Supporting Materials and Methods

Activation of FXR suppresses esophageal squamous cell carcinoma through antagonizing ERK1/2 signaling pathway

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IC50 detection

IC50 of KYSE150 and EC109 was assessed by MTT assay. Cells were seeded into 96-well plate with 4 parallel wells in one group, and cells were treated by GW4064 at different concentrations. The absorbance at 450 nm was detected after 48h of GW4064 treatment. SPSS software was used to calculate IC50.

Cell proliferation assay

Cell proliferation of KYSE150 and EC109 was assessed by MTT assay. Cells were seeded into 96-well plate with 4 parallel wells in one group, and cells were treated by GW4064 at concentrations of 0.5 μM, 1 μM and 1.5 μM. The cell proliferation was examined every 24 h. The number of viable cells was assessed by measurement of the absorbance at 450 nm.

Animals

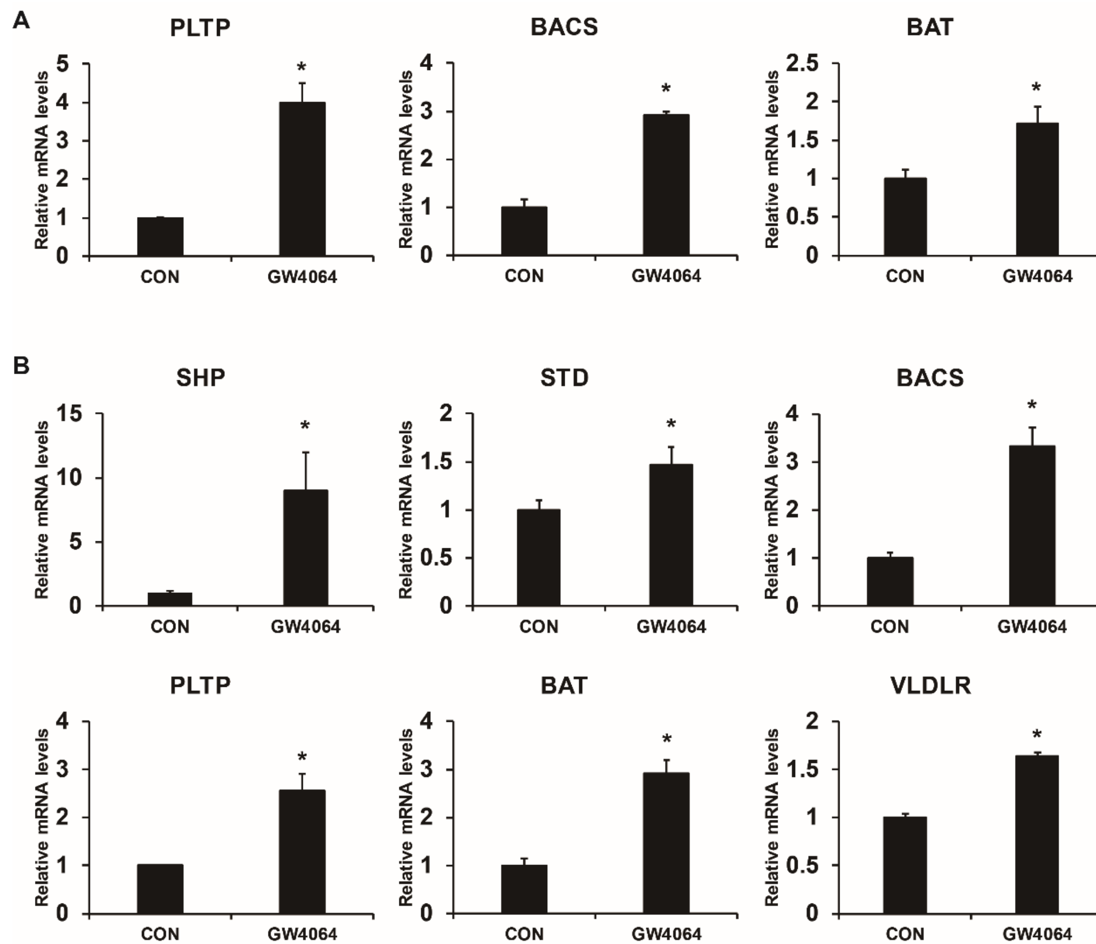
C57BL/6 WT and $FXR^{-/-}$ mice male siblings at 8 weeks of age were intraperitoneally (i.p.) injected with a single dose of 30 mg/Kg GW4064 once a day. On day 2th of treatment, 4 hours after the intraperitoneal injection, mice were sacrificed.

For *in vivo* tumorigenesis assay, Male BALB-C nude mice (4 weeks of age) were each injected subcutaneously in the left forelimb armpit with EC109 cells (4×10^6 cells per mouse, n=6-8) in a total volume of 150 μL. GW4064 was intraperitoneally injected once every three days in a concentration 1 mg/Kg, 3 mg/Kg and 30 mg/Kg (body weight). The mice were sacrificed after 7th measurement（30 days）and the tumors were removed and weighed.

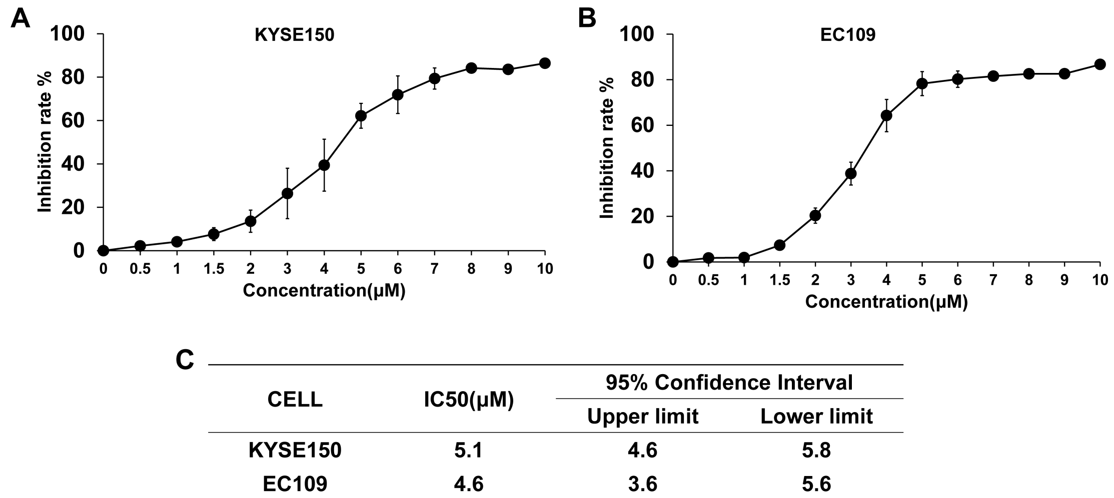
Immunohistochemistry Staining

For immunohistochemistry, a rabbit-anti-FXR antibody (diluted 1:500, Abcam) was used to evaluate the level of FXR. Tumor specimens used in the staining were treated with the principles of the Department of Pathology, Sino-Japanese Friendship

Hospital, and all of the staining methods were executed using the manufacturer's specifications.

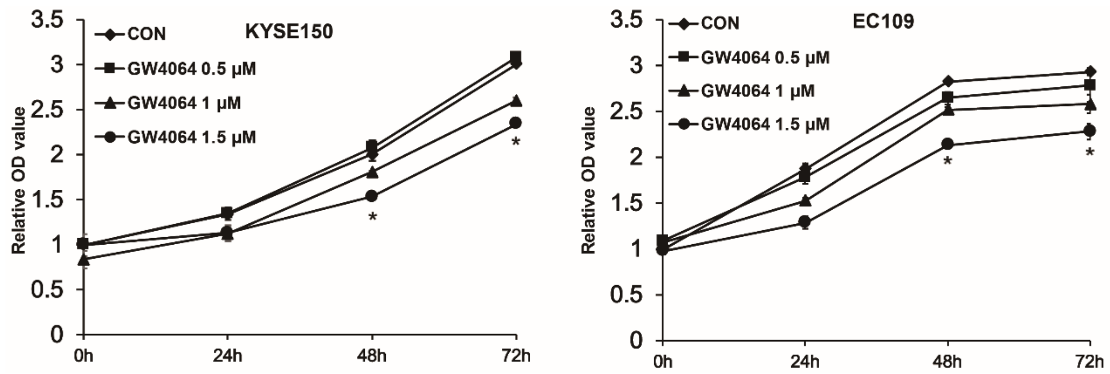


Supplementary Fig. 1. GW4064 induced FXR-related target genes in ESCC cells. (A) GW4064 induced the expression of PLTP, BACS, and BAT in KYSE150 cells. *P < 0.05. (B) GW4064 upregulated FXR-related target genes SHP, STD, BACS, PLTP, BAT, and VLDLR in EC109 cells. *P < 0.05.

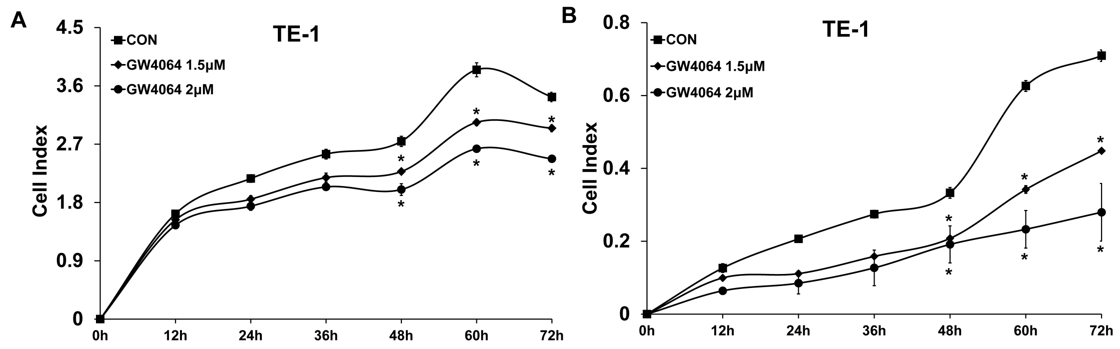


CELL	IC50(μM)	95% Confidence Interval	
		Upper limit	Lower limit
KYSE150	5.1	4.6	5.8
EC109	4.6	3.6	5.6

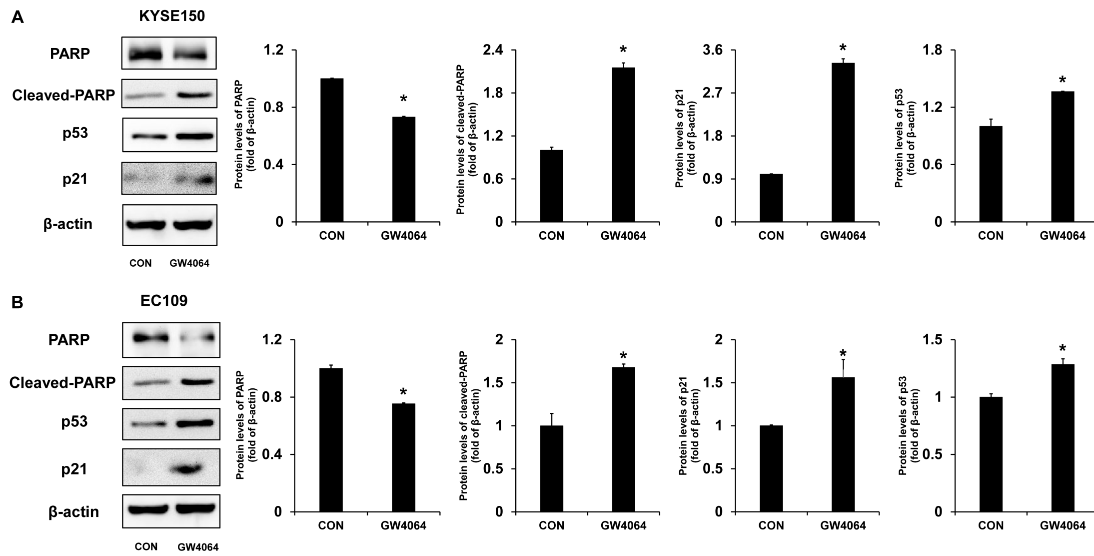
Supplementary Fig. 2. IC50 values of GW4064 against KYSE150 and EC109cell lines. (A) Inhibition curve of KYSE150 cells treated with different concentrations of GW4064 for 48 hours. (B) Inhibition curve of EC109 cells treated with different concentrations of GW4064 for 48 hours. (C) IC50 analysis of GW4064 against KYSE150 and EC109 cell lines.



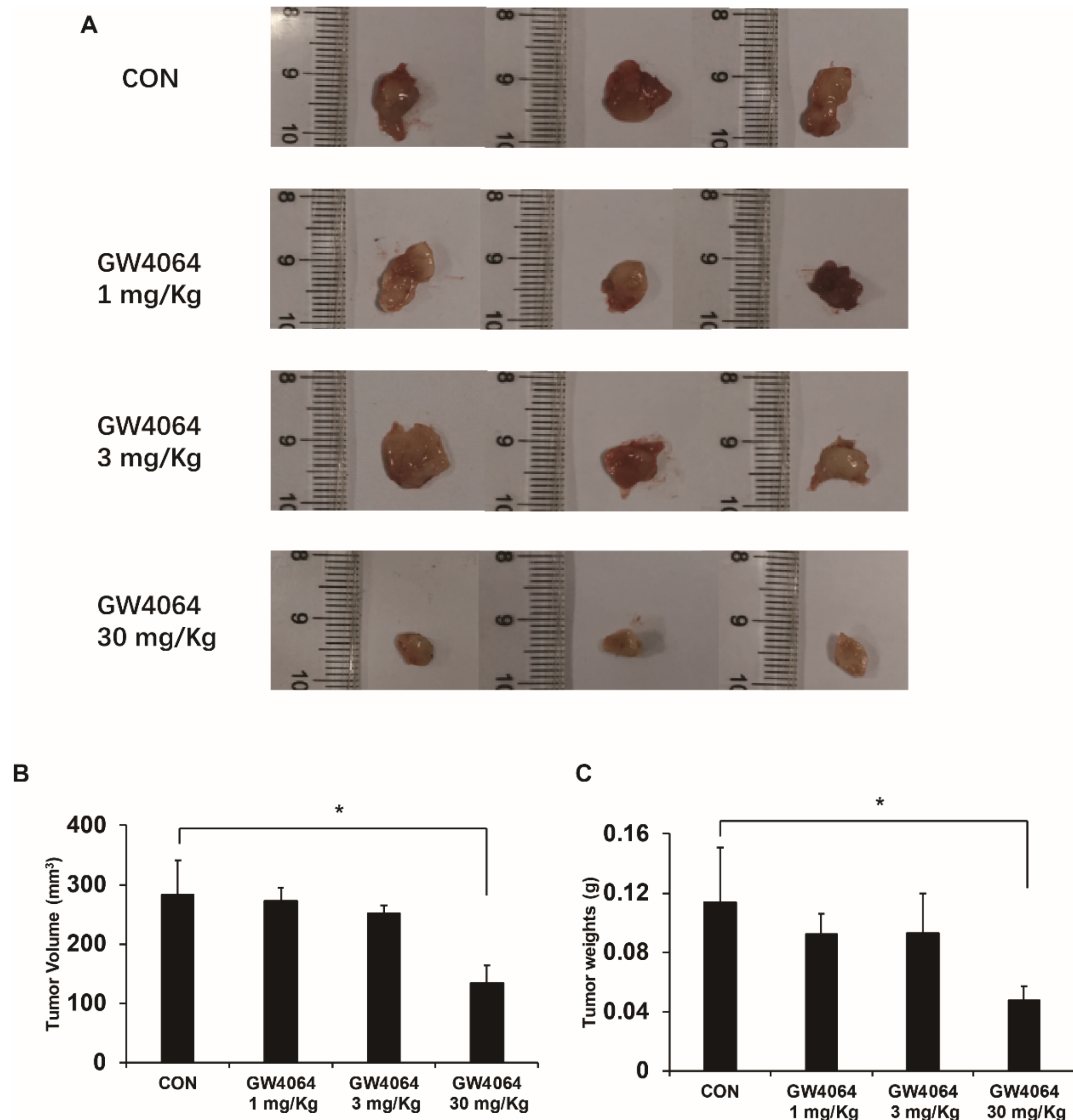
Supplementary Fig. 3. FXR activation by GW4064 showed concentration-dependent inhibitory effect on proliferation of ESCC cells (n=3). *P < 0.05 versus the control groups.



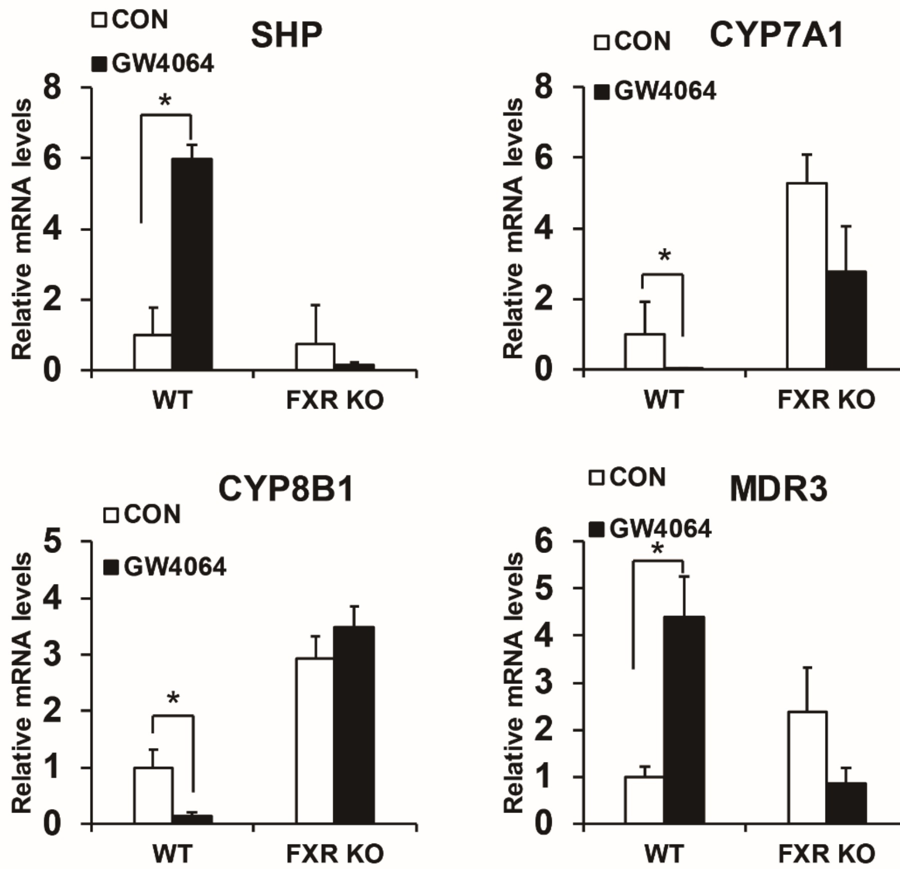
Supplementary Fig. 4. FXR agonist GW4064 impaired proliferation and migration of TE-1 cells. (A) GW4064 inhibited proliferation of TE-1 cells. Proliferation assay was performed using Real Time Cellular Analysis (RTCA). *P < 0.05. (n=5). (B) GW4064 inhibited proliferation of TE-1 cells. Migration assay was performed using Real Time Cellular Analysis (RTCA). *P < 0.05. (n=5).



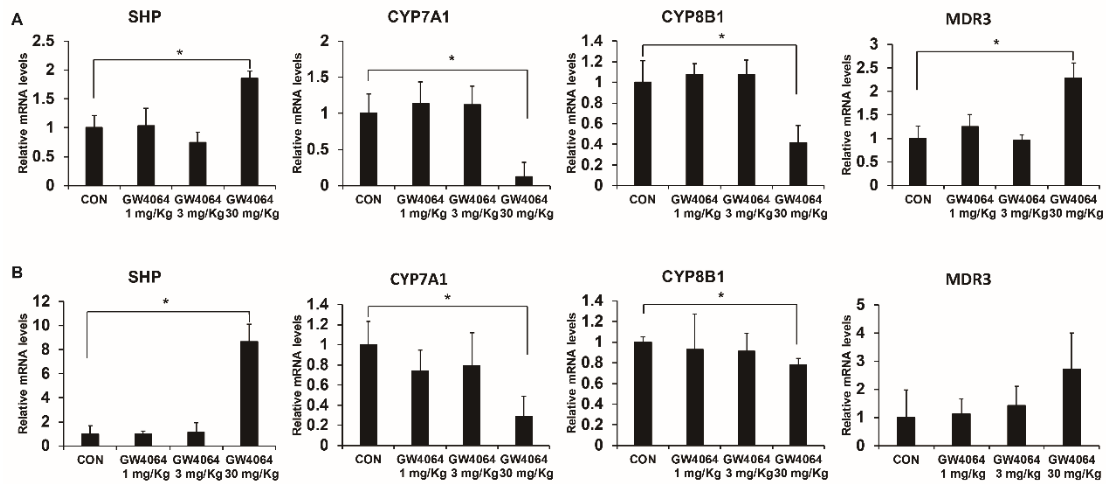
Supplementary Fig. 5. FXR agonist GW4064 promoted the expression of pro-apoptotic proteins. (A) GW4064 promoted the protein levels of Cleaved-PARP, p53 and p21 in KYSE150 cells. *P < 0.05. (n=3). (B) GW4064 promoted the protein levels of Cleaved-PARP, p53 and p21 in EC109 cells. *P < 0.05. (n=3).



Supplementary Fig. 6. 30 mg/Kg GW4064 suppressed EC109 cells-induced tumorigenesis *in vivo*. (A) Representative xenograft tumors were shown after different doses of GW4064 administered. EC109 cells were injected subcutaneously into the nude mice. 1 mg/Kg, 3 mg/Kg, and 30 mg/Kg GW4064 (body weight) was administered by intraperitoneally injected every 3 days for 7 times (n=5). (B) Tumor volume and (C) tumor weight was measured and analyzed (n=5). *$P < 0.05$.



Supplementary Fig. 7. Examination of $FXR^{-/-}$ livers reduced sensitivity to FXR activation by GW4064. WT and $FXR^{-/-}$ mice male siblings at 8 weeks of age were intraperitoneally (i.p.) injected with a single dose of 30 mg/Kg GW4064. Expression of FXR-related target gene was analyzed (n=5-6). $^{*}P < 0.05$.



Supplementary Fig. 8. Expression of FXR target genes in liver and tumor after administration of different doses of GW4064. (A) 30 mg/Kg GW4064 induced SHP and MDR3, suppressed CYP7A1 and CYP8B1 in liver (n=5). *P < 0.05. (B) 30 mg/Kg GW4064 induced SHP expression, suppressed CYP7A1 and CYP8B1 in tumors (n=5). *P < 0.05.