Supplementary Material and Methods

Transfections. PPARG expression was transiently downregulated using a predesigned siRNA duplex directed against PPARG, and a nontargeting siRNA was used as a negative control (Life Technologies Corporation, Japan). ESCC cells were transfected with the annealed siRNA for 48 hours using LipofectAMINE RNAiMAX (Invitrogen. USA).

Antibodies. Antibody which recognized PPARγ (ab19481) was purchased from Abcam (Abcam, USA). The predicted molecular weight was 58 kDa, and this antibody will recognize both PPARγ1 and PPARγ2 according to the manufacturer's datasheet (Supplementary data). This anti-body may not distinguish PPARγ1 from PPARγ2. p21Cip1 (sc-19) and phospho-p21 Tyr145 (sc-20220) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). EGFR (#2232), phospho-EGFR Tyr1068 (#2234: Western blot, #3777: IHC), ERK1/2 (#9102), phospho-ERK1/2 Thr202/Tyr204 (#4376), Akt (#9272), phospho-Akt Ser473 (#9271) and β-actin (#4967) were purchased from Cell Signaling Technology (Boston, MA, USA).

Immunohistochemical staining of PPARγ. Polyclonal rabbit anti-PPARγ antibody was diluted 1:250 with 0.1 M phosphate-buffered saline (PBS; pH 7.4). The sections were microwaved in 10 mmol/L citrate buffer (pH 6.0) for 20 min and incubated with 3% hydrogen peroxide for 5 min at 25°C to block endogenous peroxidase activity. Each sample was incubated with anti-PPARγ antibody overnight at 4°C. For linking, all sections were incubated with horseradish peroxidase-labeled polymer (Envision™+ Kit, Dako) for 60 min at 25°C. PPARγ-positivity was defined as the presence of at least weak nuclear staining in >20% of tumor cells. Absent staining or weak staining in ≤20% of tumor cells was interpreted as negative staining, according to a previous study (Ogino S. et al. 2009 Gastrolenterology).

Evaluation of p21 and Ki-67 expression. Immunohistochemistry was performed in TE-4 xenografts for p21 and Ki-67. The immunoreactivity of p21 and Ki-67 were considered positive when an intense staining was observed homogeneously in nucleus of cancer cell. The count was performed in three randomly chosen high-power fields. The percentage of positive cancer cells to total cancer cells was calculated. The average of these results was analyzed. The positive expression rate of p21 and Ki-67 of the tumors treated with troglitazone (n=8) and the tumors treated with efatutazone (n=8) were compared with the control tumors (n=8).

Cell cycle analysis. The cell cycle distribution was analyzed by flow cytometry using a BD FACS ARIA II instrument (BD Bioscience, San Jose, CA, USA), as described previously (Hirashima K. 2012 Br J Cancer). Percentages of cells in G, S and G2/M phases were calculated using FlowJo software.

Cell proliferation analysis. The water-soluble tetrazolium salt WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) (Dojin Chemicals, Tokyo, Japan) was used to analyze the effects of efatutazone on cell proliferation. ESCC cells were cultured overnight in 96-well plates ($1.0–5.0\times10^3$ cells per well). The number of surviving cells was assessed by measuring the absorbance at 450 nm (A450). Experiments performed in triplicate and more than three times, and the data are shown as the means±SE

Real-time reverse-transcription polymerase chain reaction (RT-PCR). RNA was isolated from the cell lines using an RNeasy Mini Kit (Qiagen), according to the manufacturer's protocol. The expression levels of mRNA were determined by qRT-PCR using TaqMan probes (Roche Diagnostic K.K.) and the values were normalized to those of glyceraldehyde 3-phosphate dehydrogenase (Supplementary Fig.

S9). All qRT-PCR reactions were run using the LightCycler 480 System II (Roche Diagnostics). All data obtained using real-time RT-PCR were from experiments performed in triplicate, and the data are shown as the means±SE.

Western blot analysis. The cells and tumors were lysed in RIPA buffer (25 mM Tris-HCl [pH 7.4], 100 mM NaCl with 2 mM EDTA, Halt Protease and Phosphatase Inhibitor Single-Use Cocktail [100×] [Thermo: 78442]). Equal amount of proteins were loaded onto 10% gels and separated using SDS-PAGE. The resolved proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Inc.). 10 The membranes were blocked with 5% low-fat dry milk in TBS-T [25 mM Tris (pH 7.4), 125mM NaCl, 0.4% Tween 20] for 1 hour at room temperature, following by incubation with each primary antibody overnight at 4°C. The blots were extensively washed with TBS-T and incubated with a 1:1500 dilution of peroxidase-conjugated anti-rabbit IgG antibody. The membranes were washed and detection was accomplished with an enhanced chemiluminescence reagent (GE Healthcare, Piscataway, NJ, USA). Western blot analysis was performed at least three times and the representative data was shown. Some data was supported with the quantitation of phosphorylation or protein levels.

Immunofluorescence staining. TE-4 cells were treated with efatutazone for 48 h and then fixed in 4% paraformaldehyde. Non-specific protein-binding sites were blocked with 3% bovine serum albumin (Sigma, Tokyo, Japan). Sections were permeabilized in 0.1% Triton X-100 and probed with rabbit-anti-p21 antibodies for 1 h at room temperature. The primary antibody was detected with goat-anti-rabbit IgG labeled with Alexa Fluor 488 (1:100) for 1 h at room temperature. Nuclei were detected by propidium iodide. Cells were observed using IMMERSION OIL TYPE-F (Olympus, Tokyo, Japan) and photographed with a FluoView FV300 confocal microscope (Olympus, Tokyo, Japan).

Patient follow-up after surgery. The patients underwent computed tomography (CT) or PET-CT examinations every three months after surgery. Two radiologists diagnosed whether the patients experienced recurrence. We defined the disease-free survival as the period from the day of surgery to the diagnosis of the recurrence of ESCC. The esophageal cancer-specific survival was determined as the period from the day of surgery to the day of patient death caused by ESCC recurrence. During the follow-up period for the 145 patients, there were 47 deaths, 44 recurrences and 34 esophageal

cancer-specific deaths.

The duration / schedule of drug administration in mice. The mice inoculated subcutaneously with TE-4 cells were randomized three groups; control (0.5 w/v% methylcellulose) group, troglitazone group and efatutazone group. When the tumors reached approximately 80 mm^3 in diameter, the administration of the agents were started. These agents were daily administered for 28 days and the last administration was performed three hours before the sacrifice. For the evaluation of the combination therapy, the mice inoculated subcutaneously with TE-4 cells were randomized four groups; control group, efatutazone group, cetuximab group and combination group. Efatutazone or control (0.5 w/v% methylcellulose) were daily administered and cetuximab (1 mg/injection) or placebo (PBS) was injected intraperitoneally twice a week for 38days. The last administration was performed three hours before the sacrifice.

Supplementary Figure legends

Supplementary Figure S1. The study design for selecting the ESCC patients.

Two hundred forty esophageal squamous cell carcinoma patients underwent surgical treatment at Kumamoto University Hospital between January 2000 and December 2008. Seventy-nine of the 240 underwent preoperative treatment, such as chemo-radiation therapy (n=60), chemotherapy (n=5), radiation therapy (n=10) or EMR (n=4), and were excluded from this study. One hundred forty-five of the 161 remaining patients underwent curative surgery with radical lymph node dissection. Paraffin sections of the145 ESCC patients were evaluated by immunohistochemistry in this study. *1 Endoscopic Mucosal Resection. *2 lymph node.

Supplementary Figure S2. TE-4 cells were transfected with PPARG siRNA.

A. Real-time RT-PCR for PPARG in TE-4 cells after transfection with a scrambled siRNA or three kinds of siRNA for PPARG. B. Western blot analysis for PPARγ in TE-4 cells after transfection with a scrambled siRNA or three kinds of siRNA for PPARG.

Supplementary Figure S3. *In vitro* anti-proliferative effects of efatutazone.

A. PDK4 mRNA levels after treatment with 25 μM troglitazone or 25 μM efatutazone were evaluated by real-time PCR. B. The anti-proliferative effects of treatment with the various concentrations of troglitazone or efatutazone for 72 hours were evaluated. The data are shown as the means ± SE. n.s. not significant, *P<0.05, **P<0.01.

Supplementary Figure S4. Anti-proliferative effects of efatutazone in ESCC cells occur via pAkt-p21Cip1.

A. Cell cycle analysis by flow cytometry was performed for TE-11 cells after treatment with 25 μM or 50μM efatutazone for 48 hours. An accumulation of cells in the G1 phase, and a reduction in the G2/M phases, were observed after treatment with efatutazone for 48 h. The percentage of cells in the G, S and G2/M phases were calculated using the FlowJo software program. n.s. not significant, *P<0.05, **P<0.01. B. Western blot analysis for Akt (p-Akt) and p-p21 in TE-6 cells treated with 25 μM efatutazone for 48h. D. Western blot analysis for p-Akt Ser473 in TE-8 ESCC cells which were transfected with the scrambled siRNA or PPARG siRNA, followed by treatment with control (DMSO) or 50 μM efatutazone for 48 h.

Supplementary Figure S5. *In vivo* anti-proliferative effects of efatutazone.

A. The resected TE-4 xenograft tumors are shown. The tumors were labeled according to the treatment group and the size of tumor (ex. the largest of the tumor after treatment with efatutazone was E1, the second largest was E2…). B. The mRNA expression of PDK4, p21Cip1, p27 and PLIN2 in the TE-4 tumors treated with the control (n=8) or efatutazone (n=8) were examined by real-time RT-PCR. n.s. not significant, **P<0.01

Supplementary Figure S6. Investigation using condition medium.

TE-4 cells were treated with the control (DMSO) or efatutazone (25 μM) for 48 hours, and the each culture medium was collected, then these media were named conditioned medium A and conditioned medium B, respectively. These media were adjusted to have the same concentrations of both efatutazone and DMSO by adding efatutazone to the conditioned medium A and adding DMSO to the conditioned medium B, then these media were named the conditioned medium (cond. med.) control and the cond. med. efatutazone, respectively. The TE-4 cells were then treated with these different cond. med. control and cond. med. efatutazone for 10 min. In the Fig. 5H, the TE-4 cells which had pretreated with cetuximab for 6 hours were stimulated with the cond. med. control or the cond. med. efatutazone for 10 minutes.

Supplementary Figure S7. EGFR/MAPK signaling activation.

A. Western blot analysis for EGFR (p-EGRF) in TE-4 xenografts after treatment with the control, efatutazone or troglitazone for 28 days. B. Western blot analysis for EGFR (p-EGRF), ERK (p-ERK) in three indipendent lysates from the TE-4 xenografts after treatment with efatutazone compared with the control. *P<0.05.

Supplementary Figure S8. Anti-proliferative effects of molecule targeted therapy.

A. The anti-proliferative effects of treatment for 72 hours with the various concentrations of U0126 (MEK inhibitor) were compared with the control. Treatment of TE-4 ESCC cells with 10 μM U0126 led to a 25% decrease in the proliferation of the cells. B. The growth inhibitory effects of 10μM U0126, 25μM efatutazone and the combination of the two agents were evaluated for various times. C. The anti-proliferative effects of a 72-hour treatment with the various concentrations of cetuximab (an anti-EGFR antibody) were compared with the control. The treatment with cetuximab (300 μg/ml) decreased the proliferation of the TE-4 cells by 20%. D. TE-4 cells that had been stimulated with EGF (20 ng/ml) were treated with various concentrations of cetuximab. The treatment with cetuximab (300 μg/ml) completely blocked the stimulation of EGF at a concentration of 20 ng/ml. E. The growth inhibitory

effects of cetuximab, efatutazone and efatutazone combined with cetuximab were evaluated for various times. All data represent the means ± SE of triplicate sample.

Supplementary Figure S9. The primers and probes used for real time RT-PCR.

The probes for PPARG, p21Cip1, p27, PDK4, PLIN2 and GAPDH were designed by the universal probe library system provided by Roche. The indicated probe number was used.