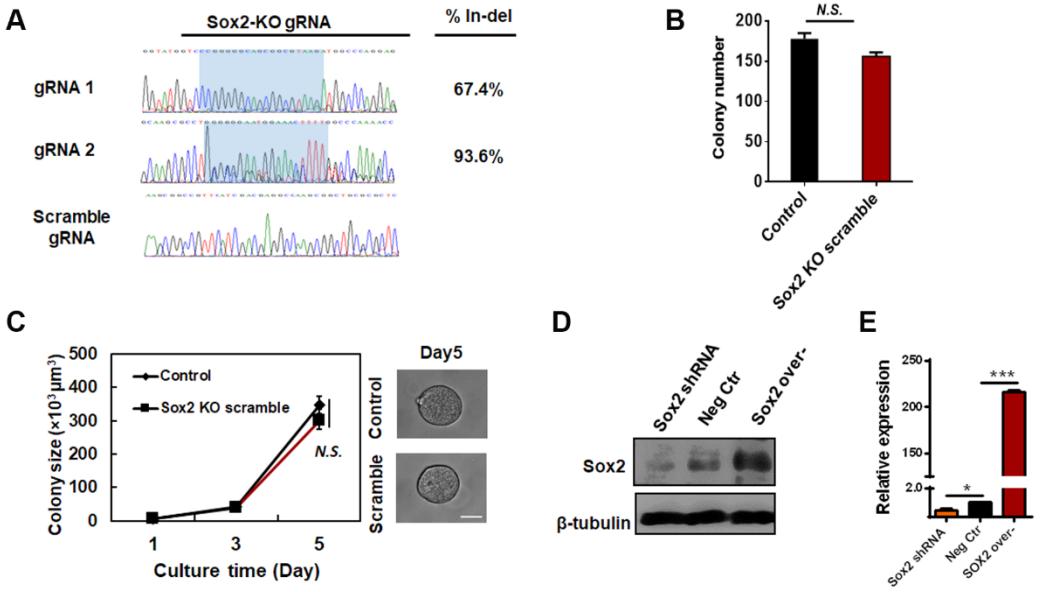

Figure S1. **Generation of Sox2 KO cell lines and transfection efficiency of Sox2 shRNA and overexpression. (A)** Genomic DNA was purified and the targeted loci analyzed by Sanger sequencing. The highlighted region indicates 20 nucleotides of the sequence recognized by two independent Sox2-knockout gRNAs and a scramble gRNA. Percent in-del (insertion-deletion) formation was estimated using TIDE analysis. **(B, C)** Colony numbers and sizes of control TRCs and Sox2 KO scramble TRCs were measured. Mean ± s.e.m.; n =15 samples; three independent experiments; N.S., not statistically significant. **(C)** Representative bright field images (right) of colonies were taken on day 5. Scale bar, 50 μm. **(D)** Cells were transfected with scramble shRNA (negative control), Sox2 shRNA, or Sox2 overexpression plasmid, and cultured in 90-Pa fibrin gels for 5 days, and then Sox2 expression was detected by western blot analysis. **(E)** Sox2 expression was quantified by real-time PCR. Mean ± s.e.m; n = 3; *P < 0.05, ***P < 0.001.

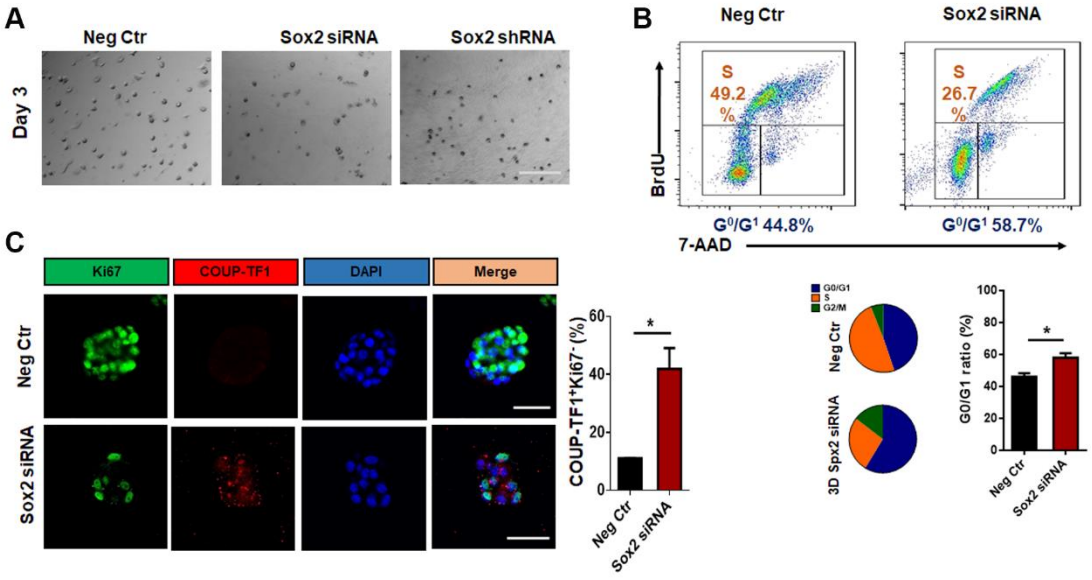

Figure S2. Sox2 knockdown using siRNA partially induces cycle arrest and increasing of dormancy marker. (A) Representative images of colonies in different conditions. Cells were transfected with negative control, Sox2 siRNA, or Sox2 shRNA, and then placed into 3D fibrin gels for three days. Scale bar, 500 μm. **(B)** Cell cycles were analyzed by flow cytometry after seeding cells in 90-Pa fibrin gels for 5 days. Quantification of cell cycle analysis. Mean ± s.e.m; n = 3 independent experiments; *P < 0.05. **(C)** Representative images (left) and quantification (right) of double immunostaining of COUP-TF1 and Ki67 under various conditions. Scale bar, 50 μm. Mean ± s.e.m; n = 10 randomly chosen view-fields from two different experiments; *P < 0.05.

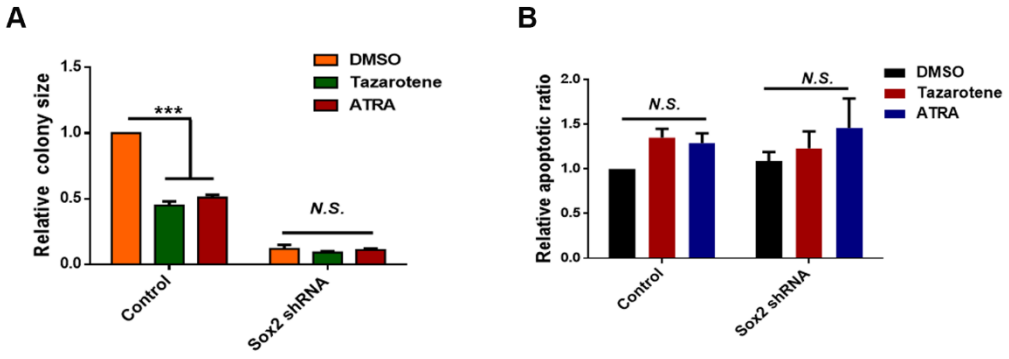

Figure S3. Melanoma cells with low levels of Sox2 do not respond to cancer drugs. Control B16 cells and B16 cells transfected with Sox2 shRNA were treated with 0.1% DMSO, Tazarotene (100 μM), or all-trans retinoic acid (ATRA) (100 μM) for 3 days in 90 Pa fibrin gels. **(A)** Relative colony size was measured by comparing the colony sizes with those from the control DMSO group, which were set to 1. **(B)** Cell apoptotic ratio was measured by flow cytometry. Mean ± s.e.m.; three independent experiments; $^{***}P <$ 0.001, *N.S.*, not statistically significant.

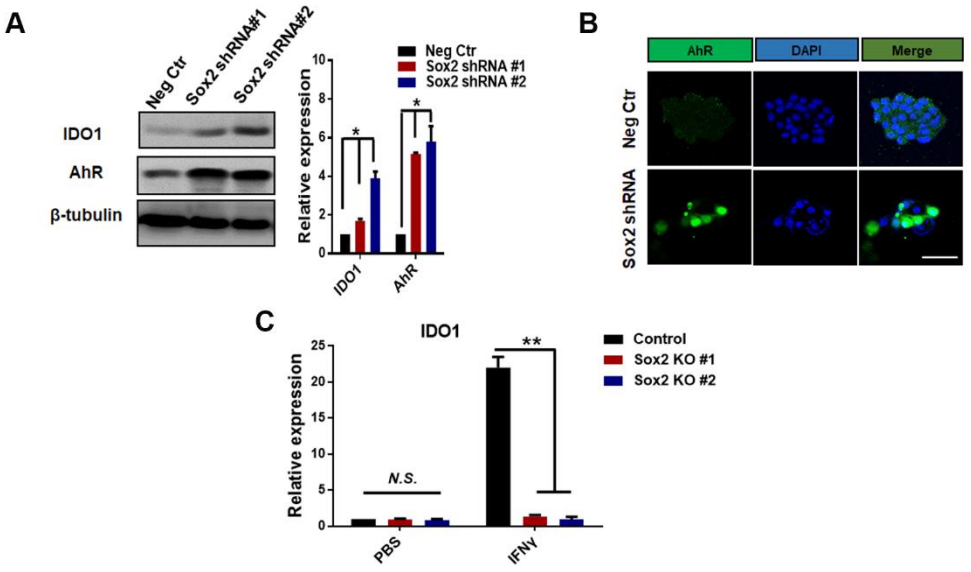

Figure S4. Silencing Sox2 in TRCs induces activation of IDO1-AhR cascade. B16 cells transfected with Sox2 shRNA#1, shRNA#2, or negative control were cultured in 90 Pa fibrin gels for 5 days. (**A**) Representative images and quantification of IDO1 or AhR expression by western blotting. (**B**) Representative images of immunostaining of AhR translocation. Scale bar, 50 μm. Two different experiments showed similar results. (**C**) Quantification of IDO1 expressions by western blotting. Mean ± s.e.m.; three independent experiments; $^{*}P < 0.05$; $^{**}P < 0.01$.

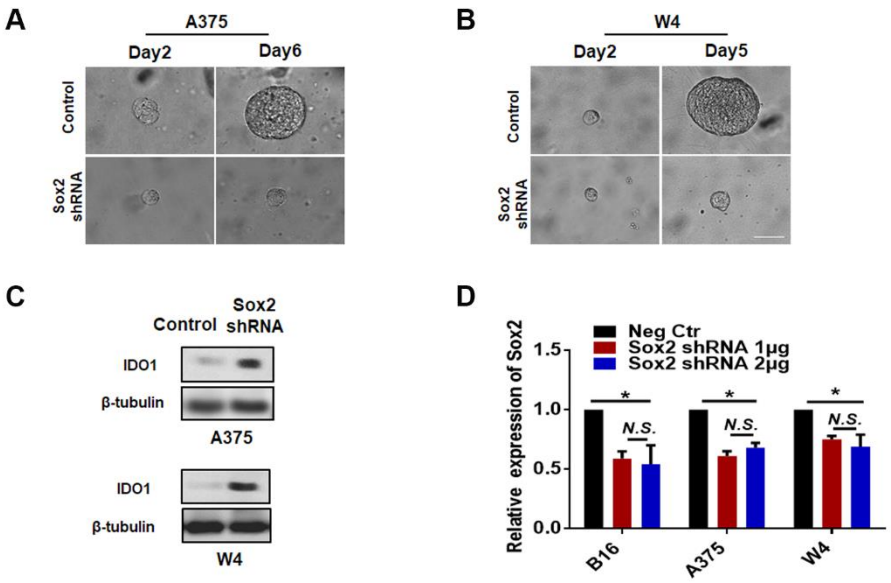

Figure S5. Silencing Sox2 induces dormancy in embryonic stem cells and human melanoma cells. The human malignant melanoma cell line A375 and murine embryonic stem cell line W4 were transfected with Sox2 shRNA or scrambled shRNA (negative control). The cells were cultured in 90-Pa fibrin gels or on 2D rigid plastic for 5 or 6 days. **(A and B)** Brightfield images of colonies on days 2, 5, or 6. Scale bar, 50 μm. **(C)** Representative images of IDO1 by western blotting. **(D)** Total mRNAs were extracted for quantitative analysis of Sox2 expression by real-time PCR. Mean ± s.e.m.; three independent experiments; $^{*}P < 0.05$; $^{**}P < 0.01$.

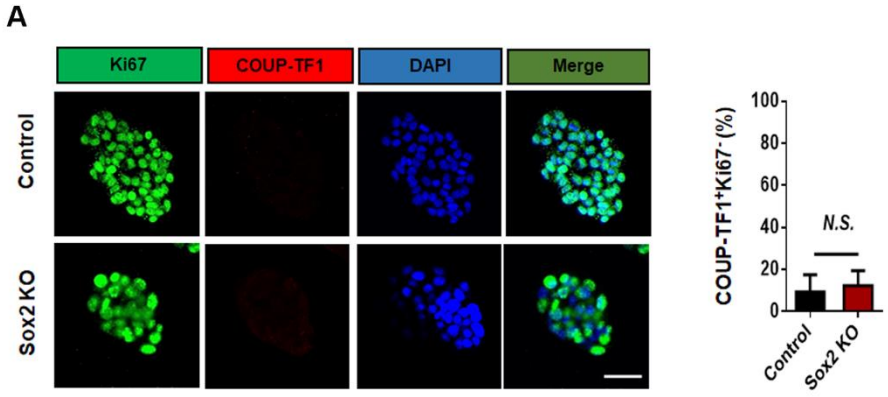

Figure S6. Knockout of Sox2 does not induce the increasing of dormancy marker.

(A) Representative images (left) and quantification (right) of double immunostaining of COUP-TF1 and Ki67 for control TRCs or Sox2 KO TRCs. Scale bar, 50 μm. Mean ± s.e.m; n = 10 randomly chosen view-fields from two different experiments; N.S., not statistically significant.

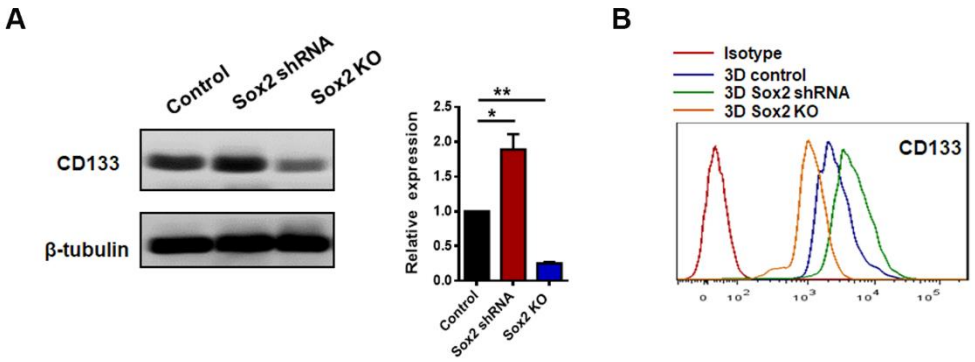

Figure S7. CD133 expression decreases in Sox2 KO TRCs and slightly increases in Sox2 shRNA TRCs. (A) B16 cells transfected with Sox2 shRNA, control B16 cells, and Sox2 KO cells were cultured in 90-Pa fibrin gels for 5 days, and then the proteins were extracted for western blot analysis of CD133 expression. Left, representative blotting images; Right, quantitative data. Mean ± s.e.m; n = 3; *P < 0.05, **P < 0.01. **(B)** The expression of CD133 was analyzed by flow cytometry. Data are representative of three independent experiments.

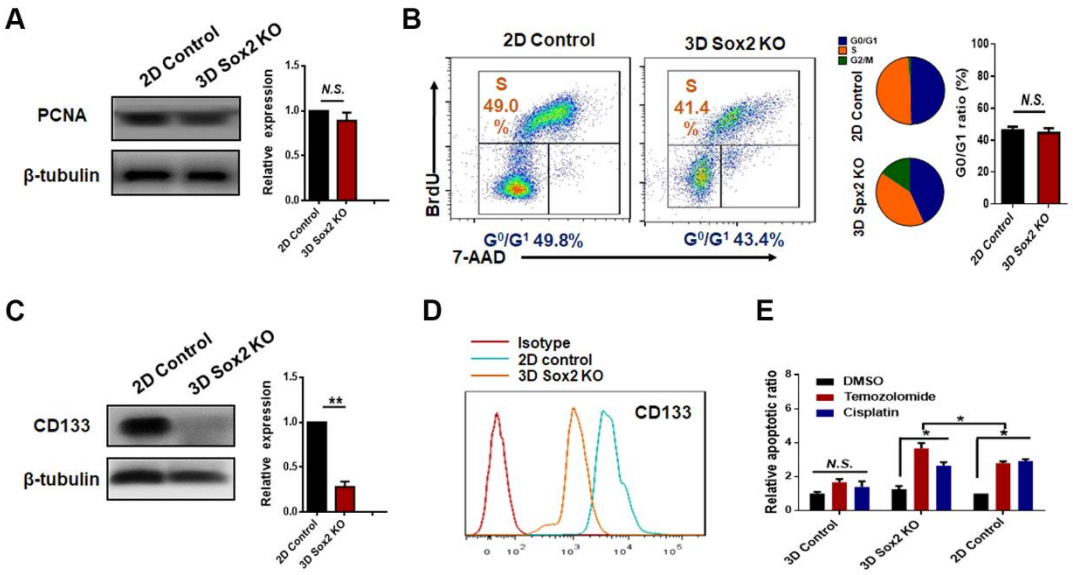

Figure S8. Sox2 KO TRCs and 2D control cells exhibit similar proliferation patterns but different CD133 expression or drug resistance. Sox2 KO cells were cultured in 90-Pa fibrin gels for 5 days and 2D B16 control cells were cultured on rigid plastic 6-well plates. **(A)** The proteins were extracted for western blot analysis of PCNA expression. Left, representative blotting images; Right, quantitative data. Mean ± s.e.m; n = 3; N.S., not statistically significant. **(B)** Cell cycles were analyzed by flow cytometry. Left, representative images; Right, quantitative data. Mean ± s.e.m; n = 3; N.S., not statistically significant. **(C)** The proteins were extracted for western blot analysis of CD133 expression. Left, representative blotting images; Right, quantitative data. Mean ± s.e.m; n = 3; **P < 0.01. **(D)** The expression of CD133 was analyzed by flow cytometry. Data are representative of three independent experiments. (E) Cells were cultured for 48 h and then treated with 0.1% DMSO, Temozolomide (500 μM), or Cisplatin (100 μM). Cell apoptotic ratio was measured by flow cytometry. Mean ± s.e.m.; n = 3 independent experiments. N.S., not statistically significant. *P < 0.05.

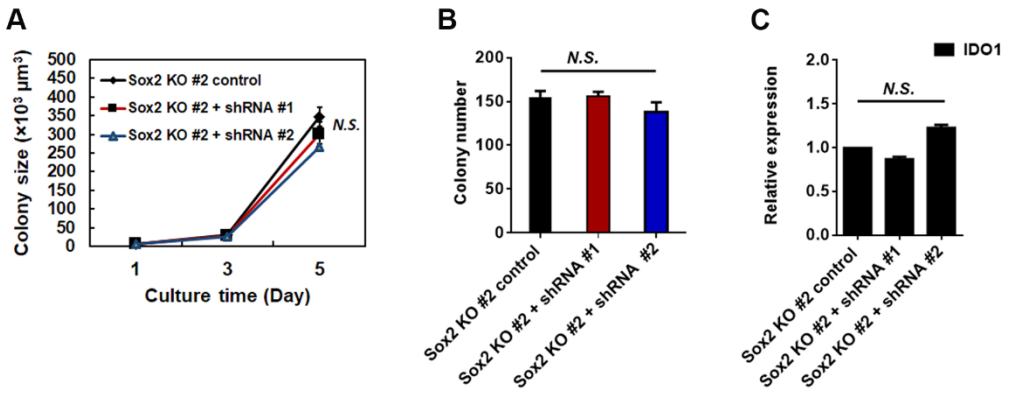

Figure S9. Silencing of Sox2 in Sox2 KO cell line does not induce dormancy. Sox2 KO cells were transfected with Sox2 shRNA#1, Sox2 shRNA#2, or negative control, and then seeded in 90-Pa fibrin gels for 5 days. **(A)** Colony size was measured for 5 days. **(B)** Colony numbers were counted on day 5. Mean ± s.e.m.; three independent experiments, *N.S.*, statistically significant. **(C)** Total mRNAs were extracted for quantitative analysis of IDO1 expression by real-time PCR. Mean ± s.e.m.; three independent experiments, *N.S.*, not statistically significant.

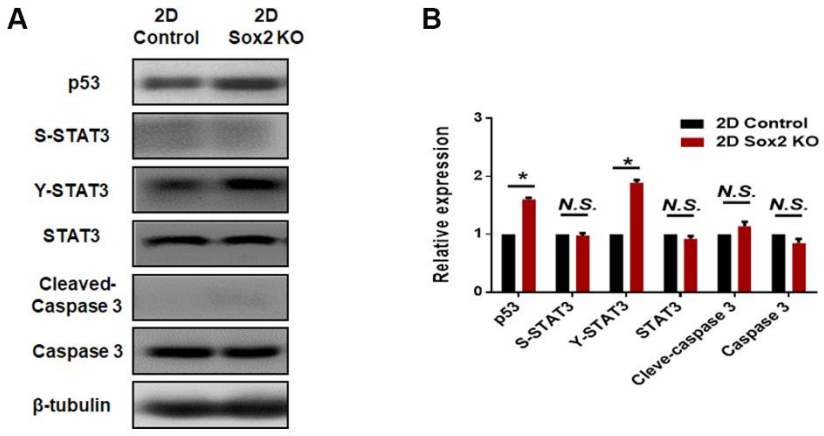

Figure S10. Knockout Sox2 on 2D rigid dishes does not activate STAT3/p53-caspase 3 pathway. (A) Western blotting analysis of p53, S-STAT3 (phospho-S727), Y-STAT3 (phospho-Y705), total STAT3, Cleaved Caspase 3, or Caspase 3 expression protein levels in control B16 cells or Sox2 KO cells by western blotting, three independent experiments. Representative blotting images. **(B)** Quantitative data of western blotting. Mean ± s.e.m; n = 3; N.S., not statistically significant. $^{*}P < 0.05$.

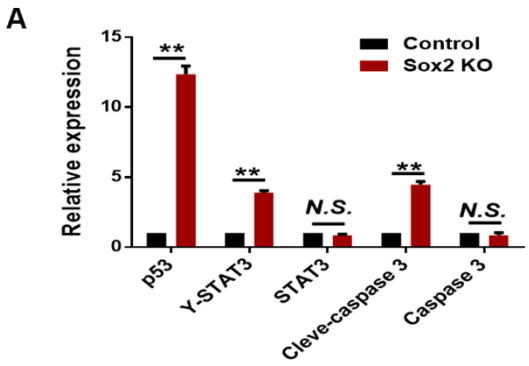

Figure S11. Quantification of p53, Y-STAT3, STAT3, Cleave-caspase 3, and Caspase 3 expression by western blotting. Melanoma tissues were collected and lysed for protein analysis. Mean ± s.e.m.; three independent experiments; $^{**}P < 0.01$, *N.S.*, not statistically significant.

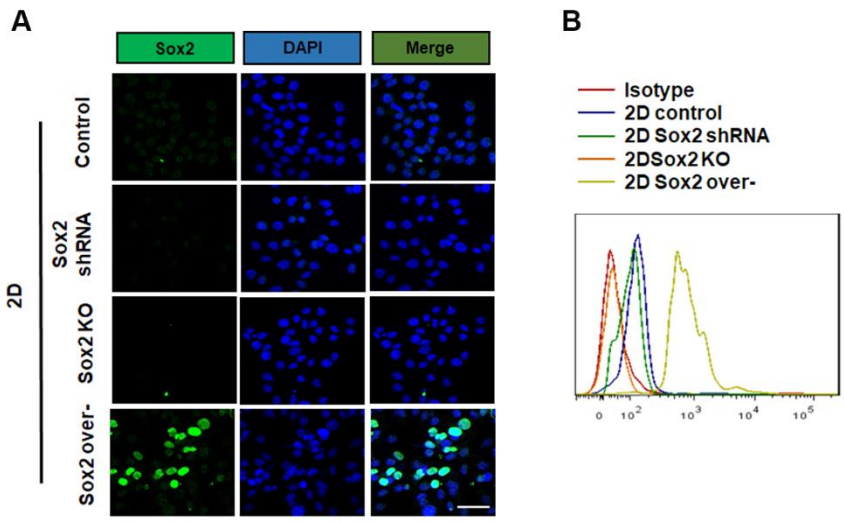

Figure S12. Sox2 expression of 2D cells under different conditions by flow-cytometry and immunofluorescence. (A) Representative images of immunostaining of Sox2 for 2D control cells, cells transfected with Sox2 shRNA, Sox2 KO cells, or cells transfected with Sox2 overexpression plasmid. Scale bar, 50 μm. Mean ± s.e.m; n = 10 randomly chosen view-fields from two different experiments. **(B)** The expression of Sox2 was analyzed by flow cytometry. Data are representative of three independent experiments.

Table S1 Sequences of primers for qPCR.

Genes		Real time PCR
Mus IDO1	5′ primer	GAGGATGCGTGACTTTG
	3′ primer	ATCAAGACTCTGGAAGATGCTG
Mus p53	5′ primer	CAAGTGAAGCCCTCCGAGTGT
	3′ primer	AGCCATAGTTGCCCTGGTAAGT
Mus p27	5′ primer	TCAAACGTGAGAGTGTCTAACG
	3′ primer	CCGGGCCGAAGAGATTTCTG
Mus p21	5′ primer	CCTGGTGATGTCCGACCTGTT
	3′ primer	CGCAACTGCTCACTGTCCAC
Mus Sox2	5′ primer	CAAGACGCTCATGAAGAAGG
	3′ primer	AGTGGGAGGAAGAGGTAACC
Mus GAPDH	5′ primer	GGAATGACGAGCACACGAGAC
	3′ primer	GCTGAACACTCCATGTACCCAA
Mus p53 promoter	5′ primer	CCAGCTTTGTGCCAGGAGTCT
	3′ primer	CAGCCAGGATGGTCCCAATGA
Mus p27 promoter	5′ primer	CACCGCCATATTGGGCAACTAA
	3′ primer	GTGAGTCGGGACAAGGGATGA

Table S2 Sequences of shRNAs.

shRNA	Sense strand sequences
Sox2 shRNA #1	GTATAACATGATGGAGACGGAGCTGAAGC
Sox2 shRNA #2	AGACGCTCATGAAGAAGGATAAGTACACG
Scramble shRNA	GCACTACCAGAGCTAACTCAGATAGTACT

Table S3 Sequences of gRNAs.

shRNA	Sense strand sequences
Sox2 gRNA #1	CCGGGGGCAGCGGCGTAAGA
Sox2 gRNA #2	AAAGTTTCCACTCCGCGCCC