Supplementary materials and methods

1. Cell lines and cell culture

TOSCa series of 8 OSCC cell lines were established from the primary tumor and metastatic lymph nodes of 4 OSCC patients at Showa University Hospital (Japan) [1]. Specifically, each cell line was derived from the primary tumor and metastatic lymph nodes of the same patient. We designated the cell line from the primary tumor and metastatic lymph nodes as P and LY, respectively (Table S1). Three commercial human OSCC cell lines (HSC3, HSC4, SAS) were purchased from the Japanese Cancer Research Resources Bank (JCRB). Cell lines were maintained in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham with L-glutamine (DMEM/F12) (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA) and 1% penicillin/streptomycin (Thermo Fisher Scientific) at 37°C in a humidified atmosphere with 5% (v/v) CO2.

2. Antibodies

Primary antibodies: mouse monoclonal anti-desmoglein3 (5G11; Abcam, Cambridge, MA), rabbit monoclonal anti-EGFR (EP38Y; Abcam).

Secondary antibodies: Cy3-donkey anti-mouse IgG (AP192C; Chemicon, Temecula,

CA), FITC-donkey anti-rabbit IgG (711-095-152; Jackson immunoResearch Lab Inc., West Grove, PA).

3. RNA extraction and real-time qPCR

Total RNA was extracted from the OSCC cell lines using RNeasy Micro Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized according to the manufacturer's protocol of PrimerScript RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan). Real-time qPCR was performed on an ABI 7000 thermocycler (Thermo Fisher Scientific) using SYBR Premix Ex Taq (Perfect Real Time, Takara) according to the manufacturer's instructions. The following primers were used:

DSG3 forward primer: 5′-CCTGTGCAGCAGCCTGGTAA-3′.

DSG3 reverse primer: 5′-CTCATGCATAAGCAGAGGCACAA-3′.

EGFR forward primer: 5′-CATCCAGGGCCCAACTGTGAG-3′.

EGFR reverse primer: 5′-CAGTGGAAGCCTTGAAGCAGAA-3′.

GAPDH forward primer: 5′-GCACCGTCAAGGCTGAGAAC-3′.

GAPDH reverse primer: 5′-TGGTGAAGACGCCAGTGGA-3′.

Relative gene expression quantity was normalized to GAPDH signals. All analyses were performed in triplicate.

4. WST-1 Cell proliferation assay

Cell growth was analyzed using the Premix WST-1 Cell Proliferation Assay System (Takara) [2]. OSCC cells were seeded onto 96-well plates. After 24 h culture, reagents (Ca, C-mab, siRNAs) were added to wells at several concentrations and incubated for additional 48 h. WST-1 reagent (10 μl) was added to each well and cells were incubated for an additional 2 h. Cell viability was measured by the colorimetric technique based on the cleavage of tetrazolium salts. Absorbance at 450 nm was measured using a microplate reader (Bio-Rad Laboratories). All assays were performed in triplicate.

5. C-mab and Calcium administration

C-mab was purchased from Bristol-Myers Squibb (Princeton, NJ) and used at concentration of 5μg/ml. Ca chloride was purchased from Wako Pure Chemical (Osaka, Japan) and added to the culture medium at final concentrations of 1, 8, and 16mM. After 48 h incubation with C-mab and Ca, real-time qPCR analysis and WST-1 cell proliferation assay were performed to evaluate DSG3 and EGFR expressions and cell viability.

6. Immunofluorescence

OSCC cell lines in DMEM/F12 containing 10% FBS and 8mM Ca were plated in 4-well chamber slides (Nalgen Nunc international, Naperville, IL). Cells were fixed in PBS containing 4% paraformaldehyde for 10 minutes at 4°C. For cell permeabilization, a 10 minutes incubation with PBS containing 0.1% Triton X-100 was used. Cells were blocked in PBS containing 1% BSA and 3% normal donkey serum for 60 minutes at room temperature, followed by incubation with primary antibodies in blocking solution for 90 minutes at room temperature. After two washes in PBS, cells were incubated with appropriately conjugated secondary antibodies diluted 1:100 or 1:200 in blocking solution for 30 minutes at room temperature. Nuclear staining was performed with 46-diamidino-2-phenyl (DAPI) (Sigma-Aldrich).

7. Statistical analysis

Statistical analyses were conducted using Student t-test or Mann-Whitney U test for the comparison of two sets of data, and Kruskal-Wallis test for multiple comparisons. Data were presented in mean ±S.D. All statistical tests were two-tailed, with a *P* value

of <0.05 considered to be statistically significant. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0). More precisely, this program is a modified version of the R Commander (version 1.6-3) designed to add statistical functions frequently used in biostatistics.

References.

[1] Nakamura E, Kozaki K, Tsuda H, et al. Frequent silencing of a putative tumor suppressor gene melatonin receptor 1 A (MTNR1A) in oral squamous-cell carcinoma. *Cancer Sci.* 2008;99(7):1390-1400.

[2] Cook JA, Mitchell JB. Viability measurements in mammalian cell systems. *Anal Biochem.* 1989;179(1):1-7.

Figure S1

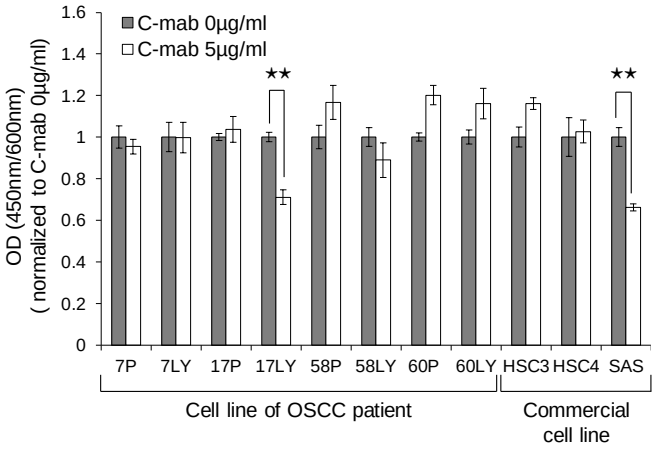
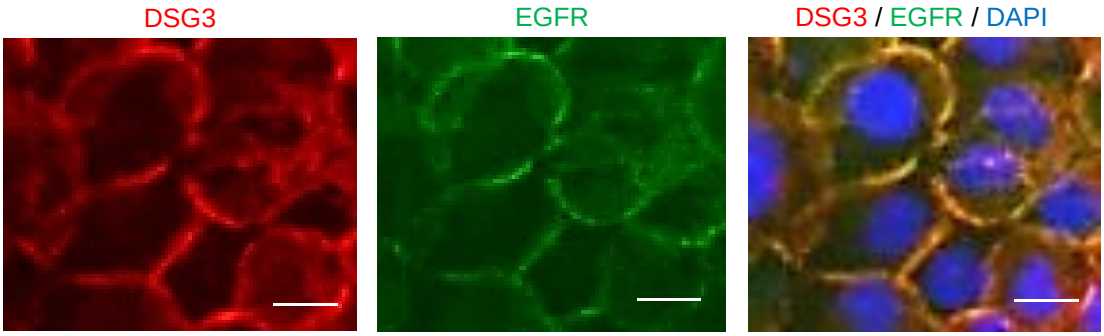

C-mab 0μg/ml
C-mab 5μg/ml
OD (450nm/600nm)
(normalized to C-mab 0μg/ml)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
★★
★★
7P
7LY
17P
17LY
58P
58LY
60P
60LY
HSC3
HSC4
SAS
Cell line of OSCC patient
Commercial cell line

Figure S2



Supplementary table

Table S1.

Profile of cell lines derived from the primary tumor and metastatic lymph nodes of 4 OSCC patients.

Cell line name	Age	Gender	Primary site	Primary tumor	Metastatic lymph node
TOSCa-7	80	Male	Gingiva	7P	7LY
TOSCa-17	69	Female	Gingiva	17P	17LY
TOSCa-58	65	Male	Tongue	58P	58LY
TOSCa-60	82	Female	Gingiva	60P	60LY