SUPPLEMENTARY MATERIALS AND METHODS

Immunohistochemistry

After deparaffinization and dehydration, FFPE sections (5 μm thick) were placed in sodium citrate buffer (10 mM, pH 6.0) at 121°C for 10 min. After incubation in 3% hydrogen peroxide for 10 min, the sections were then incubated with goat serum (Vector Laboratories Inc., Burlingame, CA) for 1 h. They were then incubated with an anti-human RACGAP1 antibody (A-6, dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°Covernight. Envision reagents (Dako REAL EnVision Detection System; peroxidase/DAB1, DakoCytomation, Glostrup, Denmark) were used to detect the binding antibody.

Quantitative real-time PCR and relative expression level

We performed amplification under the following conditions: 95°C for 10 min, then 45 cycles of 95°C for 15 s and 60°C for 1 min. After amplification, the products were subjected to a temperature gradient in the range of 60–95°C at 0.3°C/s under continuous fluorescence monitoring to produce a melting curve for the products.

Western blotting analysis

At 48 h after transfection, total cell lysates were prepared in RIPA buffer (F015, BioDynamics Laboratory Inc. Japan). An aliquot of 10 μg of total cellular protein was separated on c-PAGEL (c520L, ATTO Corp., Japan), and then blotted onto a piece of polyvinylidene difluoride (PVDF) membrane (WSE-4050, ATTO Corp., Japan). Non-specific bindings were blocked for 1 h at room temperature. The membrane was subjected to specific primary antibodies against RACGAP1 (A-6, Santa Cruz Biotechnology, Santa Cruz, CA, 1:2000, 75kDa), and anti-actin (C4, MP Biomedicals, Solon, OH, 1:5000, 42 kD), and incubated 15 minutes in room temperature. After

3 times of wash by TBST buffers, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 15 minutes at room temperature, the protein band was visualized by a chemiluminescence imaging system (WSE-6100, LuminoGraph I, ATTO Corp., Japan).

Cell proliferation assay

Cell proliferation was evaluated with a WST-8 colorimetric assay kit (Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan). Forty-eight h after the transfection of EC cell lines with RACGAP1 siRNA or control siRNA, cells were seeded in 96-well flat-bottomed microtiter plates (5×10^3 cells/well), in a final volume of 100 μL culture medium per well, and incubated in a humidified atmosphere. After 0–72 h culture, 10 μL Cell Counting Kit-8 reagent was added to each well, and the plates were incubated for 2 h in a humidified atmosphere. The absorbance of each well was measured by SoftMax Pro (Molecular Devices Corp., Sunnyvale, CA) at a wavelength of 450 nm. Each independent experiment was performed three times.

Colony formation assay

EC cell lines transfected with RACGAP1 siRNA or negative control siRNA were seeded in six-well plates at 5×10^2 cells per well. After 2 weeks, the plates were washed with phosphate-buffered saline, fixed with 2 ml methanol per well, and stained for 30 min with 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO). After removal of the crystal violet, the plates were immersed in tap water, and the number of colonies with >50 cells was counted.

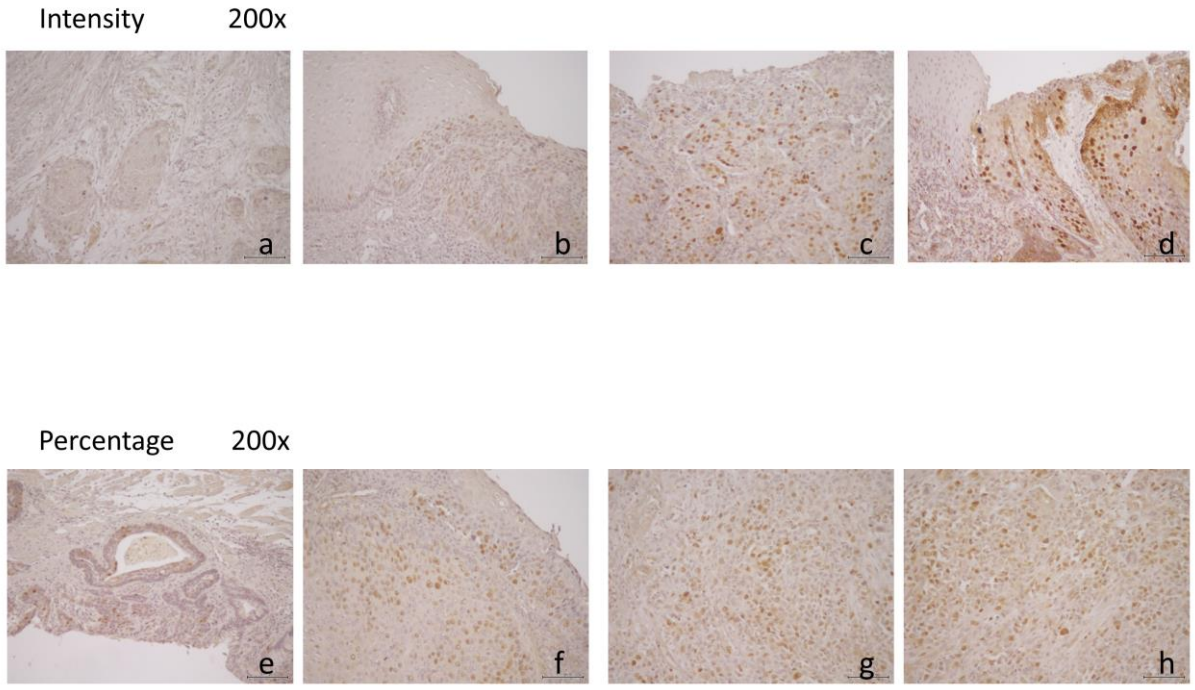
Wound scratch assay

EC cell lines transfected with RACGAP1 siRNA or negative control siRNA were seeded in six-well plates at 1×10^6 cells per well. When cells reached 100% confluency in plates containing 10% fetal bovine serum (FBS), a wound was created with a sterile 200 µl pipette tip. Cells were then incubated with RPMI 1640 medium containing 3% FBS at 37°C for 12 h. Images were acquired every 3 h by an Olympus IX71 microscope (Olympus America) at ×40 magnification. Data were analyzed using ImageJ software (NIH, Bethesda, MD).

Invasion and migration assay

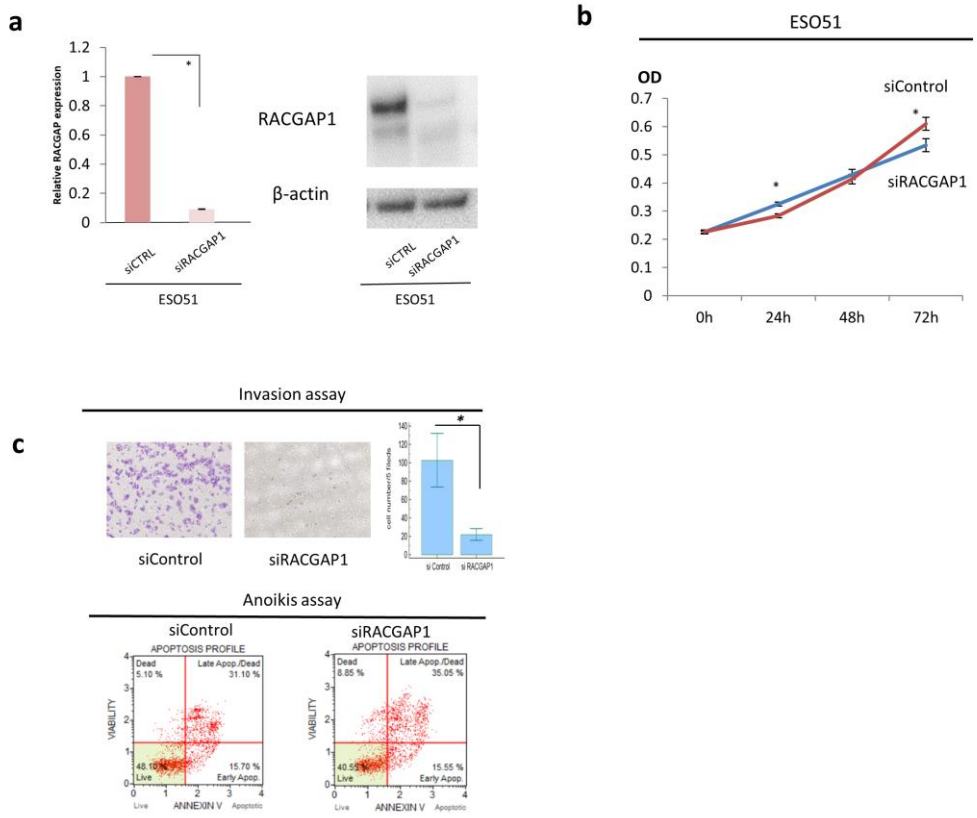
EC cell lines transfected with RACGAP1 siRNA or negative control siRNA (2.5×10^5 cells per chamber) were seeded in the top chamber (Corning® BioCoat™ Control Inserts, Corning, NY, USA) with 500 µl serum-free medium, and 750 µl complete media with 10% FBS was added to the lower chamber. KYSE150 cell were incubated for 48 h and TE8 cells were incubated 36 h at 37°C in 5% CO_2, respectively. The media from the chamber and the transwell were then removed, and the chamber was gently wiped with a cotton swab. Cells on the lower surface of the membrane were stained with Diff-Quick stain (Sysmex, Kobe, Japan) and mounted on glass slides. The number of cells migrating from the underside of the membrane was counted in five microscopic fields at ×200 magnification. The assays were performed in triplicate. The procedure for the cell invasion assay was similar, except that the transwell membranes were pre-coated with Matrigel (Corning® BioCoat™ Matrigel® Invasion Chambers).

Supplementary Figure 1



Supplementary Figure 1. Representative expression of RACGAP1 in EC tissue. Intensity scores 0–3 correspond to **a**–**d**, scores 1–4 correspond to **e**–**h**. Original magnification ×200.

Supplementary Figure 2



Supplementary Figure 2. (a) After transfection (48 h), RACGAP1 expression was significantly reduced in ESO51 cell using western blotting analysis. **(b)** The MTT assay. SiControl cells temporary increased compared with siRACGAP1 cells after 24 hours' incubation, however cell proliferation was finally reduced following RACGAP1 knockdown, compared with siControl cells after 72 hours' incubation. *P<0.05. **(c)** Cell invasion and anoikis assay. Upper panel shows cell invasion assay. The invasion ability was significantly suppressed by the down-regulation of RACGAP1 relative to siControl cells; Lower panel shows anoikis assay. Anoikis assays showed a significant increase in the apoptosis rate in ESO51 cell after RACGAP1 siRNA transfection. *P<0.05.