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YAP signaling induces PIEZO1 expression to promote oral squamous cell carcinoma cell proliferation

A short running title: YAP signaling induces PIEZO1 expression to promote OSCC cell growth

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DNA microarray analysis was carried out in the current study, and the raw data reported in this study was deposited in NCBI GEO under accession number (GSE150044).

For Peer Review

Abstract

Most cancer cells are exposed to extracellular environments, such as an increase in extracellular matrix (ECM) stiffness with genetic transformation of cancer cells and soluble signals consisting of growth factors and cytokines. It is therefore conceivable that changes in the tumorous extracellular environments would affect tumor cell behavior. The Hippo pathway reportedly responds to the extracellular environment and regulates the nuclear localization of the transcription co-activator, yes-associated protein (YAP)/transcriptional co-activator with PDZ-binding motif (TAZ). Inactivation of the Hippo pathway with nuclear translocation of YAP/TAZ leads to stimulate cell proliferation. Its pathway also regulates gene expression, but the precise molecule meditating the cell-proliferating effect of YAP signaling on oral squamous cell carcinoma (OSCC) is unclear. First, we examined the effects of YAP signaling on OSCC tumorigenesis. Loss-of-function experiments using siRNA or an inhibitor, and immunohistochemical analyses of tissue specimens obtained from OSCC patients demonstrated that YAP signaling was involved in OSCC cell proliferation. Second, we identified Piezo-type mechanosensitive ion channel component 1 (PIEZO1), a Ca²⁺ channel, as a transcriptional target of YAP signaling and showed that an elevated PIEZO1 expression was required for PIEZO1 agonist-dependent Ca²⁺ entry and cell proliferation in OSCC cells. Furthermore, experiments using three-dimensional culture and suspension culture revealed that PIEZO1, the expression of which is regulated by YAP signaling, was involved in OSCC cellular growth. Finally, YAP overexpression in the nucleus and/or cytoplasm was immunohistochemically detected in tumor lesions with high

frequent expression of both PIEZO1 and Ki-67 but not in non-tumor regions of OSCC specimens. These results suggest that the YAP/PIEZO1 axis promotes OSCC cell growth.

Key words: OSCC, YAP, PIEZO1, Proliferation, Three-dimensional culture, Suspension culture

For Peer Review

Introduction

Oral squamous cell carcinoma (OSCC) is the 11th-most common cancer in the world [1], with over 300,000 new cases and 145,000 deaths every year [2]. The 5-year survival rate of OSCC patients is approximately 50% [3]. Although treatments for OSCC, including surgical resection, radiotherapy and chemotherapy, have been improving, the rate of recurrence is 20%-40%, resulting in a reduced survival rate [4,5]. Therefore, a novel cancer therapy based on the molecular mechanisms underlying OSCC tumorigenesis is awaited.

Tumor cells are exposed to the extracellular environments, such as ECM and soluble signals consisting of growth factors and cytokines [6]. Clinically, matrix stiffening is a prominent hallmark of the tumor microenvironment. In OSCC, stiffness is an important clinical feature. Recent studies showed novel mechanotransduction mechanisms, in which an increase in tumorous ECM stiffness drove tumor progression through the activation of intracellular signaling, such as $\beta 1$ integrin-FAK signaling and RHOA-AKT-p300 signaling in breast cancer and liver cancer, respectively [7,8]. We also demonstrated that transient receptor potential vanilloid 4 (TRPV4), a Ca^{2+} channel, responds to the extracellular stiffness, which in turn promoting OSCC cell proliferation through CaMKII-AKT activation [9]. These reports indicate that tumorous extracellular environments could affect tumor cell behavior through several mechanotransduction machineries.

The Hippo pathway responds to the extracellular environments and stimulates cell proliferation, through regulating the nuclear localization of major downstream effectors, yes-associated protein (YAP)/transcriptional co-activator with PDZ-binding motif (TAZ),

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6 resulting in the induction of transcriptional target expression [10-13]. Its pathway has
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8 been involved in tumorigenesis in OSCC and hepatocellular carcinoma and is associated
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10 with the poor prognosis in breast cancer, ovarian cancer and hepatocellular carcinoma
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12 [14-17], but transcriptional targets that mediate the function of the Hippo pathway are not
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14 well understood.
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18 Piezo-type mechanosensitive ion channel component (PIEZO) channels, including
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20 PIEZO1 and PIEZO2, were identified as mechanically activated ion channels [18].
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22 PIEZO1 is reportedly associated with epithelial cellular division, neural cell
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24 differentiation and vasculature development by sensing mechanical stress, such as cell
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26 density, substrate stiffness and shear stress [19-21]. Although the effects of PIEZO1 on
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28 tumorigenesis have been demonstrated [22-25], its function and expression mechanism
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30 in OSCC tumorigenesis remain unclear. Recent reports showed that the depletion of
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32 PIEZO1 expression induced nuclear exclusion of YAP in human neural stem cells and
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34 zebrafish outflow tract valve development [20,26], suggesting that PIEZO1 could act as
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36 an upstream of YAP. But, the hierarchical relationship between YAP and PIEZO1 is
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38 unclear in OSCC. We conducted a study to clarify their relationship in OSCC and
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40 elucidate the function of PIEZO1 to control OSCC cellular growth using cell lines and
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60 pathological specimens.

Materials and methods

Cell lines and reagents

Human OSCC cell lines HSC-4, HSC-3, HSC-2, SAS (Japanese Cancer Research Resources Bank), MISK81-5 and sMISK [27], human gastric cancer cell line MKN-45 (Japanese Cancer Research Resources Bank) and human lung adenocarcinoma cell line A549 [28] were used in this study. HSC-4, HSC-3, HSC-2, MISK81-5 and sMISK were maintained in α -MEM (Invitrogen, Carlsbad, CA, USA), SAS and A549 were maintained in D-MEM (Invitrogen), and MKN-45 was maintained in RPMI-1640 (Invitrogen). These culture media were supplemented with 10% FBS (Invitrogen), and contained 100 IU/ml penicillin and 100 mg/ml streptomycin (Invitrogen). All these cell lines were incubated at 37°C in a 5% CO₂ atmosphere. See supplementary Materials and methods for further details.

Knockdown of protein expression by siRNA and quantitative RT-PCR

The effects of protein knockdown by siRNA were analyzed as previously described [29]. Briefly, siRNAs (final conc. 20 nM) were transfected into OSCC cells using Lipofectamine RNAiMAX (Invitrogen). Target sequences are listed in supplementary material (Table S1). The transfected cells were then used for experiments conducted at 48 h post-transfection.

Quantitative RT-PCR was performed as described previously [29]. Primers are listed in supplementary material (Table S2).

Plasmid construction and infection using lentivirus harboring a cDNA or shRNA

The YAP^{5SA} plasmid, CSII-CMV-MCS-IRES2-Bsd/FLAG-YAP^{5SA}, was used [12]. To construct a lentiviral vector harboring shRNA, a DNA fragment containing the H1 promoter and shRNA was cloned into CS-RfA-EVBsd, which was kindly provided by Dr. H. Miyoshi (RIKEN BioResource Center, Ibaraki, Japan) [30], using Gateway technology (Invitrogen). Target sequence is #1 5'-AGAAGAAGATCGTCAAGTA-3' or #2 5'-ACCAACCTCATCAGCGACT-3'. The vectors were then transfected along with the packaging vectors, pCAG-HIV-gp and pCMV-VSV-G-RSV-Rev, into X293T cells using the Lipofectamine LTX reagent (Invitrogen) to generate lentiviruses [28]. See supplementary Materials and methods for further details.

Three-dimensional culture and suspension culture

3D culture was prepared using collagen gel culture kit Tri-D (Nippi, Inc.) or Matrigel (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. Briefly, bottom collagen gel (1 mg/ml) was poured into the dishes and incubated for polymerization for 30 min at 37°C. Then, cells mixed with 1 mg/ml collagen gel were layered onto the bottom gel. Culture medium was added on top, and then cells cultured in 3D culture were incubated at 37°C. 40 µl of Matrigel was mounted on a round coverslip and incubated for 30 min at 37°C to solidify the gel, and cells (4×10^4 cells) suspended in 1 ml of growth medium containing 2% Matrigel (v/v) were incubated for 96 h [9,31].

In suspension culture, the cells (2×10^5 cells) were cultured with growth medium in low-attachment 1.5 ml tube (Sumitomo Bakelite Co., Ltd, Tokyo, Japan) for 24 h.

Cell proliferation assay

To examine proliferative capabilities in 2D culture, 3D culture or suspension culture, CyQUANT NF assay (Invitrogen) was performed according to the manufacturer’s protocol. Then, the fluorescence intensity was measured using FlexStation 3 microplate reader (Molecular Devices, Tokyo, Japan) at 485 nm excitation/530 nm emission.

ChIP assay

ChIP assay was performed as described previously [32]. HSC-4 cells (1×10^7) were cross-linked with 1% (v/v) formaldehyde for 20 min at room temperature. The cells were lysed and sonicated to shear DNA to a size range between 200 and 1000 bp. Sheared chromatin samples were incubated for 4 h at 4°C with 10 µg of antibodies. Immunocomplexes were absorbed with salmon sperm DNA/protein A-agarose beads. See supplementary Materials and methods for further details.

Intracellular calcium measurement

Intracellular calcium measurement was performed as described previously [9]. See supplementary Materials and methods for further details.

Immunocytochemistry

Immunocytochemistry was carried out as previously described [33]. See supplementary Materials and methods for further details.

Patients and immunohistochemistry

A total of 45 patients with ages ranging from 42 to 90 years (median, 70 years) with primary OSCC diagnosed for treatment at the Department of Oral and Maxillofacial Surgery, Kyushu University Hospital, Japan, from March 2011 to February 2020 were examined in this study. The protocol for this study was approved by the ethical review board of the Local Ethical Committee of Kyushu University, Japan (#30-235). Following the initial biopsy, all specimens were fixed in 10% (v/v) formalin and embedded in paraffin blocks. Subsequently, the paraffin-embedded specimens were sliced into 4- μ m-thick sections, stained with hematoxylin-eosin (HE), and examined by three experienced pathologists to confirm the diagnoses and the histological grades. The histological grade in the OSCC was assessed according to the WHO classification [34]. The tumor extent and the clinical stages were evaluated according to the TNM classification established by the American Joint Committee on Cancer and the International Union Against Cancer (UICC) [35]. The medical records were reviewed to collect information on the clinical characteristics. The clinicopathological data of the patients with OSCC are presented in Table 1 and 2. Immunohistochemical staining was performed as previously described [9]. See supplementary Materials and methods for further details.

Microarray analysis

Microarray analyses were performed using HSC-4 cells cultured in 2D culture or suspension culture as previously described [33]. The raw data reported in this study was deposited in NCBI GEO under accession number (GSE150044). Gene Ontology (GO) analysis was performed using DAVID database (<http://david.abcc.ncifcrf.gov/>). See supplementary Materials and methods for further details.

Analysis of TCGA head and neck cancer dataset

PIEZO1, *PIEZO2*, *amphiregulin (AREG)* and *cyclin dependent kinase 6 (CDK6)* gene expression data for 518 head and neck tumor lesions and 44 non-tumor regions were obtained from The Cancer Genome Atlas (TCGA) web site (<http://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp>).

Statistical analysis

Significant differences were determined using Fisher’s exact test for Table 1 and 2. For other experiments, significant differences were determined using Student’s t test and one-way ANOVA with turkey. *P* value of < 0.05 or 0.01 was considered statistically significant.

Results

Proliferative capabilities of oral squamous cell carcinoma cells in three-dimensional and suspension culture

Inactivation of the Hippo pathway regulates YAP/TAZ nuclear translocation, thereby inducing transcriptional targets and promoting cell proliferation [10-17]. YAP/TAZ siRNA and the treatment with simvastatin, a YAP inhibitor [36,37], reduced not only the mRNA levels of *ankyrin repeat domain1* (*ANKRD1*) and *cellular communication network factor 1* (*CCN1*; also known as *CYR61*), which are transcriptional targets of YAP/TAZ, but also 2-dimensional (2D) proliferation of HSC-4 cells (supplementary material, Figure S1A). These loss-of-function experiments indicated that YAP/TAZ signaling was involved in HSC-4 cell proliferation. Next, we conducted to set up the cell culture system, which is capable of analyzing the relationship between YAP/TAZ signaling and its transcriptional targets.

Our recent study showed that HSC-4 cells respond to extracellular environments, such as 3D culture with Matrigel and 2D plastic dish culture, and exhibited lower proliferation capabilities in 3D culture than in 2D culture [9]. Consistently, the cellular growth in 3D culture with collagen gel was also suppressed compared with 2D culture in HSC-4 and HSC-3 cells (Figure 1A), indicating that extracellular conditions do indeed affect OSCC cellular proliferation capabilities, regardless of the matrix. Proliferative capabilities in suspension culture were extremely lower than 2D or 3D culture (Figure 1A). As YAP signaling could be inactivated in suspension culture [12], we carried out a microarray analysis using HSC-4 cells cultured in 2D and suspension culture. Expectedly, GO

analysis showed enrichment for previously reported the YAP/TAZ function, such as DNA replication, mitotic nuclear division, DNA replication initiation, regulation of cell cycle, cell proliferation, DNA repair, cell cycle and positive regulation of cell migration (Figure 1B and supplementary material, Table S3). Indeed, transcriptional targets of YAP/TAZ, such as *AREG*, *aurora kinase B (AURKB)*, *cyclin D1(CCND1)*, *CDK6*, *E2F transcription factor 1 (E2F1)*, *forkhead box M1(FOXMI)*, *kinesin family member 23 (KIF23)* and *minichromosome maintenance complex component 7 (MCM7)* [38-44] were listed as a down-regulated gene in suspension culture (supplementary material, Table S4). These data suggested that suspension culture could be useful to identify transcriptional targets of YAP/TAZ signaling. In suspension culture, YAP/TAZ expression was reduced in a time-dependent manner (Figure1C). Similarly, the mRNA levels of *ANKRD* and *CYR61* were reduced in **OSCC** cells in suspension culture compared with 2D culture (Figure 1D and supplementary material, **Figure S1B**). In addition, siRNA treatment for large tumor suppressor 2 (LATS2), which phosphorylates YAP/TAZ on serine residues [10], reversed the suspension culture-dependent decrease of *ANKRD1*, *CYR61*, *AURKB* and *FOXMI* expression (Figure 1E and supplementary material, Figure S1C and S1D). These results indicate that YAP/TAZ signaling may be inactivated in the suspension culture, thereby suppressing cell proliferation in OSCC cells.

PIEZO1 is a transcriptional target of YAP signaling in oral squamous cell carcinoma cells

PIEZO channels, including PIEZO1 and PIEZO2, were identified as mechanically

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6 activated ion channels [18]. The Cancer Genome Atlas (TCGA) dataset showed that
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8 *PIEZO1* and *PIEZO2* mRNA were elevated in tumor lesions compared with in non-tumor
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10 regions, but *PIEZO2* mRNA expression levels were lower than that of *PIEZO1* in head
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12 and neck region (supplementary material, Figure S2A). Although *PIEZO1* has been
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14 demonstrated both positive [22,23] and negative [24,25] effects on tumorigenesis, its
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16 function and expression mechanism in OSCC tumorigenesis remain unclear. Therefore,
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18 the function of *PIEZO1* in OSCC tumorigenesis was investigated in the current study.
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21 Previous report showed YAP activation in OSCC tumor lesion [37]. Consistently, the
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23 expression of *AREG* or *CDK6* was elevated in tumor lesions. Since a significant
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25 correlation between *PIEZO1* and *AREG* or *CDK6* was observed (supplementary material,
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27 Figure S2A), it is possible that *PIEZO1* expression is correlated with YAP signaling. It
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29 has been reported that *PIEZO1* could act as an upstream of YAP in the developmental
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31 stages [20,26]. Therefore, we conducted to clarify the relationship between YAP/TAZ
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33 signaling and *PIEZO1* in OSCC cells in the subsequent experiments.
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41 *PIEZO1* expression was decreased in 3D culture compared with 2D culture in HSC-4
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43 cells (Figure 2A), and was also reduced in suspension culture (Figure 2B and
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45 supplementary material, Figure S2B). Importantly, knockdown of *LATS2* rescued the
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47 suspension culture-dependent reduction of the expression of *PIEZO1* in HSC-4 cells
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49 (Figure 2C), suggesting that YAP/TAZ signaling may act as an upstream of *PIEZO1*. In
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51 addition, two different YAP/TAZ siRNAs reduced *PIEZO1* expression in HSC-4 and
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53 HSC-3 cells (Figure 2D and supplementary material, Figure S2C). To elucidate the
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55 function of YAP, HSC-4 and HSC-3 cells expressing mock or an active YAP mutant
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YAP^{5SA}, in which five possible phosphorylation serine residues are changed to alanine [12,45], were generated by lentiviral transduction. In YAP^{5SA}-expressing OSCC cells, the expression of PIEZO1, as well as *ANKRD1* and *CYR61*, was elevated compared with mock-expressing cells, indicating that YAP activation induces PIEZO1 expression (Figure 2E and supplementary material, Figure S2C and S2D). We conducted to find the putative binding sites of TEA domain transcription factors 4 (TEAD4), which is a major transcription factor in the Hippo pathway regulating the expression of transcriptional targets associated with YAP/TAZ [46], using Cistrome Data Browser database (<http://cistrome.org/db/#/>) in human *PIEZO1* gene. The database showed that ChIP-seq peaks of TEAD4 and active histone modifications, such as H3K27ac and H3K9ac, were hardly detected in the region upstream from the TSS of *PIEZO1* gene. In contrast, three ChIP-seq peaks of TEAD1 or TEAD4, which are commonly detected in multiple cell lines and contain putative binding sites of four TEAD genes (data not shown), were found within the *PIEZO1* gene body. Site 1 is located within exon 1, and site 2 and site 3 were located between exons 1 and 2. In addition, these sites overlapped the peaks of H3K27ac and H3K9ac, and exhibited high enhancer activity region using epilogs database (<https://epilogs.altius.org>) (supplementary material, Figure S2E). ChIP assay revealed that TEAD4 indeed bound to *PIEZO1* gene more dominantly through sites 2 and 3, as well as to promoter region of *ANKRD1* as a positive control [47], than in site 1 (Figure 2F and supplementary material, Figure S2E). Furthermore, YAP^{5SA} also formed a complex with sites 2 and 3 (Figure 2F). Previous report showed that YAP activity is mediated via TEADs binding to not only promoters but also enhancers of the

transcriptional targets [47]. Therefore, the current TEAD4 binding sites could be involved in *PIEZO1* expression mechanism. These results suggest that *PIEZO1* is a possible transcriptional target of YAP signaling.

***PIEZO1* expression is involved in agonist-induced ERK1/2 and p38 MAPK activation**

PIEZO1 or *PIEZO2* expression in cancer cell lines was analyzed using Cancer Cell Line Encyclopedia (<https://portals.broadinstitute.org/ccle>) dataset, which was viewed by cBioportal (<https://www.cbioportal.org>). In HSC-2, HSC-3 and HSC-4 cells, *PIEZO1* was expressed to the similar levels to other cancer cells, while *PIEZO2* expression was little expressed in many cancer cells (supplementary material, Figure S3A). We also measured *PIEZO1* expression in six human OSCC cell lines and a control gastric cancer cell line. HSC-4 and HSC-3 highly expressed *PIEZO1* (Figure 3A), while *PIEZO2* mRNA level was low in OSCC cell lines, compared with A549 cells (supplementary material, Figure S3B). Treatment with Yoda1, a specific *PIEZO1* agonist [48,49], increased the intracellular Ca^{2+} influx in a dose-dependent manner in HSC-4 cells (Figure 3B and supplementary material, Figure S3C). Two different siRNAs knocked down *PIEZO1* mRNA and protein, but not *PIEZO2* mRNA expression (Figure 3C and supplementary material, Figure S3D). Importantly, *PIEZO1* knockdown did not change the mRNA levels of YAP transcriptional targets, such as *ANKRD1* and *CYR61*, and YAP/TAZ nuclear localization, indicating that YAP signaling would not be regulated by *PIEZO1* in OSCC cells (supplementary material, Figure S3D). Knockdown of *PIEZO1*

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6 suppressed the Yoda1-dependent Ca^{2+} influx (Figure 3D and supplementary material,
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8 Figure S3E). Since Yoda1 treatment stimulated ERK1/2 and p38 MAPK activation in
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10 human bone marrow-derived mesenchymal stem cells [50], the effect of PIEZO1 on its
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12 activation was examined in HSC-4 cells. PIEZO1 knockdown reduced Yoda1-induced
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14 ERK1/2 and p38 MAPK phosphorylation, but did not affect the AKT phosphorylation
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16 (Figure 3E). Supporting our results, previous report revealed that gain-of-function
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18 mutations in PIEZO1 increased Ca^{2+} concentrations with ERK1/2 activation, indicating
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20 that the expression of PIEZO1 channel activates intracellular signaling [51]. These results
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22 suggest that PIEZO1 may function as an ion channel and its expression is involved in
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24 agonist-dependent ERK1/2 and p38 MAPK activation in OSCC cells.
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33 **PIEZO1 expression is required for cell proliferation in oral squamous cell** 34 **carcinoma cells**

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36 As ERK1/2 and p38 MAPK activation is involved in cell proliferation [52,53], the effect
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38 of PIEZO1 on cell proliferation was examined in OSCC cells. Knockdown of PIEZO1
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40 decreased the ratio of Ki-67-positive or PCNA-positive cells and cell proliferation
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42 capability in HSC-4 cells (Figure 4A and 4B, and supplementary material, Figure S4A),
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44 but did not increase the number of cleaved caspase-3-positive cells (supplementary
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46 material, Figure S4B). GsMTx-4, a mechanosensitive and stretch-activated ion channel
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48 inhibitor and also functions as an inhibitor of PIEZO1 [20,51], decreased cell proliferation
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50 (Figure 4C). To further clarify the function of PIEZO1, we generated HSC-4 cells stably
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52 expressing PIEZO1 #1 or #2 shRNA (Figure 4D). Based on the above results using
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siRNAs, target sequences of PIEZO1 #1 and #2 shRNAs were designed to be identical to those of PIEZO1 #1 and #2 siRNAs, respectively. In these cells, Yoda1-dependent Ca^{2+} influx was suppressed, which was consistent with our experiments using siRNAs (Figures 3D and 4E, and supplementary material, Figure S4C). In 3D culture, LATS2 knockdown or YAP^{5SA} expression increased the ratio of Ki-67- or PCNA-positive cells in control cells (Figure 4F and 4G and supplementary material, Figure S4D). Importantly, in HSC-4 cells expressing PIEZO1 #1 or #2 shRNA, LATS2 knockdown or YAP^{5SA} expression did not change the ratio of Ki-67- or PCNA-positive cells and induced the expression of *ANKRD1*, but not *PIEZO1* (Figure 4F and 4G and supplementary material, Figure S4E). These results suggest that YAP signaling promotes cell proliferation through inducing PIEZO1 expression in 3D-cultured OSCC cells.

PIEZO1 and YAP are expressed in human oral squamous cell carcinoma tissues

Immunohistochemical analyses were performed to examine PIEZO1 and YAP expression in human OSCC specimens. PIEZO1 expression was detected in 45/45 (100%) tumor lesions and 25/31 (80.6%) non-tumor regions (Figure 5A and supplementary material, Figure S5). Based on the intensity of the PIEZO1-positive signal, the samples were divided into three groups: negative, weak and strong (Figure 5A). PIEZO1 was strongly expressed in the cellular cytoplasm in 23/45 (51.1%) tumor lesions, while a strong expression was noted in 2/31 (6.5%) non-tumor regions, showing a significant difference ($p < 0.0001$; Fisher's exact test) (Figure 5A).

Based on the YAP expression and localization, specimens were divided into three

groups: negative, nucleus/cytoplasm and nucleus (supplementary material, **Figure S6A**). When YAP was expressed in nucleus/cytoplasm or nucleus, the results were defined as a YAP-positive case, in which the ratio of YAP-positive cells was more than 30% in a tumor lesion or a non-tumor region. YAP-positive cases were 43/45 (95.6%) in tumor lesions, while those were 10/31 (32.3%) in non-tumor regions (**supplementary material, Figure S6B**), showing a significant difference ($p < 0.0001$; **Fisher's exact test**). Notably, YAP was hardly detected in nucleus in non-tumor regions. Mice with tongue-specific deletion of MOB kinase activator 1A/B developed tongue carcinoma through endogenous YAP1 hyperactivation, in which YAP1 was localized in nucleus at high frequencies [37]. Therefore, we speculated that YAP might be hyperactivated in tumor lesions of our OSCC specimens. As expected, the YAP-positive expression was frequently detected in tumor lesions with Ki-67 expression but not in non-tumor regions (Figure 5B and supplementary material, **Figure S6C**), suggesting that hyperactivated YAP might promote cancer cell proliferation. Significant correlations with **the PIEZO1 intensity or the YAP expression** and localization were not shown for the clinical T (tumor size) stage, histological grade and lymph node metastasis (Table 1 and 2). **It is noteworthy that** YAP-positive cases with a strong PIEZO1 expression in tumor lesions were more frequent, but not significantly, than in non-tumor regions in consecutive sections (Figure 5B **and supplementary material, Figure S7**). Taken together, these results suggest that YAP, which might be hyperactivated in tumor lesion, likely induces PIEZO1 expression, resulting in enhanced OSCC cell proliferation.

Discussion

It is generally accepted that the changes in the tumorous extracellular environments, including ECM stiffness and exposure to soluble signals, affect tumor cell behavior [6]. We aimed to identify a molecule, which is activated by extracellular environments, to regulate OSCC tumorigenesis. In the current suspension culture and 3D culture, OSCC cell proliferation capabilities were reduced. Subsequently, we identified PIEZO1 as a transcriptional target of YAP signaling and showed that an elevated PIEZO1 expression was required for cell proliferation of OSCC cells.

To our knowledge, this is the first report to suggest that PIEZO1 might function as an oncogene-related molecule in OSCC. Similar to our immunohistochemical data, PIEZO1-positive signals were detected in the cytoplasm of human prostate carcinoma tissue [23]. In Madin-Darby canine kidney normal epithelial cells, PIEZO1 developed into large cytoplasmic aggregates in the crowded regions with increased cell density [19]. Considering these results, the cytoplasmic localization of PIEZO1 in tumor lesions would correlate with its function, but underlying mechanism is not clear. Cryo-electron microscopy and high-speed atomic force microscopy showed that PIEZO1 structural deformation gates its channel in response to mechanical stress [54], thereby inducing Ca^{2+} influx [55]. Therefore, the effects of PIEZO1 on OSCC tumorigenesis might depend on PIEZO1 expression level.

In the present study, suspension culture was used to elucidate the hierarchical relationship between YAP and PIEZO1. At present, we do not know why PIEZO1 was not suggested as a down-regulated gene in the microarray data (supplementary material,

Table S4).

Recently, it has been reported that PIEZO1 could act as an upstream of YAP in the developmental stages, such as human neural stem cell differentiation and zebrafish heart development [20,26]. In this study, PIEZO1 knockdown did not affect the expression of YAP transcriptional targets and YAP/TAZ nuclear localization. In addition, gain-of-function experiments and ChIP data demonstrated that YAP signaling promotes PIEZO1 expression in OSCC cells. Although the hierarchical relationship between YAP signaling and PIEZO1 may depend on cell context, it is noteworthy that these molecules could be a common mechanotransduction mechanism between organ formation by stem cells and tumor formation by cancer cells.

The current immunohistochemical data demonstrated YAP localization in nucleus and/or cytoplasm in tumor lesions with an increased Ki-67-positive signal, suggesting that YAP signaling might be activated with enhanced OSCC cellular growth. But underlying mechanism to activate YAP signaling is unclear. In non-tumor regions, we also confirmed a low frequency of YAP-positive expression in nucleus/cytoplasm with a decreased Ki-67-positive signal (see supplementary material, Figure S6C), but its precise function in the regions remains unclear. As the Hippo pathway regulates cell differentiation as well as cell proliferation [45], cancer therapy targeting for components of the Hippo pathway might cause side effects in the non-tumor region, at least in OSCC. If the transcriptional targets mediating the Hippo pathway in regulating cellular growth may function in tumor lesion specifically, its gene could be a molecular target for novel cancer therapy.

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6 Some genetic mutations of cancer-related genes, which are responsible for
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8 tumorigenesis and might be molecular targets of anti-tumor therapy for OSCC, have been
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10 reported [56]. Recent report also demonstrated that the resistance to phosphoinositide-3-
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12 kinase (PI3K)- α inhibitors for head and neck SCC bearing PIK3 catalytic subunit alpha
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14 (PIK3CA) mutations and amplification [57], indicating that the therapeutic options for
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16 patients with head and neck SCC are limited. Another molecular mechanism could be
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18 involved in OSCC tumorigenesis. We previously demonstrated that TRPV4, a Ca^{2+}
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20 channel, enhances OSCC cell proliferation through CaMKII-AKT activation [9]. Given
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22 the present and previous findings, it is intriguing to speculate that Ca^{2+} influx-dependent
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24 cellular growth through PIEZO1 or TRPV4 may be a potential target for anti-tumor
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26 therapy.
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33 In summary, we found that YAP, which is activated in OSCC tumor lesion, promotes
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35 cancer cell proliferation. We also demonstrated that YAP signaling induces PIEZO1
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37 expression to regulate OSCC cellular growth positively. Taken together, these results
38
39 suggest that the YAP/PIEZO1 axis contributes to OSCC cell growth.
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Author contributions

K. H. carried out experiments, analyzed data and co-wrote the manuscript. S. F. carried out experiments, conceived and wrote the manuscript. S. M. and A. K. performed ChIP experiments. Y. T. analyzed immunohistochemical data. T. K. carried out data interpretation and co-wrote the manuscript. All authors were involved in writing the paper and had final approval of the submitted and published versions.

Supporting Information

Supplementary figure legends

Figure S1. The effect of YAP/TAZ signaling on OSCC cells.

Figure S2. YAP signaling regulates PIEZO1 expression in OSCC cells.

Figure S3. The effect of PIEZO1 on YAP/TAZ signaling.

Figure S4. PIEZO1 regulates cell proliferation of OSCC cells.

Figure S5. PIEZO1 is expressed in human oral squamous cell carcinoma tissues.

Figure S6. YAP is expressed in human oral squamous cell carcinoma tissues.

Figure S7. Co-expression of PIEZO1, YAP, Ki-67 and PCNA in human oral squamous cell carcinoma tissues.

Table S1. List of siRNA sequence used in this study.

Table S2. List of quantitative RT-PCR primer used in this study.

Table S3. Gene enrichment analysis in HSC-4 cells.

Table S4. List of gene from microarray analysis data of HSC-4 cells cultured in 2D culture and suspension culture.

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Table 1. Relationship between the PIEZO1 intensity and clinicopathological characteristics of OSCC cases.

		PIEZO1 expression			P value
		Negative	Weak	strong	
Clinical T stage					
T1	7	0	3	4	N.S. ^a
T2	20	0	13	7	
T3	7	0	4	3	
T4	11	0	2	9	
Histologic grade					
Well	25	0	14	11	N.S. ^a
Moderately	16	0	7	9	
Poorly	4	0	1	3	
Lymph node metastasis					
Positive	11	0	4	7	N.S. ^a
Negative	34	0	18	16	

Abbreviations: Clinical T stage; *T1* tumor 2 cm or less in greatest dimension and 5 mm or less depth of invasion, *T2* tumor 2 cm or less in greatest dimension and more than 5 mm but no more than 10 mm depth of invasion or tumor more than 2 cm but not more than 4 cm in greatest dimension and depth of invasion no more than 10 mm, *T3* tumor more than 2 cm but not more than 4 cm in greatest dimension and depth of invasion more than 10 mm or tumor more than 4 cm in greatest dimension and not more than 10 mm depth of invasion, *T4* (*T4a* and *T4b*): *T4a* (*lip*) tumor invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin (of the chin or nose), *T4a* (*oral cavity*) tumor more than 4 cm in greatest dimension and more than 10 mm depth of invasion or

tumor invades through the cortical bone of the mandible or maxilla or involves the maxillary sinus, or invades the skin of the face, *T4b (lip and oral cavity)* tumor invades masticator space, pterygoid plates, or skull base, or encases internal carotid artery. Histologic grade (WHO); *Grade 1* well differentiated, *Grade 2* moderately differentiated, *Grade 3* poorly differentiated.

^a Statistical analyses were performed by Fisher’s exact test. N.S.: not significant.

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Table 2. Relationship between the YAP expression and localization, and clinicopathological characteristics of OSCC cases.

		YAP expression and localization			P value
		Negative	Nucleus/cytoplasm	Nucleus	
Clinical T stage					
T1	7	0	3	4	N.S. ^a
T2	20	1	5	14	
T3	7	1	2	4	
T4	11	0	3	8	
Histologic grade					
Well	25	1	9	15	N.S. ^a
Moderately	16	1	4	11	
Poorly	4	0	0	4	
Lymph node metastasis					
Positive	11	2	4	5	N.S. ^a
Negative	34	0	9	25	

Abbreviations are same in the Table 1.

^a Statistical analyses were performed by Fisher's exact test. N.S.: not significant.

Figure Legends

Figure 1. Proliferative capabilities of oral squamous cell carcinoma cells in the three-dimensional and suspension cultures.

(A) HSC-4 cells (1×10^5 cells) and HSC-3 cells (1×10^5 cells) were seeded in 2D, 3D (collagen or Matrigel) or suspension culture in the presence of 10% FBS for 48 h, and relative cell numbers were quantified using the CyQUANT cell proliferation assay. RFU: relative fluorescence units. (B) Gene enrichment analysis of down-regulated genes in suspension culture comparing with 2D culture for gene ontology biological processes was carried out. (C) HSC-4 cells were cultured in 2D or suspension culture for the indicated numbers of hours. Cell lysates were probed with anti-YAP/TAZ and anti- β -actin antibodies. Molecular weight size was noted referring to molecular weight marker. (D) *ANKRD1* and *CYR61* mRNA levels in HSC-4 cells cultured in 2D or suspension culture for 24 h were measured by quantitative RT-PCR. Relative levels of *ANKRD1* and *CYR61* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with expression in 2D cells. (E) HSC-4 cells were transfected with control or LATS2 siRNA for 48 h, and then cells were cultured in 2D or suspension culture for 24 h. *ANKRD1* and *CYR61* mRNA levels were measured by quantitative RT-PCR. Relative levels of *ANKRD1* and *CYR61* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with levels in 2D control cells. Results are shown as means \pm s.d. of three independent experiments. * $P < 0.05$, ** $P < 0.01$. n.s.: not significant.

Figure 2. PIEZO1 is a transcriptional target of YAP signaling in oral squamous cell

carcinoma cells.

(A) HSC-4 cells were cultured in 2D (UT), 2D (Coat) or 3D (collagen) culture for 24 h.

PIEZO1 mRNA levels were measured by quantitative RT-PCR. Relative levels of *PIEZO1* mRNA expression were normalized to *GAPDH* and expressed as fold-changes

compared with expression in 2D (UT) cells. Cell lysates were probed with anti-PIEZO1

and anti- β -actin antibodies. Molecular weight size was noted referring to molecular

weight marker. (B) HSC-4 cells were cultured in 2D or suspension culture for the

indicated numbers of hours. *PIEZO1* mRNA levels were measured by quantitative RT-

PCR. Relative levels of *PIEZO1* mRNA expression were normalized to *GAPDH* and

expressed as fold-changes compared with expression in 2D cells. Cell lysates were probed

with anti-PIEZO1 and anti- β -actin antibodies. Molecular weight size was noted referring

to molecular weight marker. (C) HSC-4 cells were transfected with control or LATS2

siRNA for 48 h, and then cells were cultured in 2D or suspension culture for 24 h. *PIEZO1*

mRNA levels were measured by quantitative RT-PCR. Relative levels of *PIEZO1* mRNA

expression were normalized to *GAPDH* and expressed as fold-changes compared with

expression in 2D control cells. (D) HSC-4 cells were transfected with control or two

different YAP/TAZ siRNAs for 48 h, and then cells were cultured in 2D culture for 3 h.

PIEZO1 mRNA levels were measured by quantitative RT-PCR. Relative *PIEZO1* mRNA

levels were normalized by *GAPDH* and expressed as fold-changes compared with levels

in control siRNA transfected cells. (E) HSC-4 cells expressing mock or YAP^{5SA} were

cultured in 2D or suspension culture for 24 h and *PIEZO1* mRNA levels were measured

by quantitative RT-PCR (left panel). Relative levels of *PIEZO1* mRNA expression were

normalized to *GAPDH* and expressed as fold-changes compared with expression in 2D control cells. Cell lysates were probed with anti-PIEZO1, anti-YAP/TAZ and anti- β -actin antibodies (right panel). Molecular weight size was noted referring to molecular weight marker. Arrowhead indicates non-specific bands. (F) Chromatin from HSC-4 cells expressing mock or YAP^{5SA} was immunoprecipitated with anti-control IgG or anti-TEAD4 and anti-FLAG antibodies, and the precipitates were analyzed by PCR for TEAD4 binding site 1, site 2 or site 3 of the *PIEZO1* gene. White box indicates TEAD4 binding motif and site 3 has two TEAD4 binding motifs. Results are shown as means \pm s.d. of three independent experiments. * $P < 0.05$, ** $P < 0.01$. n.s.: not significant.

Figure 3. PIEZO1 expression is involved in agonist-induced ERK1/2 and p38 MAPK activation.

(A) *PIEZO1* mRNA levels were measured in OSCC cell lines (HSC-4, HSC-3, HSC-2, SAS, MISK81-5 and sMISK cells) by quantitative RT-PCR. Relative levels of *PIEZO1* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with expression in control MKN-45 cells. (B) HSC-4 cells were pre-loaded with Fluo-4-AM and were exposed without or with 1 and 10 μ M Yoda1. Then, intracellular Ca^{2+} influx at 160 sec was measured. RFU: relative fluorescence units. (C) HSC-4 cells were transfected with control or two different PIEZO1 siRNAs for 48 h, and *PIEZO1* mRNA levels were measured by quantitative RT-PCR. Relative *PIEZO1* mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in control siRNA transfected cells. Cell lysates were probed with anti-PIEZO1 and anti- β -actin

antibodies. Molecular weight size was noted referring to molecular weight marker. (D) HSC-4 cells transfected with control or two different PIEZO1 siRNAs were pre-loaded with Fluo-4-AM and were exposed without or with 10 μ M Yoda1. Then, intracellular Ca^{2+} influx at 160 sec was measured. RFU: relative fluorescence units. (E) HSC-4 cells were transfected with control or two different PIEZO1 siRNAs, and treated without or with 10 μ M Yoda1 for 5 min. Cell lysates were probed with anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-p38, anti-p38, anti-phospho-AKT and anti-pan-AKT antibodies. Molecular weight size was noted referring to molecular weight marker. Results are shown as means \pm s.d. of three independent experiments. * P <0.05, ** P <0.01. n.s.: not significant.

Figure 4. PIEZO1 expression is required for cell proliferation in oral squamous cell carcinoma cells.

(A) HSC-4 cells were transfected with control or PIEZO1 #1 siRNA for 48 h. The cells were stained with anti-Ki-67 antibody and Hoechst 33342, and then Ki-67-positive cells and Hoechst 33342-stained cells were counted, respectively. Results are expressed as the percentage of Ki-67-positive cells compared with total Hoechst 33342-stained cells. (B) HSC-4 cells were transfected with control or two different PIEZO1 siRNAs. The cells were cultured in the presence of 5% FBS for the indicated numbers of days, and cell numbers were counted. (C) HSC-4 cells were cultured without or with 100 μ M GsMTx-4 in the absence of FBS for 48 h, and relative cell numbers were quantified using the CyQUANT cell proliferation assay. RFU: relative fluorescence units. (D) *PIEZO1*

mRNA levels in HSC-4 cells expressing control or PIEZO1 #1 and #2 shRNAs were measured by quantitative RT-PCR. Relative levels of *PIEZO1* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with expression in control cells. Lysates of HSC-4 cells expressing control or PIEZO1 #1 and #2 shRNAs were probed with anti-PIEZO1 and anti- β -actin antibodies. Molecular weight size was noted referring to molecular weight marker. (E) HSC-4 cells expressing control or PIEZO1 #1 and #2 shRNAs were pre-loaded with Fluo-4-AM and were exposed without or with 10 μ M Yoda1. Then, intracellular Ca^{2+} influx at 160 sec was measured. RFU: relative fluorescence units. (F) HSC-4 cells expressing control or PIEZO1 #1 and #2 shRNAs were transfected with control or LATS2 siRNA for 48 h, and then cells were cultured for 4 days in the 3D Matrigel. The cells were stained with anti-Ki-67 antibody and Hoechst 33342, and then Ki-67-positive cells and Hoechst 33342-stained cells were counted, respectively. Results are expressed as the percentage of Ki-67-positively stained cells compared with total Hoechst 33342-stained cells ($n = 13,469$). (G) HSC-4 cells expressing control or PIEZO1 #1 and #2 shRNAs were transfected with mock or YAP^{5SA}, and then cells were cultured for 4 days in the 3D Matrigel. The cells were stained with anti-Ki-67 antibody and Hoechst 33342, and then Ki-67-positive cells and Hoechst 33342-stained cells were counted, respectively. Results are expressed as the percentage of Ki-67-positively stained cells compared with total Hoechst 33342-stained cells ($n = 5,603$). Results are shown as means \pm s.d. of three independent experiments. * $P < 0.05$, ** $P < 0.01$. Scale bars, 100 μ m.

Figure 5. PIEZO1 and YAP are expressed in human oral squamous cell carcinoma tissues.

(A) Oral squamous cell carcinoma tissues ($n = 45$) were stained with anti-PIEZO1 antibody and hematoxylin. PIEZO1-positive signal intensity was classified as follows: negative; weak; strong. Percentages of three groups based on PIEZO1-positive signal intensity in the non-tumor regions and the tumor lesions are shown in the panel. Boxes show enlarged images. (B) Oral squamous cell carcinoma tissues ($n = 45$) were stained with anti-PIEZO1, anti-YAP and anti-Ki-67 antibodies, and hematoxylin. Solid boxes show enlarged images. Numbers of cases with YAP-positive in non-tumor region or tumor lesion, and PIEZO1 intensity strong or negative/weak was shown. Scale bars, 500 μm (A), 100 μm (B).

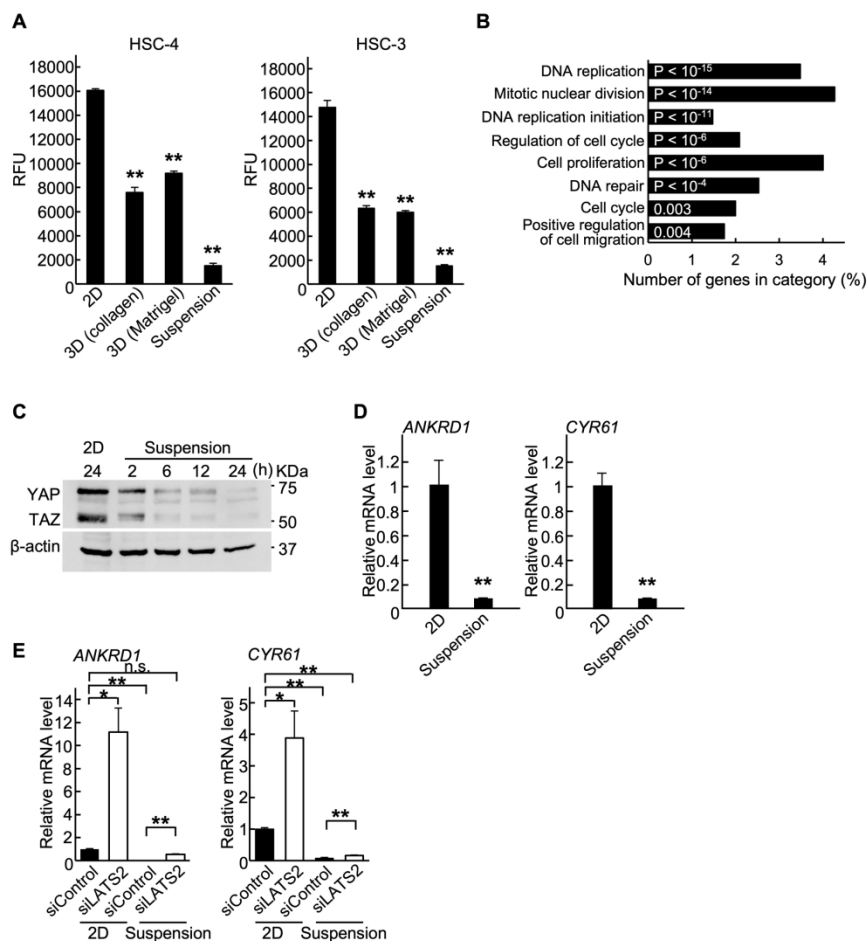


Figure 1. Proliferative capabilities of oral squamous cell carcinoma cells in the three-dimensional and suspension cultures.

190x254mm (300 x 300 DPI)

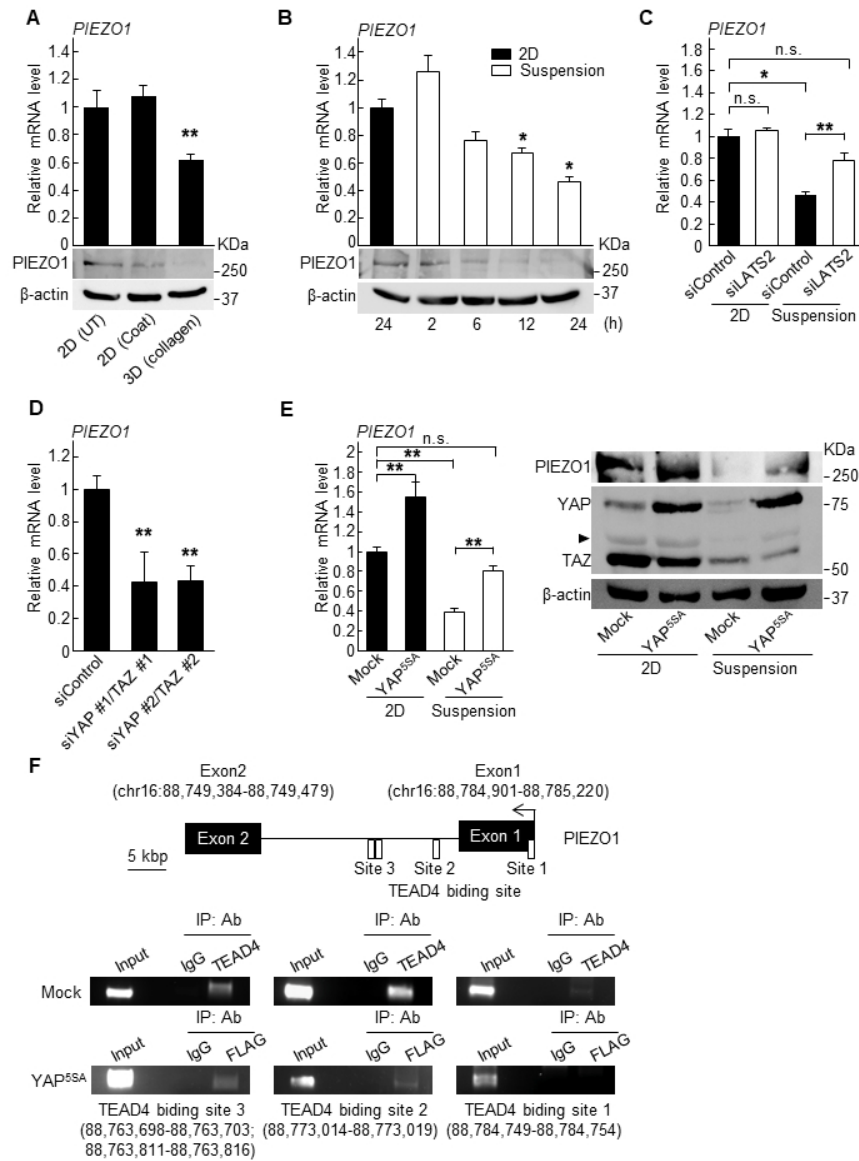


Figure 2. PIEZO1 is a transcriptional target of YAP signaling in oral squamous cell carcinoma cells.

190x254mm (96 x 96 DPI)

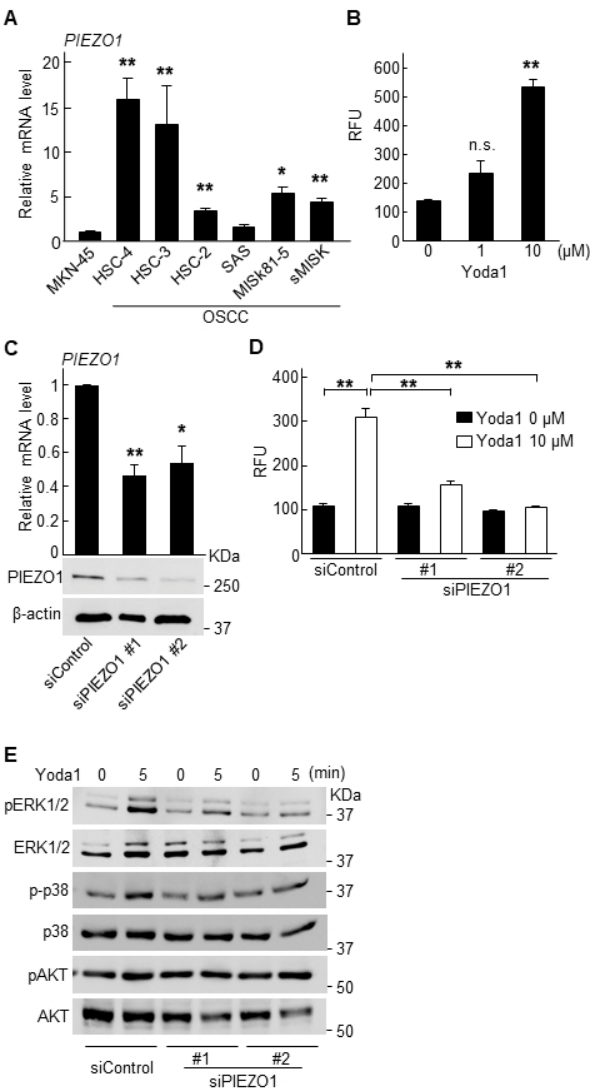


Figure 3. PIEZO1 expression is involved in agonist-induced ERK1/2 and p38 MAPK activation.

190x254mm (96 x 96 DPI)

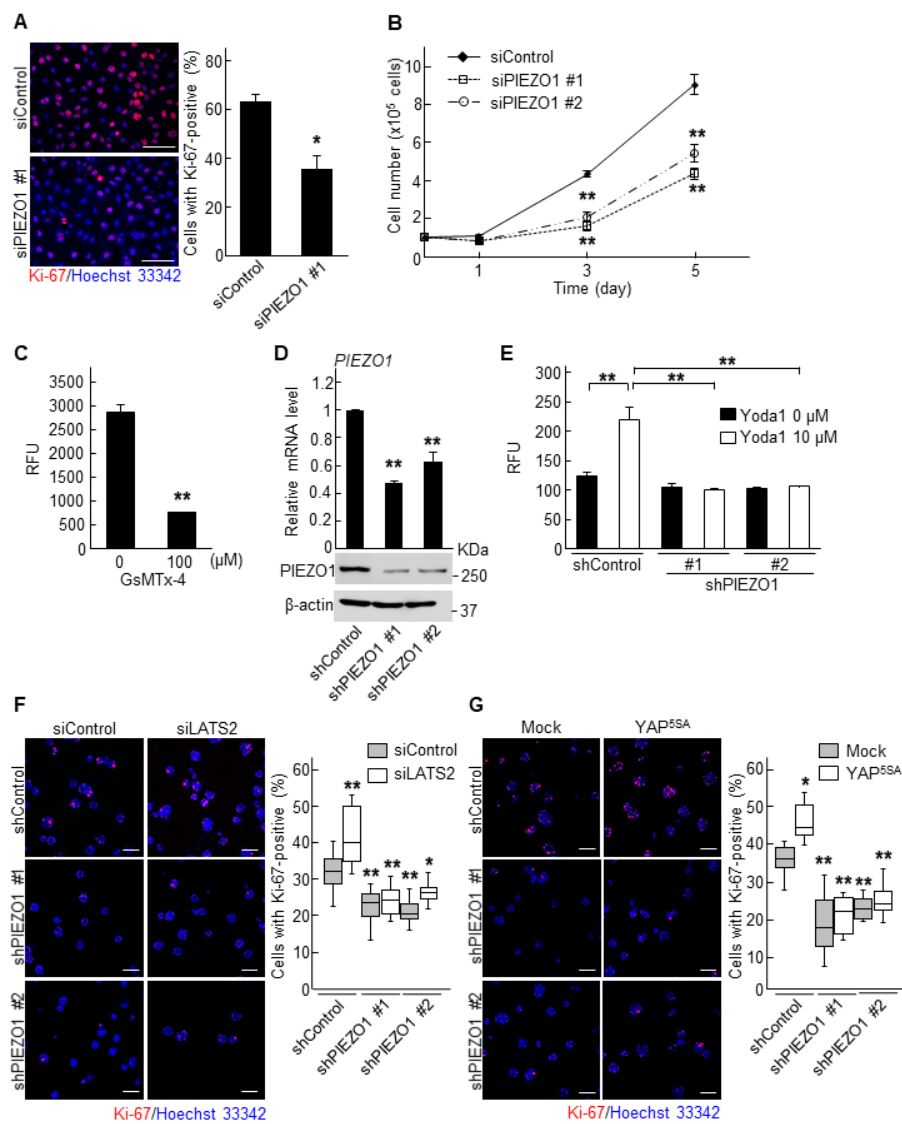


Figure 4. PIEZO1 expression is required for cell proliferation in oral squamous cell carcinoma cells.

190x254mm (96 x 96 DPI)

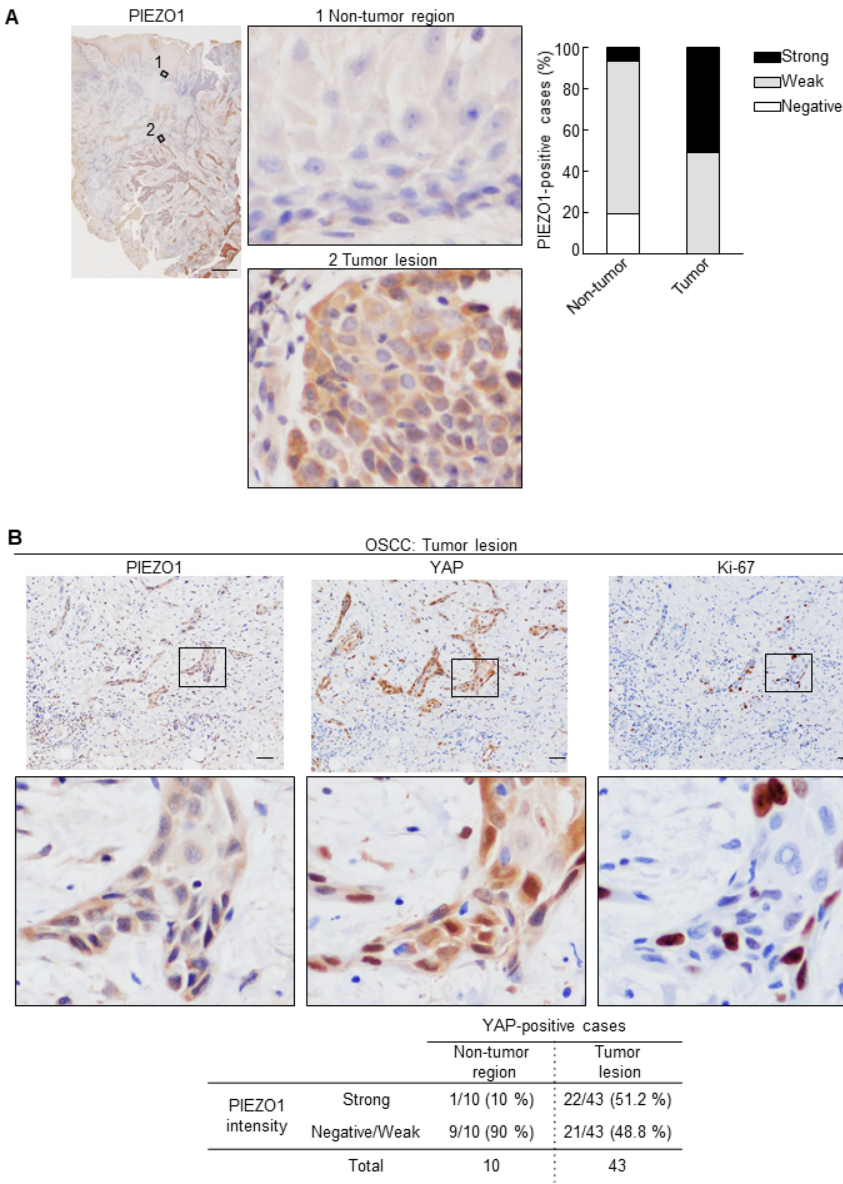


Figure 5. PIEZO1 and YAP are expressed in human oral squamous cell carcinoma tissues.

190x254mm (96 x 96 DPI)

Supporting Information

YAP signaling induces PIEZO1 expression to promote oral squamous cell carcinoma cell proliferation

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Supplementary Materials and methods

Cell lines and reagents

Anti-PIEZO1 polyclonal antibody (15939-1-AP) (for western blotting and immunohistochemistry) was obtained from Proteintech (Rosemont, IL, USA). Anti-YAP/TAZ (8418S) (for western blotting and immunocytochemistry), anti-phospho-ERK1/2 (4370S), anti-ERK1/2 (4695S), anti-phospho-AKT (4060S), anti-pan-AKT (4691S) (for western blotting) and anti-cleaved caspase-3 (9664S) (for immunocytochemistry) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-Ki-67 (ab15580) and anti-PCNA (ab92552) (for immunocytochemistry and immunohistochemistry) antibodies were from Abcam (Cambridge, UK). Anti-YAP (sc-101199) (for immunohistochemistry) antibody was from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-phospho-p38 (612280) and anti-p38 (612168) (for western blotting) antibodies were from BD Biosciences (San Jose, CA, USA). Anti-β-actin (A5441) (for western blotting) antibody was from Sigma-Aldrich (Steinheim, Germany). Type I collagen solution was purchased from Nippi, Inc. (Tokyo, Japan). Phalloidin, and Simvastatin and Yoda1 were from Invitrogen and Sigma-Aldrich, respectively. GsMTx-4 was from Abcam.

Plasmid construction and infection using lentivirus harboring a cDNA or shRNA

To generate OSCC cells that stably express YAP^{5SA} or PIEZO1 shRNA, parental cells (5 × 10⁴ cells/well in a 12-well plate) were treated with lentivirus and 10 µg/ml polybrene.

The cells were then centrifuged at $1080 \times g$ for 1 h, and incubated for another 24 h. The cells that demonstrated stable expression of YAP^{5SA}, PIEZO1 #1 or #2 shRNA, were selected and maintained in culture medium containing 5 $\mu\text{g/ml}$ Blasticidin S (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) [1].

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described previously [2]. HSC-4 cells expressing Mock or YAP^{5SA} were cross-linked with 1% (v/v) formaldehyde for 20 min at room temperature. The cells were lysed with sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% (w/v) SDS) and sonicated to shear DNA to a size range between 200 and 1000 bp. Sheared chromatin samples were diluted in ChIP dilution buffer (16.7 mM Tris-HCl (pH 8.0), 167 mM NaCl, 1.2 mM EDTA, and 1.1% (w/v) Triton X-100) supplemented with protease inhibitors, and precleared with salmon sperm DNA/protein A-agarose (Millipore) and incubated for 4 h at 4°C with 10 μg of anti-TEAD4 (ab58310, Abcam), anti-FLAG (F1804, Sigma) or negative control IgG (5415S, Cell Signaling Technology). Immunocomplexes were absorbed with salmon sperm DNA/protein A-agarose beads, and washed once with high salt buffer (20 mM Tris-HCl (pH 8.1), 500 mM NaCl, 0.1% (w/v) SDS, 1% (w/v) TritonX-100, and 2 mM EDTA), once with LiCl buffer (10 mM Tris-HCl (pH 8.1), 0.25 M LiCl, 1 mM EDTA, 1% (w/v) deoxycholic acid, and 1% (w/v) Nonidet P-40), and three times with TE buffer (10 mM Tris-HCl (pH 8.1), and 1 mM EDTA). Immune complexes extracted in elution buffer (1% (w/v) SDS and 100 mM NaHCO_3) were incubated for 4 h at 65°C to revert DNA-protein

cross-links. Then the DNA was extracted by incubation in proteinase K (final concentration of 50 mg/ml) buffer for 1 h at 45°C. The purified DNA was used in PCR to assess the presence of target sequences. Forward and reverse primers were as follows: fragment containing TEAD4-binding site 1, 5'-GCGCTCATGCTTCAGGAATGC-3' and 5'-CTGCTGGCTGGTGAGTGG-3; TEAD4-binding site 2, 5'-TGCCTGGAAGACTGTGTCAG-3' and 5'-AATGAGACCCAGGAGGAAGGAG-3; TEAD4-binding site 3, 5'-TAGGTGCTCCCTGACACTCTCTG-3' and 5'-CCAAAGCCTTCATTTTCAGGA-3; ANKRD1-binding site, 5'-GAGGGGAGGACAAGCTAACC-3' and 5'-CGATGTGATCACCACCAAAG-3 [3].

Intracellular calcium measurement

To examine agonist-dependent Ca²⁺ influx, cells were loaded with Calcium Kit-Fluo-4 AM (FUJIFILM Wako) for 1 h at 37°C according to the manufacturer's instructions [4]. The measurements were performed with FlexStation 3 microplate reader using the 'Flex' read mode, and images at 485 nm excitation/525 nm emission were collected every 2 sec. After the cells were stimulated by compound, fluorescence measurements were carried out for 5 min. And then, we confirmed the vitality of the cells, in which propidium iodide (PI) (Wako) was not stained (data not shown).

Immunocytochemistry

HSC-4 cells were fixed in 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature. After cells were permeabilized in PBS containing 0.5% (w/v) Triton X-100 and 40 mg/ml BSA (Wako) for 30 min at room temperature, and then blocked with 0.2% BSA to prevent non-specific binding for 30 min at room temperature. The cells were incubated with primary antibodies (used at 1:100 for Ki-67, used at 1:300 for PCNA, used at 1:400 for cleaved caspase-3) at 4°C overnight, and then incubated with secondary antibodies for 3 h at room temperature. Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Dojindo, Kumamoto, Japan) or Hoechst 33342 (Dojindo) for 15 min at room temperature [5]. The samples were viewed with Z-X800 fluorescence microscope (Keyence, Osaka, Japan).

Immunohistochemistry

Immunohistochemical staining was performed on 5- μ m-thick paraffin sections. Antigen retrieval, elimination of the endogenous peroxide activity, and blocking were carried out as previously described [4]. Then the sections were reacted with each primary antibody (used at 1:250 for PIEZO1, used at 1:100 for YAP, used at 1:300 for Ki-67, used at 1:500 for PCNA) at 4°C overnight. The sections were incubated with secondary antibody (Histofine Simple Stain MAX PO, Nichirei, Tokyo, Japan) for 1 h at RT. The immunoreactivity was visualized with a solution of DAB substrate solution. Subsequently, the sections were counterstained with hematoxylin.

Microarray analysis

Microarray analyses were performed using HSC-4 cells cultured in 2D culture or suspension culture as previously described [5]. 1 µg of total RNA was used for microarray analysis. The mRNA expression profile was produced by Cell Innovator Inc. (Fukuoka, Japan) using gene microarray technology (SurePrint G3 Human Gene Expression Microarray 8 × 60 K v3, Agilent Technologies, Santa Clara, CA, USA). The raw data reported in this study was deposited in NCBI GEO under accession number (GSE150044). Gene Ontology (GO) analysis was performed using DAVID database (<http://david.abcc.ncifcrf.gov/>).

SUPPLEMENTAL REFERENCES

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Supplementary Figure Legends

Supplementary Figure 1. The effect of YAP/TAZ signaling on OSCC cells.

(A) HSC-4 cells were transfected with control or two different YAP/TAZ siRNAs for 48 h. Cell lysates were probed with anti-YAP/TAZ and anti- β -actin antibodies (upper left panel). Molecular weight size was noted referring to molecular weight marker. *ANKRD1* and *CYR61* mRNA levels in the cells were also measured by quantitative RT-PCR. Relative levels of *ANKRD1* and *CYR61* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with levels in control siRNA transfected cells (upper middle panels). The cells were cultured in the presence of 5% FBS for 48 h, and relative cell numbers were quantified using the CyQUANT cell proliferation assay (upper right panel). RFU: relative fluorescence units. HSC-4 cells were cultured without or with 1 and 2 μ M Simvastatin. The cells were cultured in the presence of 5% FBS for the indicated numbers of days, and cell numbers were counted (lower left panel). *ANKRD1* and *CYR61* mRNA levels in the cells were also measured by quantitative RT-PCR. Relative levels of *ANKRD1* and *CYR61* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with levels in control siRNA transfected cells (lower right panel). (B) *ANKRD1* and *CYR61* mRNA levels in HSC-3 and MISC81-5 cells cultured in 2D or suspension culture for 24 h were measured by quantitative RT-PCR. Relative levels of *ANKRD1* and *CYR61* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with expression in 2D cells. (C) HSC-4 cells were transfected with control or LATS2 siRNA for 48 h, and *LATS2* mRNA levels were measured by quantitative RT-PCR. Relative *LATS2* mRNA levels were normalized

by *GAPDH* and expressed as fold-changes compared with levels in control siRNA transfected cells. (D) HSC-4 cells were transfected with control or LATS2 siRNA for 48 h, and then cells were cultured in 2D or suspension culture for 24 h. *AURKB* and *FOXMI* mRNA levels were measured by quantitative RT-PCR. Relative levels of *AURKB* and *FOXMI* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with levels in 2D control cells. Results are shown as means \pm s.d. of three independent experiments. * $P < 0.05$, ** $P < 0.01$. n.s.: not significant.

Supplementary Figure 2. YAP signaling regulates PIEZO1 expression in OSCC cells.

(A) The expression of *PIEZO1*, *PIEZO2*, *AREG* or *CDK6* in 518 tumor lesions and 44 non-tumor regions in head and neck region was evaluated using TCGA dataset. Correlation between *PIEZO1* and *AREG* or *CDK6* mRNA was examined in the tumor lesion. RPKM, reads per kilobase per million reads. Spearman's rank rho was used to calculate the "r" correlation coefficient. (B) *PIEZO1* mRNA levels in HSC-3 and MISC81-5 cells cultured in 2D or suspension culture for 24 h were measured by quantitative RT-PCR. Relative levels of *PIEZO1* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with expression in 2D cells. (C) HSC-3 cells were transfected with control or two different YAP/TAZ siRNAs for 48 h, and then cells were cultured in 2D culture for 3 h. *PIEZO1* mRNA levels were measured by quantitative RT-PCR. Relative *PIEZO1* mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in control siRNA transfected cells (left panel). HSC-3 cells expressing mock or YAP^{SSA} were cultured in 2D or suspension culture

for 24 h and *PIEZO1* mRNA levels were measured by quantitative RT-PCR. Relative levels of *PIEZO1* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with expression in 2D control cells (right panel). (D) HSC-4 cells expressing mock or YAP^{5SA} were cultured in 2D or suspension culture for 24 h, and *ANKRD1* and *CYR61* mRNA levels were measured by quantitative RT-PCR. Relative levels of *ANKRD1* and *CYR61* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with expression in 2D control cells. (E) Genomic views of TEAD1, TEAD4, H3K27ac and H3K9ac ChIP enrichment at human *PIEZO1* gene obtained from Cistrome Data Browser database. The *PIEZO1* gene enhancer region contains three predicted TEAD4-binding sites. Chromatin from HSC-4 cells expressing mock was immunoprecipitated with anti-control IgG or anti-TEAD4 antibody, and the precipitates were analyzed by PCR for TEAD4 binding site of the *ANKRD1* gene promoter. Results are shown as means \pm s.d. of three independent experiments. * $P<0.05$, ** $P<0.01$. n.s.: not significant.

Supplementary Figure 3. The effect of PIEZO1 on YAP/TAZ signaling.

(A) The expression of *PIEZO1* and *PIEZO2* in cell lines was evaluated using Cancer Cell Line Encyclopedia dataset. (B) *PIEZO2* mRNA levels were measured in OSCC cell lines (HSC-4, HSC-3, HSC-2, SAS, MISK81-5 and sMISK cells), A549 and MKN-45 cells by quantitative RT-PCR. Relative levels of *PIEZO2* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with expression in control A549 cells. (C) HSC-4 cells were pre-loaded with Fluo-4-AM and were exposed without or with 1

and 10 μ M Yoda1. Then, intracellular Ca^{2+} influx was measured. RFU: relative fluorescence units. (D) HSC-4 cells were transfected with control or two different PIEZO1 siRNAs for 48 h, and *PIEZO2*, *ANKRD1* and *CYR61* mRNA levels were measured by quantitative RT-PCR. Relative *PIEZO2*, *ANKRD1* and *CYR61* mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in control siRNA transfected cells (upper panels). HSC-4 cells were transfected with control or two different PIEZO1 siRNAs for 48 h, and the cells were stained with anti-YAP/TAZ antibody, phalloidin and Hoechst 33342. Cells with nuclear YAP/TAZ are expressed as the percentage of YAP/TAZ-positively stained cells compared with total Hoechst 33342-stained cells ($n = 1,516$) (lower panels). (E) HSC-4 cells transfected with control or two different PIEZO1 siRNAs were pre-loaded with Fluo-4-AM and were exposed without or with 10 μ M Yoda1. Then, intracellular Ca^{2+} influx was measured. RFU: relative fluorescence units. Results are shown as means \pm s.d. of three independent experiments. Scale bars, 100 μ m. n.s.: not significant.

Supplementary Figure 4. PIEZO1 regulates cell proliferation of OSCC cells.

(A) HSC-4 cells were transfected with control or PIEZO1 #1 siRNA for 48 h. The cells were stained with anti-PCNA antibody and Hoechst 33342, and then PCNA-positive cells and Hoechst 33342-stained cells were counted, respectively. Results are expressed as the percentage of PCNA-positive cells compared with total Hoechst 33342-stained cells. (B) HSC-4 cells were transfected with control or PIEZO1 #1 siRNA for 48 h. The cells were stained with anti-cleaved caspase-3 antibody and DAPI. (C) HSC-4 cells expressing

control or PIEZO1 #1 and #2 shRNAs were pre-loaded with Fluo-4-AM and were exposed without or with 10 μ M Yoda1. Then, intracellular Ca^{2+} influx was measured. RFU: relative fluorescence units. (D) HSC-4 cells expressing control or PIEZO1 #1 and #2 shRNAs were transfected with control or LATS2 siRNA for 48 h (left panels) or with mock or YAP^{5SA} expression (right panels), and then cells were cultured for 4 days in the 3D Matrigel. The cells were stained with anti-PCNA antibody and Hoechst 33342, and then PCNA-positive cells and Hoechst 33342-stained cells were counted, respectively. Results are expressed as the percentage of PCNA-positively stained cells compared with total Hoechst 33342-stained cells ($n = 14,242$). (E) HSC-4 cells expressing PIEZO1 #1 and #2 shRNAs were transfected with control or LATS2 siRNA for 48 h (left panels) or with mock or YAP^{5SA} expression (right panels), and then cells were cultured in 2D or suspension culture for 24 h. *PIEZO1* or *ANKRD1* mRNA levels were measured by quantitative RT-PCR. Relative *PIEZO1* or *ANKRD1* mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in 2D control cells. Results are shown as means \pm s.d. of three independent experiments. * $P < 0.05$, ** $P < 0.01$. Scale bars, 100 μ m. n.s.: not significant.

Supplementary Figure 5. PIEZO1 is expressed in human oral squamous cell carcinoma tissues.

Oral squamous cell carcinoma tissues ($n = 45$) were stained with anti-PIEZO1 antibody and hematoxylin. PIEZO1-positive signal intensity was classified as follows: negative; weak; strong. Boxes show enlarged images. Scale bars, 100 μ m.

Supplementary Figure 6. YAP is expressed in human oral squamous cell carcinoma tissues.

(A) Oral squamous cell carcinoma tissues ($n = 45$) were stained with anti-YAP antibody and hematoxylin. YAP expression and localization were classified as follows: negative; nucleus/cytoplasm; nucleus. Boxes show enlarged images. (B) Percentages of three groups based on the YAP expression and localization in the tumor lesions and the non-tumor regions are shown in the panel. (C) Oral squamous cell carcinoma tissues ($n = 45$) were stained with anti-YAP, anti-PIEZO1 and anti-Ki-67 antibodies, and hematoxylin. Box shows enlarged images. Scale bars, 50 μm .

Supplementary Figure 7. Co-expression of PIEZO1, YAP, Ki-67 and PCNA in human oral squamous cell carcinoma tissues.

Oral squamous cell carcinoma tissues ($n = 45$) were stained with anti-PIEZO1, anti-YAP, anti-Ki-67 and anti-PCNA antibodies, and hematoxylin. Representative data ($n = 5$) are shown. Scale bars, 20 μm .

Table S1. Designs for siRNAs used in this study.

siRNA	Sequence
human YAP #1	Sense: GACAUCUUCUGGUCAGAGATT Antisense: UCUCUGACCAGAAGAUGUCTT
human YAP #2	Sense: GCUCAUUCCUCUCCAGCUUTT Antisense: AAGCUGGAGAGGAAUGAGCTT
human TAZ #1	Sense: AGGUACUUCCUCAAUCACATT Antisense: UGUGAUUGAGGAAGUACCUTT
human TAZ #2	Sense: CCCAACAGACCCGUUUCCCUGAUUUTT Antisense: AAAUCAGGGAAACGGGUCUGUUGGGTT
human LATS2	Sense: GCCACGACUUAUUCUGGAATT Antisense: UUCCAGAAUAAGUCGUGGCTT
human PIEZO1 #1	Sense: AGAAGAAGAUCGUCAAGUATT Antisense: UACUUGACGAUCUUCUUCUTT
human PIEZO1 #2	Sense: CCAACCUCAUCAGCGACUUTT Antisense: AAGUCGCUGAUGAGGUUGGTT
Randomized control	Sense: CAGUCGCGUUUGCGACUGGTT Antisense: CCAGUCGCAAACGCGACUGTT

Table S2. Quantitative RT-PCR primers used in this study.

Gene	Primers
human LATS2	Forward 5'-GCGATTCGTTTGCGTCCTAC-3' Reverse 5'-CCGGGGAAAAGGTAGTCCAC-3'
human PIEZO1	Forward 5'-AGGAACAGGCAGGACAGCTA-3' Reverse 5'-GCGTGTCAGCTCATCCACTA-3'
human PIEZO2	Forward 5'-GACGACTCTGCAGGCAAGAA-3' Reverse 5'-AGAAGAGCAGCAGAAATCGGG-3'
human ANKRD1	Forward 5'-ACGCCAAAGACAGAGAAGGA-3' Reverse 5'-TTCTGCCAGTGTAGCACCAG-3'
human CYR61	Forward 5'-TCCGAGGTGGAGTTGACGAG-3' Reverse 5'-AGCACTGGGACCATGAAGTTG-3'
human AURKB	Forward 5'-CCTCCCTGAGGAGGAAGACA-3' Reverse 5'-TGCACCACAGATCCACCT TC-3'
human FOXM1	Forward 5'-GTTTATCAGTGCTGCTAGCTGAGG-3' Reverse 5'-CAGGCTGGATTTCTTCCTCCTTG-3'
human GAPDH	Forward 5'-GCACCGTCAAGGCTGAGAAC-3' Reverse 5'-TGGTGAAGACGCCAGTGGA-3'

Table S3. Gene enrichment analysis in HSC-4 cells.

Term	Count	%	<i>p</i> -Value
DNA replication	40	3.487358	1.53E-16
G1/S transition of mitotic cell cycle	32	2.789887	7.64E-16
rRNA processing	46	4.010462	1.28E-15
Mitotic nuclear division	49	4.272014	4.49E-15
Cell division	59	5.143854	8.91E-15
Sister chromatid cohesion	30	2.615519	5.89E-14
DNA replication initiation	17	1.482127	1.00E-12
Regulation of transcription involved in G1/S transition of mitotic cell cycle	11	0.959024	1.07E-07
Regulation of cell cycle	24	2.092415	1.42E-07
Cell proliferation	46	4.010462	1.46E-07
Chromosome segregation	17	1.482127	3.99E-07
De novo' IMP biosynthetic process	6	0.523104	2.44E-06
DNA unwinding involved in DNA replication	7	0.610288	3.94E-06
Purine nucleotide biosynthetic process	7	0.610288	1.58E-05
Purine ribonucleoside monophosphate biosynthetic process	7	0.610288	2.80E-05
Mitotic sister chromatid segregation	9	0.784656	3.03E-05
Telomere maintenance via recombination	10	0.87184	3.08E-05
Response to drug	35	3.051439	3.63E-05
Response to unfolded protein	11	0.959024	5.44E-05
DNA repair	29	2.528335	5.73E-05
DNA synthesis involved in DNA repair	10	0.87184	6.71E-05
DNA strand elongation involved in DNA replication	7	0.610288	7.45E-05
Pyrimidine nucleoside biosynthetic process	5	0.43592	1.10E-04
Spindle organization	7	0.610288	1.14E-04
Organ regeneration	11	0.959024	1.50E-04
Cellular response to hypoxia	16	1.394943	1.62E-04
rRNA transcription	6	0.523104	2.46E-04
Sprouting angiogenesis	8	0.697472	2.46E-04
Ribosomal large subunit biogenesis	8	0.697472	2.46E-04
Strand displacement	8	0.697472	3.21E-04
Canonical glycolysis	8	0.697472	3.21E-04
Response to hypoxia	22	1.918047	3.34E-04
G2/M transition of mitotic cell cycle	19	1.656495	3.56E-04
Positive regulation of gene expression, epigenetic	12	1.046207	3.87E-04
Response to starvation	9	0.784656	4.10E-04
DNA replication checkpoint	5	0.43592	4.72E-04

Double-strand break repair via homologous recombination	13	1.133391	5.05E-04
Mitotic metaphase plate congression	9	0.784656	6.12E-04
Positive regulation of establishment of protein localization to telomere	5	0.43592	8.14E-04
Positive regulation of telomerase RNA localization to Cajal body	6	0.523104	8.16E-04
Maturation of LSU-rRNA	6	0.523104	8.16E-04
Mitotic spindle organization	8	0.697472	8.26E-04
Regulation of signal transduction by p53 class mediator	17	1.482127	9.05E-04
Maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	8	0.697472	0.001244
Positive regulation of telomere maintenance via telomerase	8	0.697472	0.001244
Chaperone mediated protein folding requiring cofactor	5	0.43592	0.0013
Purine nucleobase biosynthetic process	4	0.348736	0.001392
Cellular response to DNA damage stimulus	23	2.005231	0.001706
CENP-A containing nucleosome assembly	9	0.784656	0.001733
DNA duplex unwinding	9	0.784656	0.002021
Double-strand break repair	11	0.959024	0.002456
Toxin transport	8	0.697472	0.002563
Ribosome biogenesis	8	0.697472	0.002563
Cell cycle	23	2.005231	0.0029
Spliceosomal snRNP assembly	7	0.610288	0.003093
Cytokinesis	9	0.784656	0.003567
Mitotic cytokinesis	7	0.610288	0.003727
Protein sumoylation	15	1.307759	0.003749
Protein folding in endoplasmic reticulum	5	0.43592	0.003892
Regulation of cyclin-dependent protein serine/threonine kinase activity	8	0.697472	0.00411
Cellular response to gamma radiation	6	0.523104	0.004234
Positive regulation of cell migration	20	1.743679	0.004437
Response to testosterone	7	0.610288	0.004452
DNA damage checkpoint	7	0.610288	0.004452
Positive regulation of mitotic cell cycle spindle assembly checkpoint	4	0.348736	0.004493
Maturation of SSU-rRNA	5	0.43592	0.005221
Mitotic cell cycle checkpoint	7	0.610288	0.006207
Lactation	8	0.697472	0.006279
Protein heterotetramerization	8	0.697472	0.006279
Regulation of chromosome segregation	4	0.348736	0.006905
Positive regulation of protein localization to Cajal body	4	0.348736	0.006905

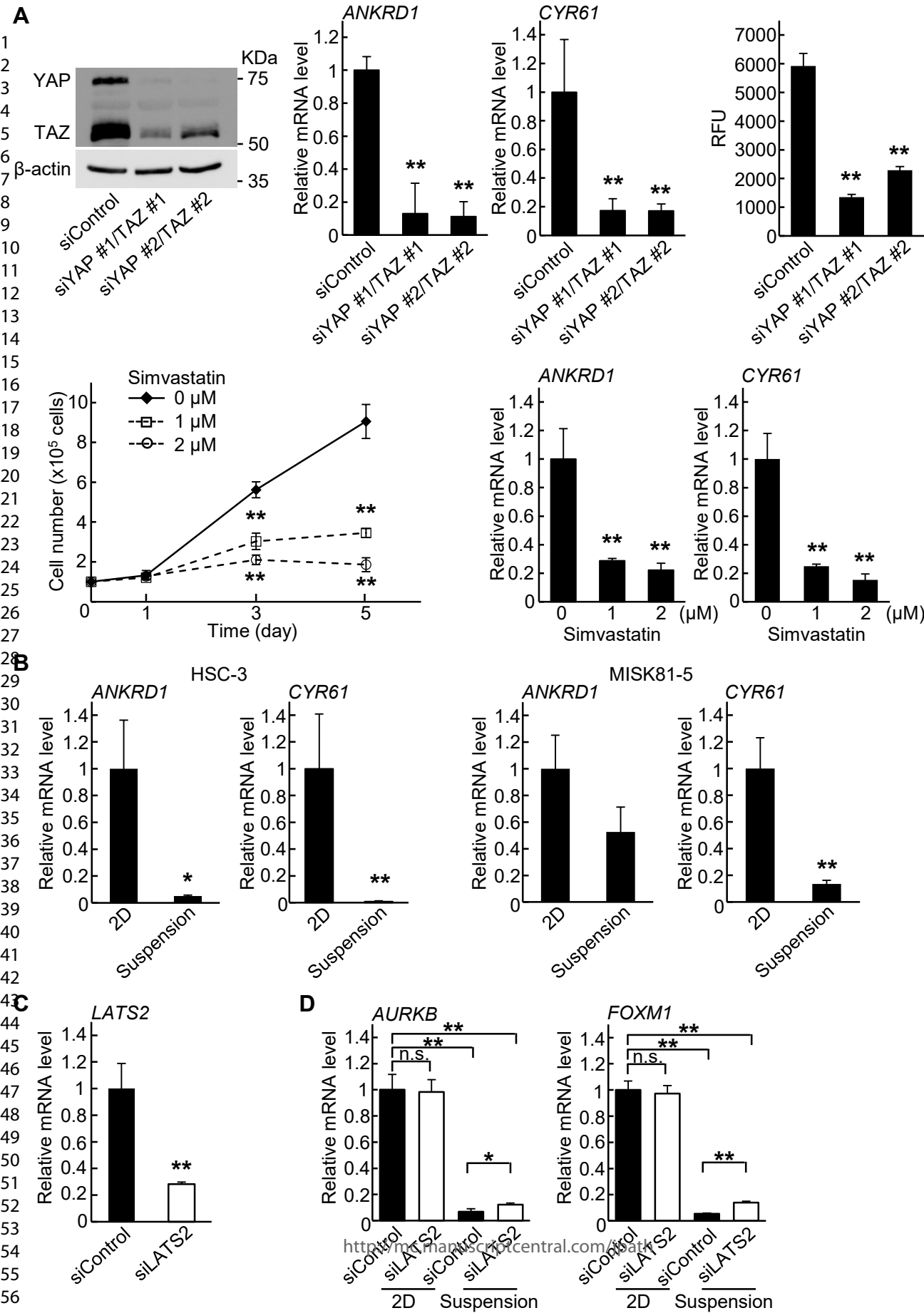
Mitotic spindle midzone assembly	4	0.348736	0.006905
Endonucleolytic cleavage in ITS1 to separate SSU-rRNA from 5.8S rRNA and LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	4	0.348736	0.006905
Negative regulation of B cell apoptotic process	4	0.348736	0.006905
Regulation of dendrite development	4	0.348736	0.006905
Cellular response to UV	8	0.697472	0.008136
Mitotic nuclear envelope disassembly	8	0.697472	0.008136
Histone H3-K9 dimethylation	3	0.261552	0.008212
De novo' UMP biosynthetic process	3	0.261552	0.008212
Epithelial to mesenchymal transition	7	0.610288	0.008417
Ribosomal small subunit biogenesis	5	0.43592	0.008719
Cerebral cortex development	9	0.784656	0.009245
Base-excision repair	7	0.610288	0.009711
Cholesterol metabolic process	10	0.87184	0.00975
Attachment of spindle microtubules to kinetochore	4	0.348736	0.009951
Maturation of LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	4	0.348736	0.009951
ATF6-mediated unfolded protein response	4	0.348736	0.009951
Protein maturation by protein folding	4	0.348736	0.009951
Nucleosome assembly	14	1.220575	0.010831
Negative regulation of transcription, DNA-templated	40	3.487358	0.010879
DNA biosynthetic process	6	0.523104	0.010982
DNA recombination	11	0.959024	0.01261
Positive regulation of vascular endothelial growth factor production	6	0.523104	0.0129
Microtubule polymerization	4	0.348736	0.013658
Microtubule depolymerization	4	0.348736	0.013658
Interstrand cross-link repair	8	0.697472	0.014507
Peptidyl-arginine N-methylation	3	0.261552	0.015843
scaRNA localization to Cajal body	3	0.261552	0.015843
Kidney development	11	0.959024	0.015931
Negative regulation of DNA replication	5	0.43592	0.016355
Nucleoside metabolic process	5	0.43592	0.016355
DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest	9	0.784656	0.016662
Positive regulation of telomerase activity	6	0.523104	0.017387
Double-strand break repair via nonhomologous end joining	9	0.784656	0.018223
Mitotic spindle assembly checkpoint	5	0.43592	0.019601
G2 DNA damage checkpoint	5	0.43592	0.019601
Glutamine metabolic process	5	0.43592	0.019601

Intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator	6	0.523104	0.022801
Negative regulation of transcription from RNA polymerase II promoter	52	4.533566	0.023118
Metaphase plate congression	4	0.348736	0.023124
PERK-mediated unfolded protein response	4	0.348736	0.023124
Positive regulation of fibroblast proliferation	8	0.697472	0.023821
Histone H3-K9 trimethylation	3	0.261552	0.025478
AMP biosynthetic process	3	0.261552	0.025478
Ribonucleoside monophosphate biosynthetic process	3	0.261552	0.025478
Double-strand break repair via break-induced replication	3	0.261552	0.025478
Cellular response to xenobiotic stimulus	3	0.261552	0.025478
Regulation of striated muscle tissue development	3	0.261552	0.025478
Response to inorganic substance	3	0.261552	0.025478
Response to activity	7	0.610288	0.025493
DNA replication-dependent nucleosome assembly	6	0.523104	0.025874
Positive regulation of neutrophil chemotaxis	5	0.43592	0.027211
Positive regulation of protein complex assembly	5	0.43592	0.027211
Chaperone-mediated protein complex assembly	4	0.348736	0.028894
Protein stabilization	14	1.220575	0.029622
Protein folding	17	1.482127	0.031226
NLS-bearing protein import into nucleus	5	0.43592	0.031591
Negative regulation of myoblast differentiation	5	0.43592	0.031591
Regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	5	0.43592	0.031591
Transmembrane receptor protein tyrosine kinase signaling pathway	11	0.959024	0.031708
Epithelial cell differentiation	9	0.784656	0.032184
Glycolytic process	6	0.523104	0.032782
Negative regulation of smooth muscle cell migration	4	0.348736	0.035351
Oxidation-reduction process	43	3.74891	0.036266
Meiotic nuclear division	5	0.43592	0.036359
Regulation of mitotic nuclear division	5	0.43592	0.036359
Cellular response to interleukin-4	5	0.43592	0.036359
Binding of sperm to zona pellucida	6	0.523104	0.036627
Tetrahydrofolate biosynthetic process	3	0.261552	0.03688
DNA replication, synthesis of RNA primer	3	0.261552	0.03688
Endonucleolytic cleavage in 5'-ETS of tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	3	0.261552	0.03688
Positive regulation of exit from mitosis	3	0.261552	0.03688
De novo' pyrimidine nucleobase biosynthetic process	3	0.261552	0.03688

Histone H4-R3 methylation	3	0.261552	0.03688
Response to cold	6	0.523104	0.040738
Mitotic spindle assembly	6	0.523104	0.040738
Microtubule bundle formation	5	0.43592	0.041519
Response to cadmium ion	5	0.43592	0.041519
Positive regulation of phosphorylation	5	0.43592	0.041519
Nucleobase-containing small molecule interconversion	5	0.43592	0.041519
Cell migration	16	1.394943	0.041694
Protein refolding	4	0.348736	0.042483
Response to copper ion	4	0.348736	0.042483
Positive regulation of angiogenesis	12	1.046207	0.042934
Response to ionizing radiation	7	0.610288	0.044815
Nucleobase-containing compound metabolic process	7	0.610288	0.044815
Adherens junction organization	6	0.523104	0.045117
Chromatin silencing at rDNA	6	0.523104	0.045117
Response to endoplasmic reticulum stress	9	0.784656	0.045757
Regulation of cellular response to heat	9	0.784656	0.045757
tRNA methylation	5	0.43592	0.047072
Positive regulation of protein tyrosine kinase activity	5	0.43592	0.047072
DNA replication-independent nucleosome assembly	5	0.43592	0.047072
Box C/D snoRNP assembly	3	0.261552	0.049836
Axon ensheathment	3	0.261552	0.049836
Strand invasion	3	0.261552	0.049836

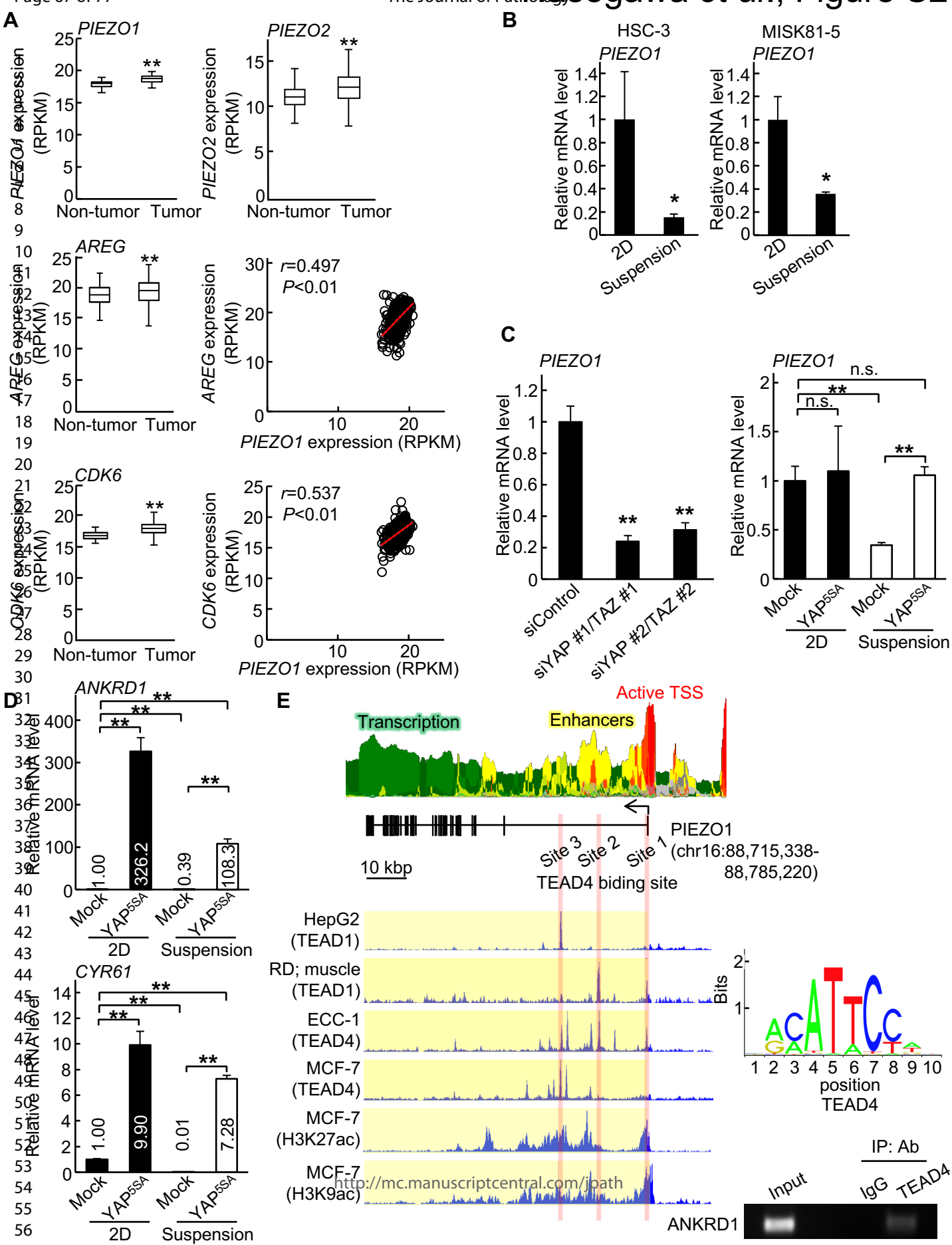
Table S4. List of gene from microarray analysis data of HSC-4 cells cultured in 2D culture and suspension culture.

Gene Symbol	EntrezGeneID	Ratio 2D culture vs suspension culture
AREG	374	0.17
AURKB	9212	0.04
CCND1	595	0.52
CDK6	1021	0.43
E2F1	1869	0.05
FOXM1	2305	0.10
KIF23	9493	0.08
MCM7	4176	0.07
PIEZO1	9780	0.94
PIEZO2	63895	NULL



Supplementary Figure 1. The effect of YAP/TAZ signaling on OSCC cells.

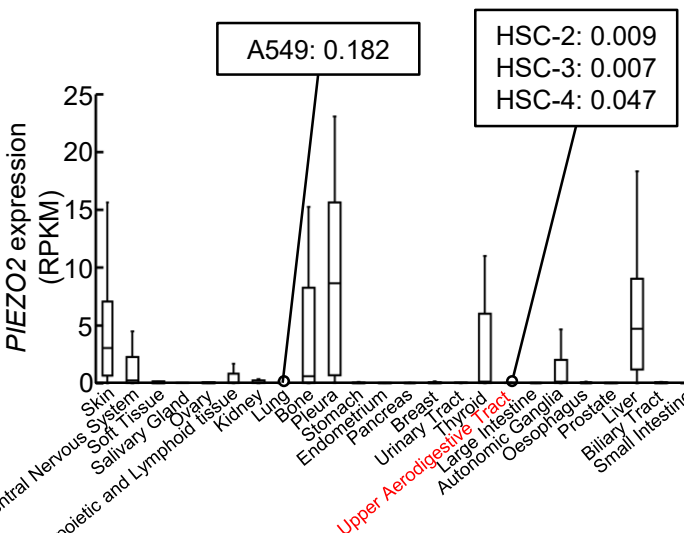
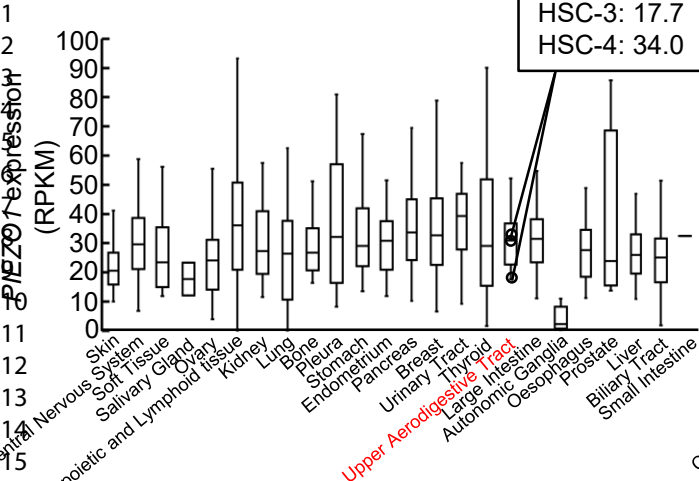
(A) HSC-4 cells were transfected with control or two different YAP/TAZ siRNAs for 48 h. Cell lysates were probed with anti-YAP/TAZ and anti- β -actin antibodies (upper left panel). Molecular weight size was noted referring to molecular weight marker. *ANKRD1* and *CYR61* mRNA levels in the cells were also measured by quantitative RT-PCR. Relative levels of *ANKRD1* and *CYR61* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with levels in control siRNA transfected cells (upper middle panels). The cells were cultured in the presence of 5% FBS for 48 h, and relative cell numbers were quantified using the CyQUANT cell proliferation assay (upper right panel). RFU: relative fluorescence units. HSC-4 cells were cultured without or with 1 and 2 μ M Simvastatin. The cells were cultured in the presence of 5% FBS for the indicated numbers of days, and cell numbers were counted (lower left panel). *ANKRD1* and *CYR61* mRNA levels in the cells were also measured by quantitative RT-PCR. Relative levels of *ANKRD1* and *CYR61* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with levels in control siRNA transfected cells (lower right panel). (B) *ANKRD1* and *CYR61* mRNA levels in HSC-3 and MISK81-5 cells cultured in 2D or suspension culture for 24 h were measured by quantitative RT-PCR. Relative levels of *ANKRD1* and *CYR61* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with expression in 2D cells. (C) HSC-4 cells were transfected with control or LATS2 siRNA for 48 h, and *LATS2* mRNA levels were measured by quantitative RT-PCR. Relative *LATS2* mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in control siRNA transfected cells. (D) HSC-4 cells were transfected with control or LATS2 siRNA for 48 h, and then cells were cultured in 2D or suspension culture for 24 h. *AURKB* and *FOXMI* mRNA levels were measured by quantitative RT-PCR. Relative levels of *AURKB* and *FOXMI* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with levels in 2D control cells. Results are shown as means \pm s.d. of three independent experiments. * $P < 0.05$, ** $P < 0.01$. n.s.: not significant.



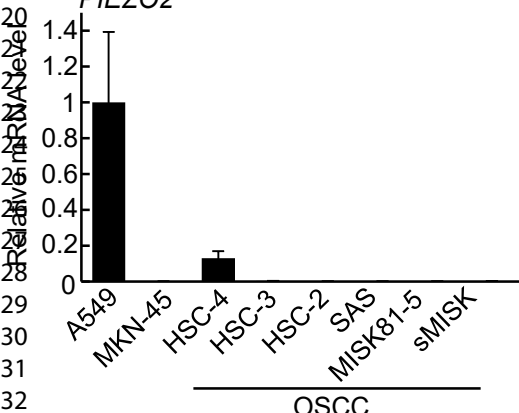
Supplementary Figure 2. YAP signaling regulates PIEZO1 expression in OSCC cells.

(A) The expression of *PIEZO1*, *PIEZO2*, *AREG* or *CDK6* in 518 tumor lesions and 44 non-tumor regions in head and neck region was evaluated using TCGA dataset. Correlation between *PIEZO1* and *AREG* or *CDK6* mRNA was examined in the tumor lesion. RPKM, reads per kilobase per million reads. Spearman's rank rho was used to calculate the "r" correlation coefficient. (B) *PIEZO1* mRNA levels in HSC-3 and MISK81-5 cells cultured in 2D or suspension culture for 24 h were measured by quantitative RT-PCR. Relative levels of *PIEZO1* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with expression in 2D cells. (C) HSC-3 cells were transfected with control or two different YAP/TAZ siRNAs for 48 h, and then cells were cultured in 2D culture for 3 h. *PIEZO1* mRNA levels were measured by quantitative RT-PCR. Relative *PIEZO1* mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in control siRNA transfected cells (left panel). HSC-3 cells expressing mock or YAP^{5SA} were cultured in 2D or suspension culture for 24 h and *PIEZO1* mRNA levels were measured by quantitative RT-PCR. Relative levels of *PIEZO1* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with expression in 2D control cells (right panel). (D) HSC-4 cells expressing mock or YAP^{5SA} were cultured in 2D or suspension culture for 24 h, and *ANKRD1* and *CYR61* mRNA levels were measured by quantitative RT-PCR. Relative levels of *ANKRD1* and *CYR61* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with expression in 2D control cells. (E) Genomic views of TEAD1, TEAD4, H3K27ac and H3K9ac ChIP enrichment at human *PIEZO1* gene obtained from Cistrome Data Browser database. The *PIEZO1* gene enhancer region contains three predicted TEAD4-binding sites. Chromatin from HSC-4 cells expressing mock was immunoprecipitated with anti-control IgG or anti-TEAD4 antibody, and the precipitates were analyzed by PCR for TEAD4 binding site of the *ANKRD1* gene promoter. Results are shown as means \pm s.d. of three independent experiments. * $P < 0.05$, ** $P < 0.01$. n.s.: not significant.

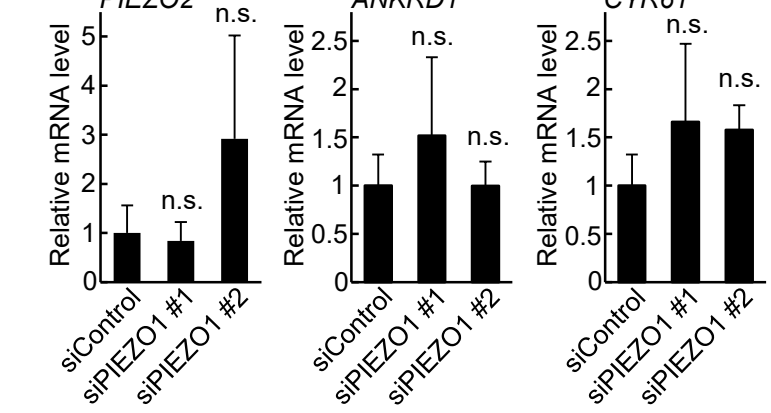
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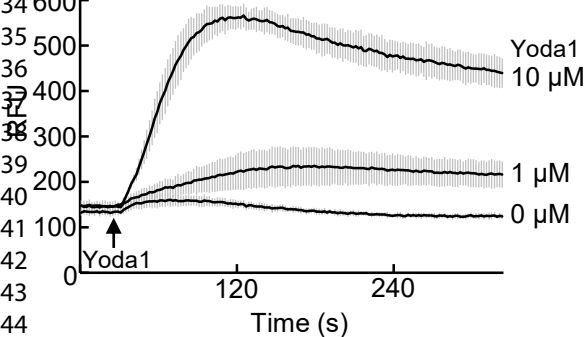
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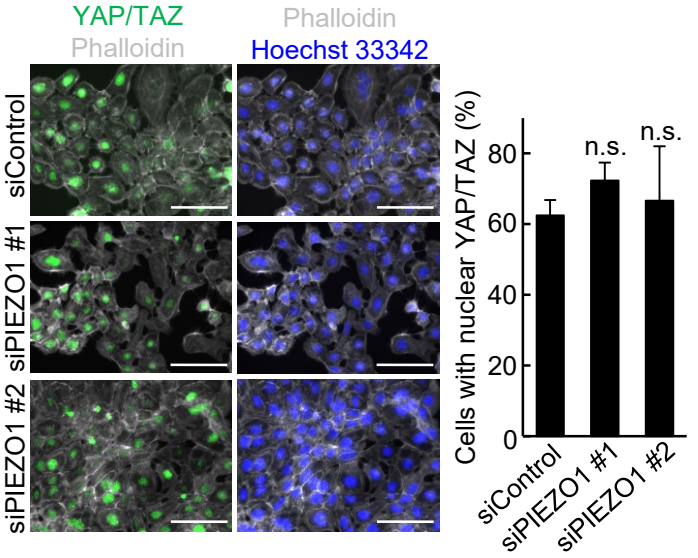
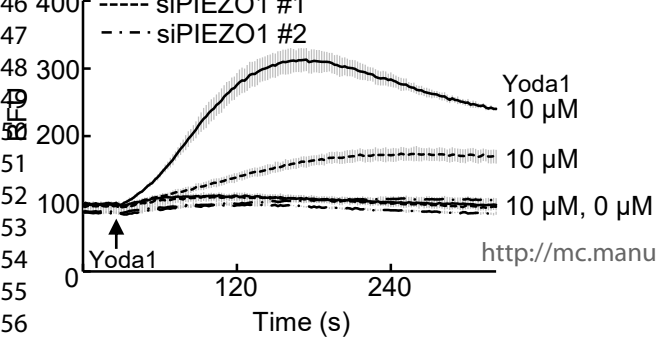
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C



E



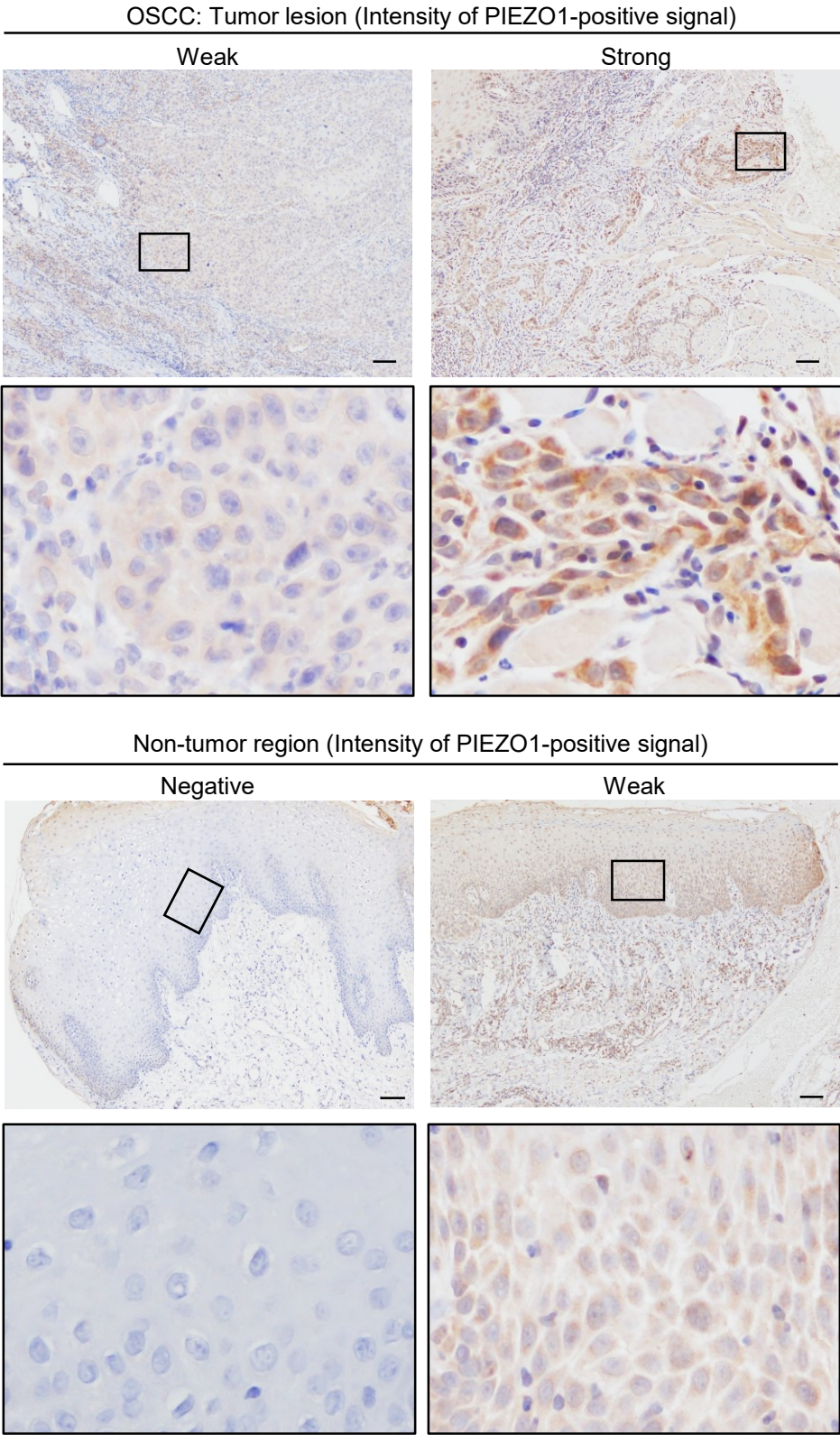
Supplementary Figure 3. The effect of PIEZO1 on YAP/TAZ signaling.

(A) The expression of *PIEZO1* and *PIEZO2* in cell lines was evaluated using Cancer Cell Line Encyclopedia dataset. (B) *PIEZO2* mRNA levels were measured in OSCC cell lines (HSC-4, HSC-3, HSC-2, SAS, MISK81-5 and sMISK cells), A549 and MKN-45 cells by quantitative RT-PCR. Relative levels of *PIEZO2* mRNA expression were normalized to *GAPDH* and expressed as fold-changes compared with expression in control A549 cells. (C) HSC-4 cells were pre-loaded with Fluo-4-AM and were exposed without or with 1 and 10 μ M Yoda1. Then, intracellular Ca^{2+} influx was measured. RFU: relative fluorescence units. (D) HSC-4 cells were transfected with control or two different *PIEZO1* siRNAs for 48 h, and *PIEZO2*, *ANKRD1* and *CYR61* mRNA levels were measured by quantitative RT-PCR. Relative *PIEZO2*, *ANKRD1* and *CYR61* mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in control siRNA transfected cells (upper panels). HSC-4 cells were transfected with control or two different *PIEZO1* siRNAs for 48 h, and the cells were stained with anti-YAP/TAZ antibody, phalloidin and Hoechst 33342. Cells with nuclear YAP/TAZ are expressed as the percentage of YAP/TAZ-positively stained cells compared with total Hoechst 33342-stained cells ($n = 1516$) (lower panels). (E) HSC-4 cells transfected with control or two different *PIEZO1* siRNAs were pre-loaded with Fluo-4-AM and were exposed without or with 10 μ M Yoda1. Then, intracellular Ca^{2+} influx was measured. RFU: relative fluorescence units. Results are shown as means \pm s.d. of three independent experiments. Scale bars, 100 μ m. n.s.: not significant.



Supplementary Figure 4. PIEZO1 regulates cell proliferation of OSCC cells.

(A) HSC-4 cells were transfected with control or PIEZO1 #1 siRNA for 48 h. The cells were stained with anti-PCNA antibody and Hoechst 33342, and then PCNA-positive cells and Hoechst 33342-stained cells were counted, respectively. Results are expressed as the percentage of PCNA-positive cells compared with total Hoechst 33342-stained cells. (B) HSC-4 cells were transfected with control or PIEZO1 #1 siRNA for 48 h. The cells were stained with anti-cleaved caspase-3 antibody and DAPI. (C) HSC-4 cells expressing control or PIEZO1 #1 and #2 shRNAs were pre-loaded with Fluo-4-AM and were exposed without or with 10 μ M Yoda1. Then, intracellular Ca^{2+} influx was measured. RFU: relative fluorescence units. (D) HSC-4 cells expressing control or PIEZO1 #1 and #2 shRNAs were transfected with control or LATS2 siRNA for 48 h (left panels) or with mock or YAP^{5SA} expression (right panels), and then cells were cultured for 4 days in the 3D Matrigel. The cells were stained with anti-PCNA antibody and Hoechst 33342, and then PCNA-positive cells and Hoechst 33342-stained cells were counted, respectively. Results are expressed as the percentage of PCNA-positively stained cells compared with total Hoechst 33342-stained cells ($n = 14,242$). (E) HSC-4 cells expressing PIEZO1 #1 and #2 shRNAs were transfected with control or LATS2 siRNA for 48 h (left panels) or with mock or YAP^{5SA} expression (right panels), and then cells were cultured in 2D or suspension culture for 24 h. *PIEZO1* or *ANKRD1* mRNA levels were measured by quantitative RT-PCR. Relative *PIEZO1* or *ANKRD1* mRNA levels were normalized by *GAPDH* and expressed as fold-changes compared with levels in 2D control cells. Results are shown as means \pm s.d. of three independent experiments. * $P < 0.05$, ** $P < 0.01$. Scale bars, 100 μ m. n.s.: not significant.

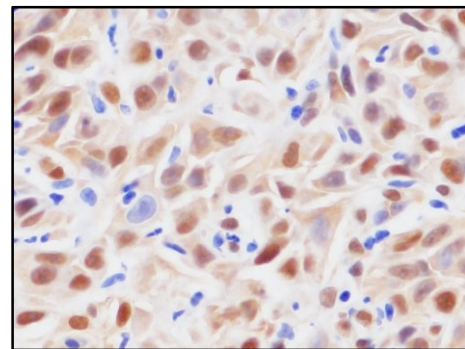
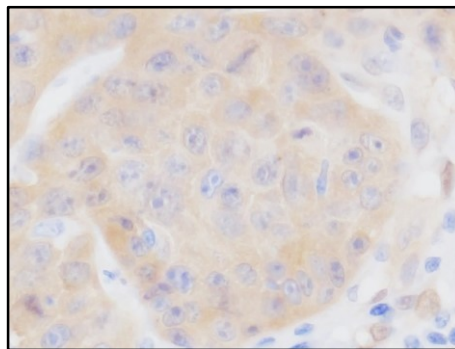
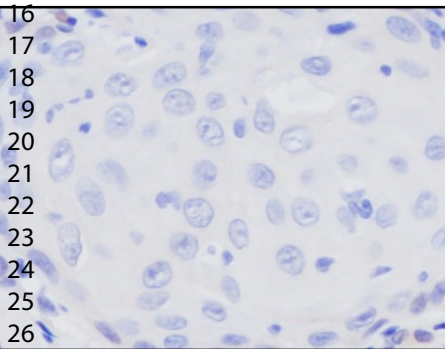
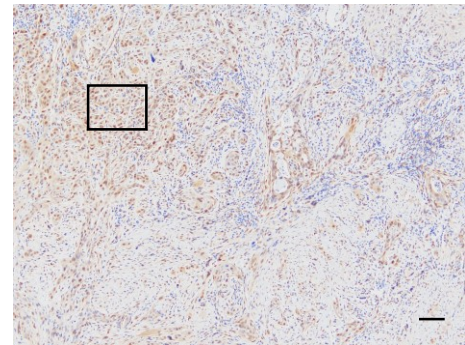
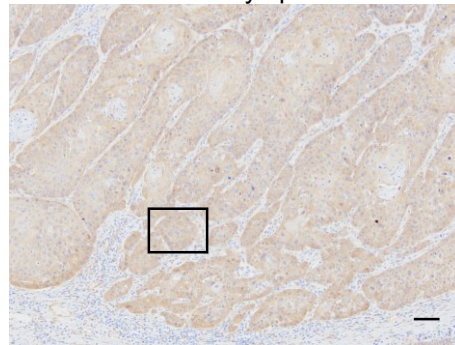
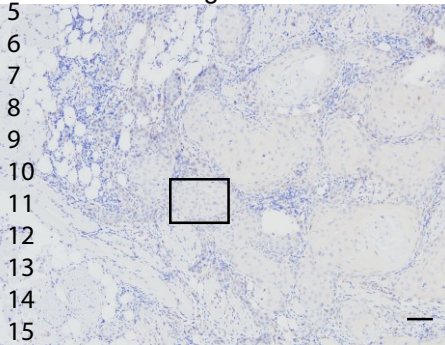


OSCC: Tumor lesion (Expression and localization of YAP-positive signal)

Negative

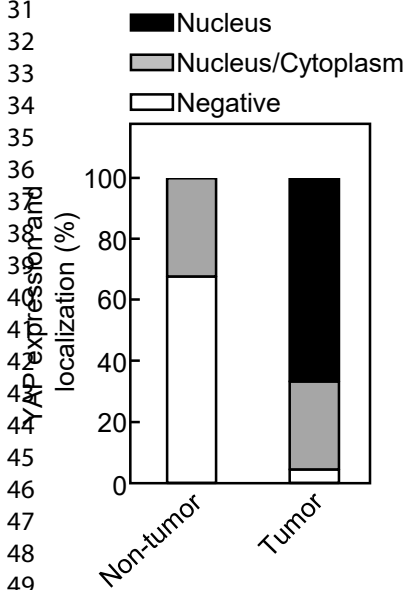
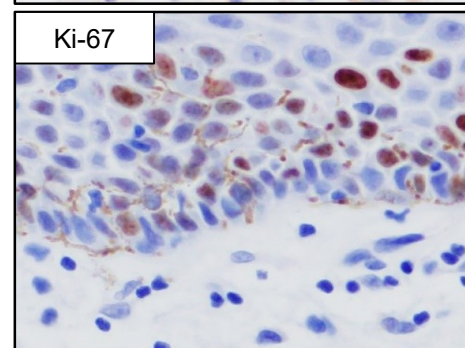
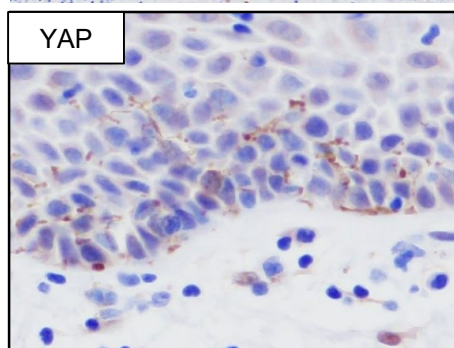
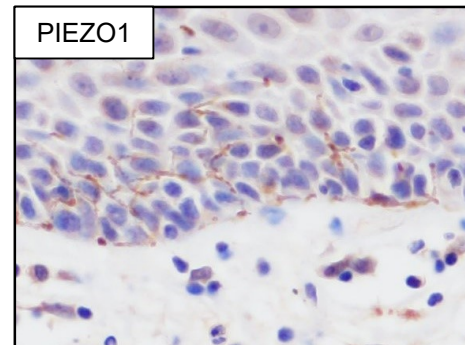
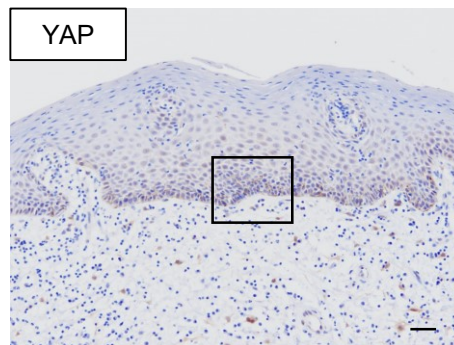
Nucleus/Cytoplasm

Nucleus



C

Non-tumor region



1 **Supplementary Figure 6.** YAP is expressed in human oral squamous cell carcinoma tissues.
2 (A) Oral squamous cell carcinoma tissues ($n = 45$) were stained with anti-YAP antibody and
3 hematoxylin. YAP expression and localization were classified as follows: negative;
4 nucleus/cytoplasm; nucleus. Boxes show enlarged images. (B) Percentages of three groups based
5 on the YAP expression and localization in the tumor lesions and the non-tumor regions are
6 shown in the panel. (C) Oral squamous cell carcinoma tissues ($n = 45$) were stained with anti-
7 YAP, anti-PIEZO1 and anti-Ki-67 antibodies, and hematoxylin. Box shows enlarged images.
8 Scale bars, 50 μ m.
9

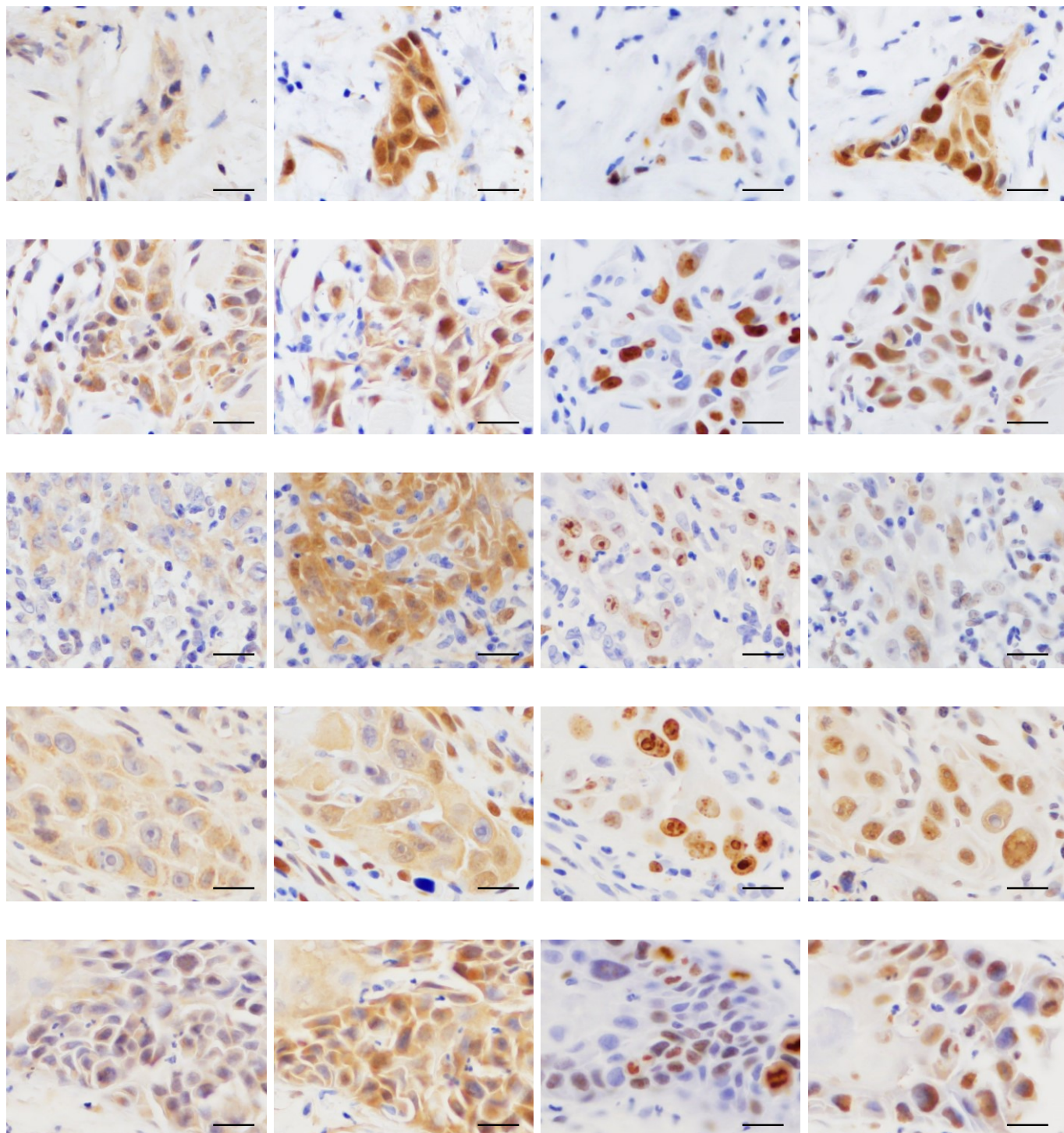
OSCC: Tumor lesion

PIEZO1

YAP

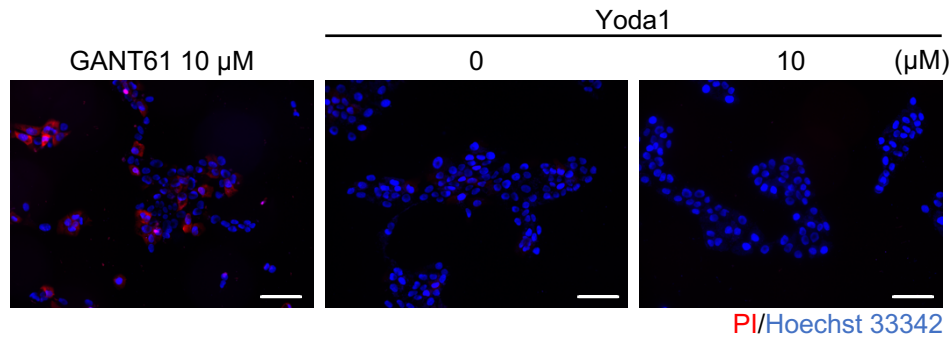
Ki-67

PCNA



Supplementary Figure 7. Co-expression of PIEZO1, YAP, Ki-67 and PCNA in human oral squamous cell carcinoma tissues.

Oral squamous cell carcinoma tissues ($n = 45$) were stained with anti-PIEZO1, anti- YAP, anti- Ki-67 and anti-PCNA antibodies, and hematoxylin. Representative data ($n = 5$) are shown. Scale bars, 20 μm.



Response to Reviewer #1 Figure 1. The vitality of the cells treated with Yoda1. HSC-4 cells were treated with 10 μ M GANT61 in the absence of FBS for 24 h, or without or with 10 μ M Yoda1 in the presence of 10% FBS for 5 min (see Figure 3B). The cells were stained with Propidium Iodide (PI) and Hoechst 33342. GANT61, a GLI-1 and GLI-2 inhibitor, was treated as a positive control [1]. Scale bars, 100 μ m.

[1] Mikami Y, Fujii S, Nagata K, et al. GLI-mediated Keratin 17 expression promotes tumor cell growth through the anti-apoptotic function in oral squamous cell carcinomas. *J Cancer Res Clin Oncol* 2017; **143**: 1381-1393.