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# Prostaglandin $E_2$ receptor EP4 regulates cell migration through Orai1

Kohei Osawa[1,2] | Masanari Umemura[1] | Rina Nakakaji[1,2] | Ryo Tanaka[1] | Rafikul Md Islam[1] | Akane Nagasako[1] | Takayuki Fujita[1] | Utako Yokoyama[1,3] | Toshiyuki Koizumi[2] | Kenji Mitsudo[2] | Yoshihiro Ishikawa[1]

[1]Cardiovascular Research Institute, Yokohama City University Graduate School of Medicine, Yokohama, Japan

[2]Department of Oral and Maxillofacial Surgery, Yokohama City University Graduate School of Medicine, Yokohama, Japan

[3]Department of Physiology, Tokyo Medical University Graduate School of Medicine, Tokyo, Japan

**Correspondence**
Masanari Umemura and Yoshihiro Ishikawa, Cardiovascular Research Institute, Yokohama City University School of Medicine, Yokohama, Japan.
Emails: umemurma@yokohama-cu.ac.jp (M.U.); yishikaw@med.yokohama-cu.ac.jp (Y.I.)

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## Abstract

The EP4 prostanoid receptors are one of four receptor subtypes for prostaglandin $E_2$ ($PGE_2$). Therefore, EP4 may play an important role in cancer progression. However, little information is available regarding their function per se, including migration and the cellular signaling pathway of EP4 in oral cancer. First, we found that mRNA and protein expression of EP4 was abundantly expressed in human-derived tongue squamous cell carcinoma cell lines HSC-3 and OSC-19. The EP4 agonist (ONO-AE1-437) significantly promoted cell migration in HSC-3 cells. In contrast, knockdown of EP4 reduced cell migration. Furthermore, we confirmed that knockdown of EP4 suppressed metastasis of oral cancer cells in the lungs of mice in vivo. Therefore, we focused on the mechanism of migration/metastasis in EP4 signaling. Interestingly, EP4 agonist significantly induced intracellular $Ca^{2+}$ elevation not in only oral cancer cells but also in other cells, including normal cells. Furthermore, we found that EP4 activated PI3K and induced $Ca^{2+}$ influx through Orai1 without activation of store depletion and stromal interaction molecule 1 (STIM1). Immunoprecipitation showed that EP4 formed complexes with Orai1 and TRPC1, but not with STIM. Moreover, the EP4 agonist ONO-AE1-437 phosphorylated ERK and activated MMP-2 and MMP-9. Knockdown of Orai1 negated EP4 agonist-induced ERK phosphorylation. Taken together, our data suggested that EP4 activated PI3K and then induced $Ca^{2+}$ influx from the extracellular space through Orai1, resulting in ERK phosphorylation and promoting cell migration. Migration is regulated by EP4/PI3K/Orai1 signaling in oral cancer.

**KEYWORDS**
calcium, EP4, Orai1, oral cancer, PI3K

## 1 | INTRODUCTION

Oral cancer is the sixth most common cancer.[1] Oral cavity cancer comprises 52% of head and neck cancer cases, and 90% of these are squamous cell carcinoma.[2] Presence of cervical lymph node metastasis is among the most important prognostic factors in oral cancer. Despite advances in the treatment of oral cancer, such as surgery, chemotherapy, and radiation, overall 5-year relative

Osawa and Umemura contributed equally to this work.

survival rate is 63% in the USA.[3] Surgery is the most established modality for initial definitive treatment of oral cancer. However, extended surgery for patients with locally advanced oral squamous cell carcinoma might result in the loss of oral function, including dysphagia, dysarthria and masticatory disturbance. Therefore, more effective treatment with fewer complications for oral cancer needs to be developed.

It is well known that rheumatoid arthritis patients taking aspirin, a COX inhibitor, for a long time have a lower incidence of colorectal cancer.[4] We previously reviewed that inhibition of COX-2 has anticancer effects on colorectal cancer, lung cancer, cervical cancer, breast cancer, prostate cancer and esophageal cancer.[5] COX-2 produces prostaglandin $E_2$ ($PGE_2$). $PGE_2$ is one of the major products generated by the actions of COX on arachidonic acid. It has been established that $PGE_2$ is an important mediator of fever, pain and inflammation. $PGE_2$ functions mainly through G protein-coupled PGE receptors designated EP1, EP2, EP3 and EP4.[6]

The EP4 receptor is one of four receptor subtypes for $PGE_2$. Stimulation of EP4 receptors usually increases intracellular cyclic AMP (cAMP) levels and activates protein kinase A (PKA) through stimulatory G protein (Gs).[6] We previously reviewed that $PGE_2$ and EP4 regulate cell migration or metastasis of colorectal cancer, lung cancer, breast cancer, prostate cancer, ovarian cancer and renal cancer.[5] However, little information is available regarding the function and cellular signaling pathway of EP4 per se, including migration in oral cancer. In cellular signaling of migration/metastasis, previous reports showed that EP4 mediated cell migration/metastasis through PBK/Akt (protein kinase B), cAMP, ERK or β-arrestin 1 in lung cancer, breast cancer and renal cancer.[5] In contrast, there are few reports on the relationships between EP4 and $Ca^{2+}$ signaling in human eosinophil and mouse neuroblastoma (Neuro-2a) cells.[7,8] Therefore, despite intracellular $Ca^{2+}$ signaling regulating diverse cellular functions, including proliferation and cell migration, its role in normal cells is not yet fully understood, let alone in cancer cells, including oral cancer. In particular, little information is available regarding how EP4 mediates the concentration of intracellular $Ca^{2+}$; therefore, we focused on the relationship between EP4 and $Ca^{2+}$ signaling in oral cancer.

Store-operated $Ca^{2+}$ entry (SOCE) is a major mechanism of $Ca^{2+}$ import from the extracellular to the intracellular space.[9] SOCE occurs in both nonexcitable and excitable cells. Physiologically, binding of agonists to G protein-coupled receptor (GPCR) (Gq) or immunoreceptors such as T-cell, B-cell and Fc receptor coupled to phospholipase C (PLC) isoforms, such as PLCβ causes cleavage of acidic lipid phosphatidylinositol 4,5-bisphosphate (PIP2) into the soluble head group inositol-1,4,5-trisphosphate ($IP_3$) and membrane-bound diacylglycerol (DAG).[10] Activation of $IP_3$ receptors on the endoplasmic reticulum (ER) causes a rapid and transient release of $Ca^{2+}$ from the ER store. The resulting decrease of $Ca^{2+}$ concentration in the ER is sensed by the EF-hand motif of stromal interaction molecules (STIM). STIM then translocate to the plasma membrane, where they interact with Orai, highly $Ca^{2+}$ selective channels located on the plasma membrane (PM), leading to $Ca^{2+}$ influx from the extracellular space to restore the $Ca^{2+}$ concentration in ER.[11] SOCE plays important roles in gene expression, proliferation, migration, fluid secretion, mast cell degranulation, platelet aggregation and T-cell activation.[12] We previously showed that SOCE contributes to melanoma progression.[13] However, little information is available regarding the relationship between EP4 signaling and $Ca^{2+}$ signaling in both normal cells and cancer cells.

Therefore, in the present study, we evaluated the mechanism of EP4 and $Ca^{2+}$ signaling in the migration of oral cancer. Herein, we showed that EP4 formed complexes with Orai1 and transient receptor potential canonical 1 (TRPC1), activated PI3K and induced $Ca^{2+}$ influx from the extracellular space through Orai1 without activation of store depletion and STIM1, resulting in ERK phosphorylation and then promoting cell migration in oral cancer cells. Nevertheless, EP4 is coupled to Gα and Gαi.

## 2 | MATERIALS AND METHODS

### 2.1 | Reagents

LY294002 was purchased from Cell Signaling. Prostaglandin E2 ($PGE_2$) and PKI-(14-22)-amide were purchased from Calbiochem. U0126, YM58483 and Xestospongin C were purchased from Sigma-Aldrich. ONO-AE1-437 and ONO-AE3-208 were kindly provided by Ono Pharmaceutical Co., Ltd.[14-16]

### 2.2 | Cell lines

Human oral squamous cell carcinoma cell lines HSC-3 and OSC-19 were purchased from Health Science Research Resources Bank (Japan Health Sciences Foundation).[17,18] The human glioblastoma cell line LN229 was purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank. The human metastatic mammary carcinoma cell line MCF-7 (MCF-7, JCRB0134) was purchased from JCRB Cell Bank.[19] The human cardiac fibroblast adult cell line (No. 6330) (HCF) was purchased from ScienCell Research Laboratories.[19,20]

### 2.3 | Cell viability assay

Cell proliferation assays were carried out with a commercial Cell Proliferation Assay Kit (ATCC) using XTT.[19]

### 2.4 | Short-hairpin RNA transduction

HSC-3 cells were subjected to transduction with EP4 shRNA, Orai1 shRNA, STIM1 shRNA and scramble control shRNA. Transductions

with lentivirus Sigma-Aldrich and SCBT (Santa Cruz Biotechnology) were carried out as previously described.[13,21]

## 2.5 | Reverse transcriptase-polymerase chain reaction

Isolation of total RNA, generation of cDNA and RT-PCR analysis were carried out as previously described.[22,23] Sequences of the specific primers were as follows: EP4 (forward, 5′-CCGGC GGTGATGTTCATCTT-3′; reverse, 5′-CCCACATACCAGCGTGTA GAA-3′), MMP-2 (forward, 5′-CGACCACAGCCAACTACGAT-3′; reverse, 5′-GTCAGGAGAGGCCCCATAGA-3′), MMP-9 (forward, 5′-TGTACCGCTATGGTTACACTCG-3′; reverse, 5′-GGCAGGGACA GTTGCTTCT-3′) and GAPDH (forward, 5′-CCCATCACCATCTT CCAGGAGCG-3′; reverse, 5′-GGCAGGGATGATGTTCTGGAGAGCC-3′).

## 2.6 | Western blotting

Western blot analyses were carried out as previously described.[19,20] The following primary antibodies were used for immunoblotting: phospho-ERK, STIM1 and GAPDH obtained from CST (Cell Signaling Technology), ERK and calpain obtained from Santa Cruz

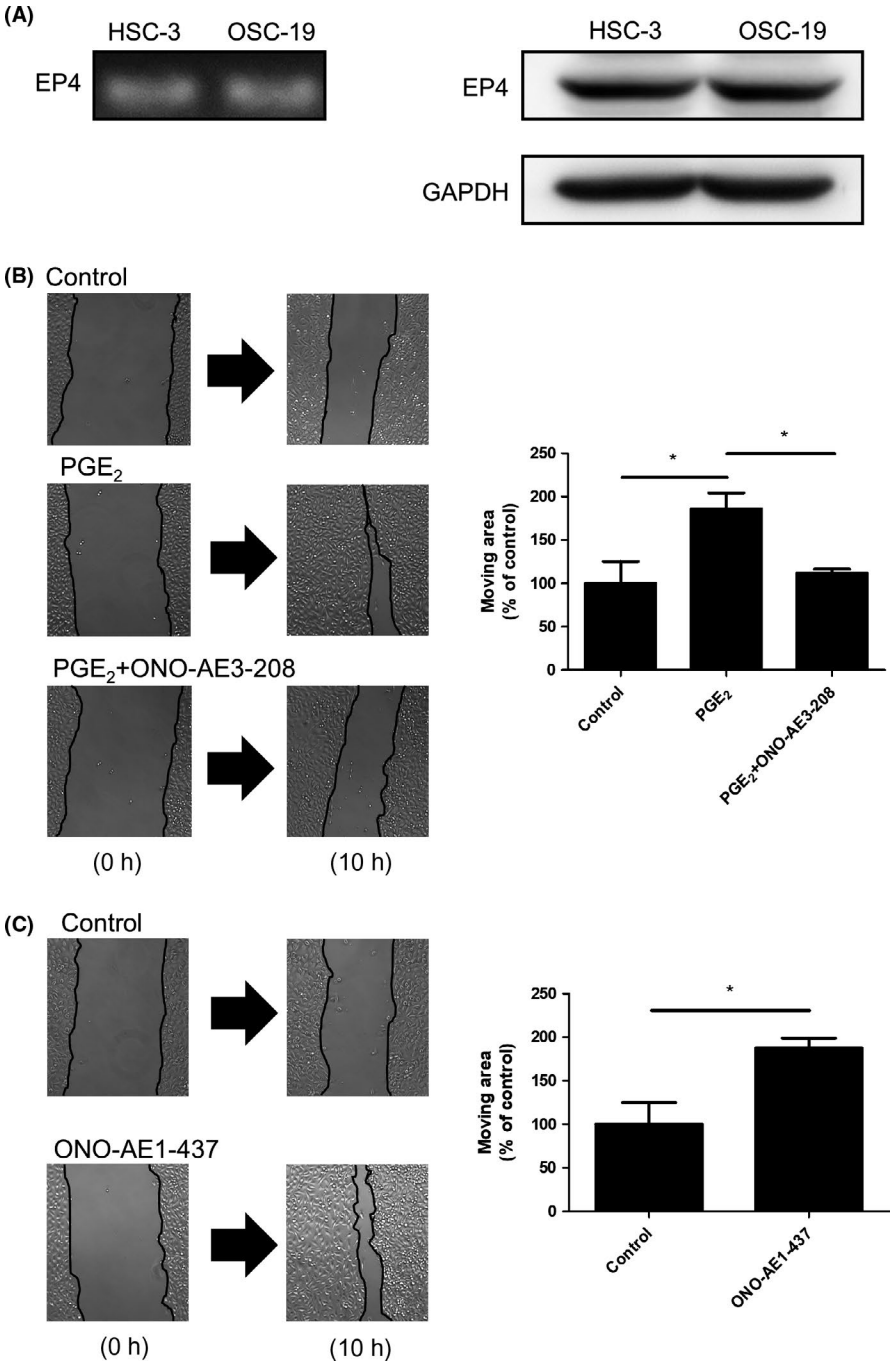



**FIGURE 1** EP4 was expressed and involved in cell migration in oral cancer cell lines. A, mRNA expression of EP4 in oral cancer cell lines (HSC-3, OSC-19) (left). Protein expression of EP4 in HSC-3 and OSC-19 (right). B, Representative images and quantification of the scratch assay in the presence of prostaglandin E $(PGE)_2$ without or with the EP4 antagonist ONO-AE3-208 for 10 h (*$P$ < .05, n = 4). C, EP4 agonist, ONO-AE1-437 enhanced the migration of oral cancer cells (*$P$ < .05, n = 4)

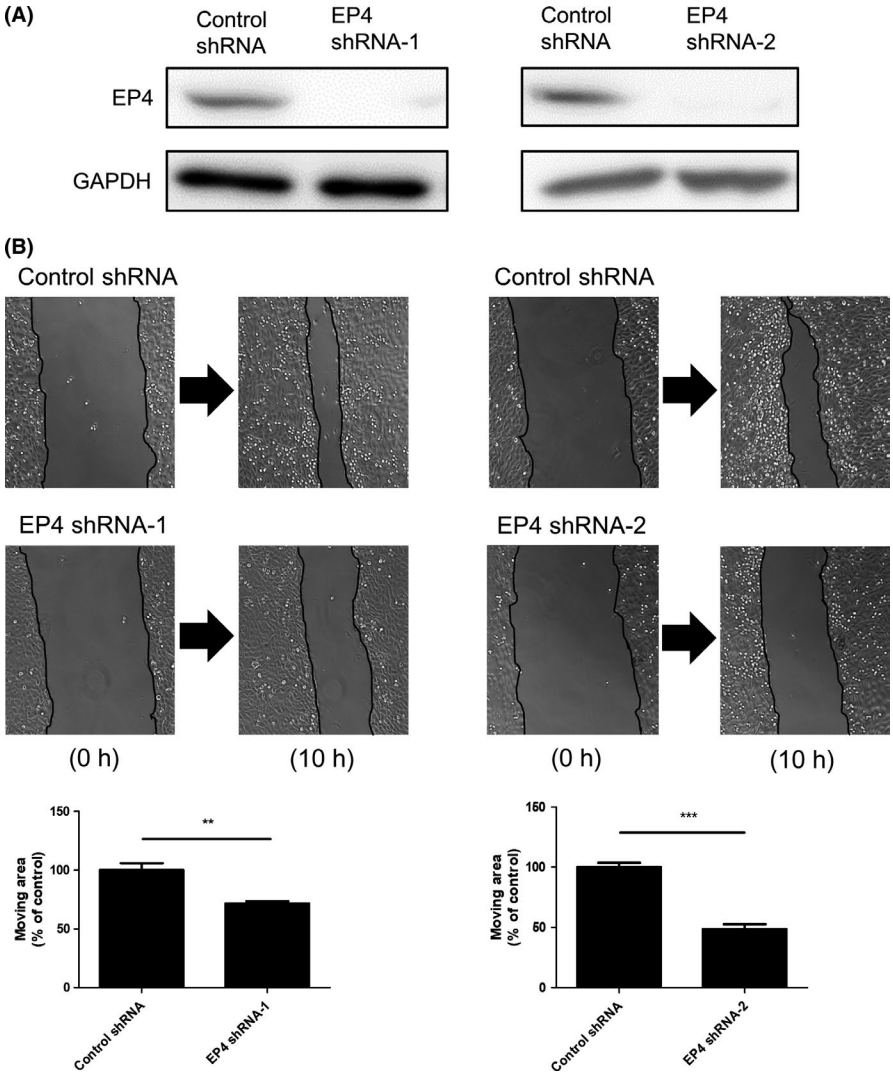



**FIGURE 2** EP4 regulated the migration of oral cancer cells. A, Western blot analysis showed that EP4 was significantly reduced by shRNA transduction with lentivirus in HSC-3 (EP4 shRNA-1 and EP4 shRNA-2). Representative pictures and quantification of the scratch assay. B, The moving area was decreased by the ablation of EP4 in HSC-3 (**$P$ < .01, ***$P$ < .001, n = 4)

Biotechnology, Orai1 and β-actin obtained from Sigma-Aldrich, α-spectrin obtained from Millipore, and EP4 obtained from Cayman.

## 2.7 | Scratch assay

Scratch assay analyses were carried out as previously described.[13] Bright-field images were captured (Eclipse Ti; Nikon Corporation) and analyzed.

## 2.8 | Lung colonization assay

HSC-3 cells transfected with a scramble control shRNA or EP4 shRNA (EP4 shRNA-1 and EP4 shRAN-2) lentivirus were harvested and injected ($2 \times 10^6$ cells/0.2 mL) into the tail veins of Balb/c Slc-nu/nu mice (female, 4-5 weeks old, 4 mice/group) (SLC).[13,24] Five weeks after the injection of the cells, metastatic colonies on the surfaces of the lungs were fixed with formalin and counted under a dissection microscope.

## 2.9 | Fluorescence imaging of intracellular $Ca^{2+}$

Measurement of intracellular $Ca^{2+}$ concentration was done as previously described with some modifications.[13,25] HSC-3 cells were incubated with 2 μM 1-(6-amino-2-[5-carboxy-2-oxazolyl]-5-benzofuranyloxy)-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid (fura-2) (Dojindo Laboratories)/HEPES buffer for 20 min at 37°C. Plates were measured using a microplate reader (PerkinElmer).

## 2.10 | Immunoprecipitation

Cells were grown to 90% confluence on 6-cm dishes, washed with PBS, lysed and protein was recovered by sonication with RIPA buffer (Thermo Scientific). Dynabeads-protein G for immunoprecipitation (Life Technologies) were incubated with the primary antibodies (anti-EP4 [Cayman] and anti-Orai1 [Sigma-Aldrich]) or (anti-EP4 [Cayman] and anti-TRPC1 [Abcam]) for 24 hours at 4°C.[21,26] These antibody-coated Dynabeads (Life Technologies) bound to the target proteins were separated by a magnet, and after repeated washing four times,

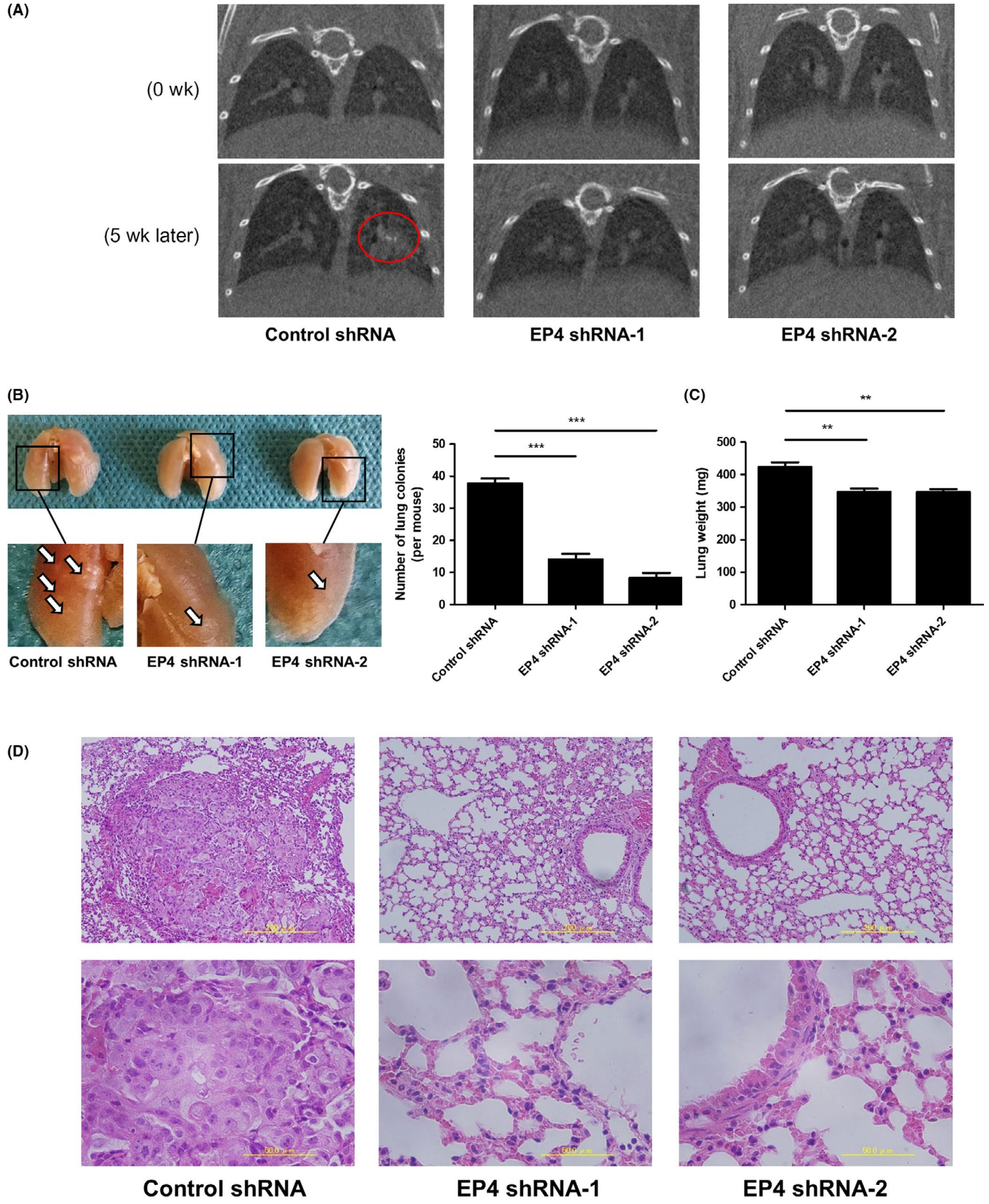



**FIGURE 3** EP4 regulates metastasis of oral cancer. A, HSC-3, oral cancer cells with knockdown of EP4 were injected into the tail vein of Balb/c nu/nu mice. Five weeks later, the lungs were removed and fixed with formalin. Representative computed tomography (CT) images of mouse lungs are shown (control shRNA, EP4 shRNA-1 and EP4 shRNA-2). B, Representative images of metastatic colonies in the lung are shown (control shRNA, EP4 shRNA-1 and EP4 shRNA-2) (left) (***$P < .001$, n = 4). The number of metastatic colonies on the lung surface was counted under a dissection microscope (right). C, Lung weights of mice are shown (**$P < .01$, n = 4). D, Representative pictures of HE staining (control shRNA, EP4 shRNA-1 and EP4 shRNA-2)

the isolated protein complexes were subjected to western blotting with the respective antibodies.

### 2.11 | Gelatin zymography

HSC-3 was stimulated with each reagent for 24 hours. Gelatin zymography was carried out using culture supernatant from HSC-3 cells.[27,28]

### 2.12 | Ethics statement

Animal experiments were carried out according to Yokohama City University guidelines for experimental animals. All experimental protocols were approved by the Animal Care and Use Committee at Yokohama City University, School of Medicine.

### 2.13 | Data analysis and statistics

Statistical comparisons among groups were carried out using Student's *t* test, one-factor analysis of variance (ANOVA) or two-way ANOVA with the Bonferroni post-hoc test. Statistical significance was set as $P < .05$. Significant differences are indicated by $^{*}P < .05$; $^{**}P < .01$; and $^{***}P < .001$; ns, not significant.

## 3 | RESULTS

### 3.1 | EP4 was expressed and involved in cell migration in human oral cancer cells

It was reported that expression levels of both COX and $PGE_2$ are elevated in cancer patients.[29] Several reports have explored whether EP4 is expressed in colorectal cancer, breast cancer, lung cancer, cervical cancer, and prostate cancer.[5] EP4 is the predominant $PGE_2$ receptor subtype in HT-29 and HCA-7 human colon cancer cell lines.[30,31] However, the expression and function of EP4 in oral cancer remain elusive. We first examined the expression of EP4 in human oral cancer cell lines. RT-PCR and western blot analysis showed that mRNA and protein expression of EP4 were expressed in HSC-3 and OSC-19, human metastatic oral cancer cell lines (Figure 1A).

EP4 regulates cell migration in colorectal cancer, lung cancer, breast cancer, ovarian cancer and renal cancer.[32-36] We next examined the role of EP4 in human oral cancer cell migration. ONO-AE3-208, an EP4 antagonist, negated $PGE_2$-induced cell migration (Figure 1B). In contrast, ONO-AE1-437, an EP4 agonist, promoted cell migration (Figure 1C). In our experiment, we confirmed that the optimal concentration of ONO-AE1-437 was 1 μmol/L. We also confirmed that the reagents used in the scratch assay did not affect cell proliferation by themselves (Figure S1A).

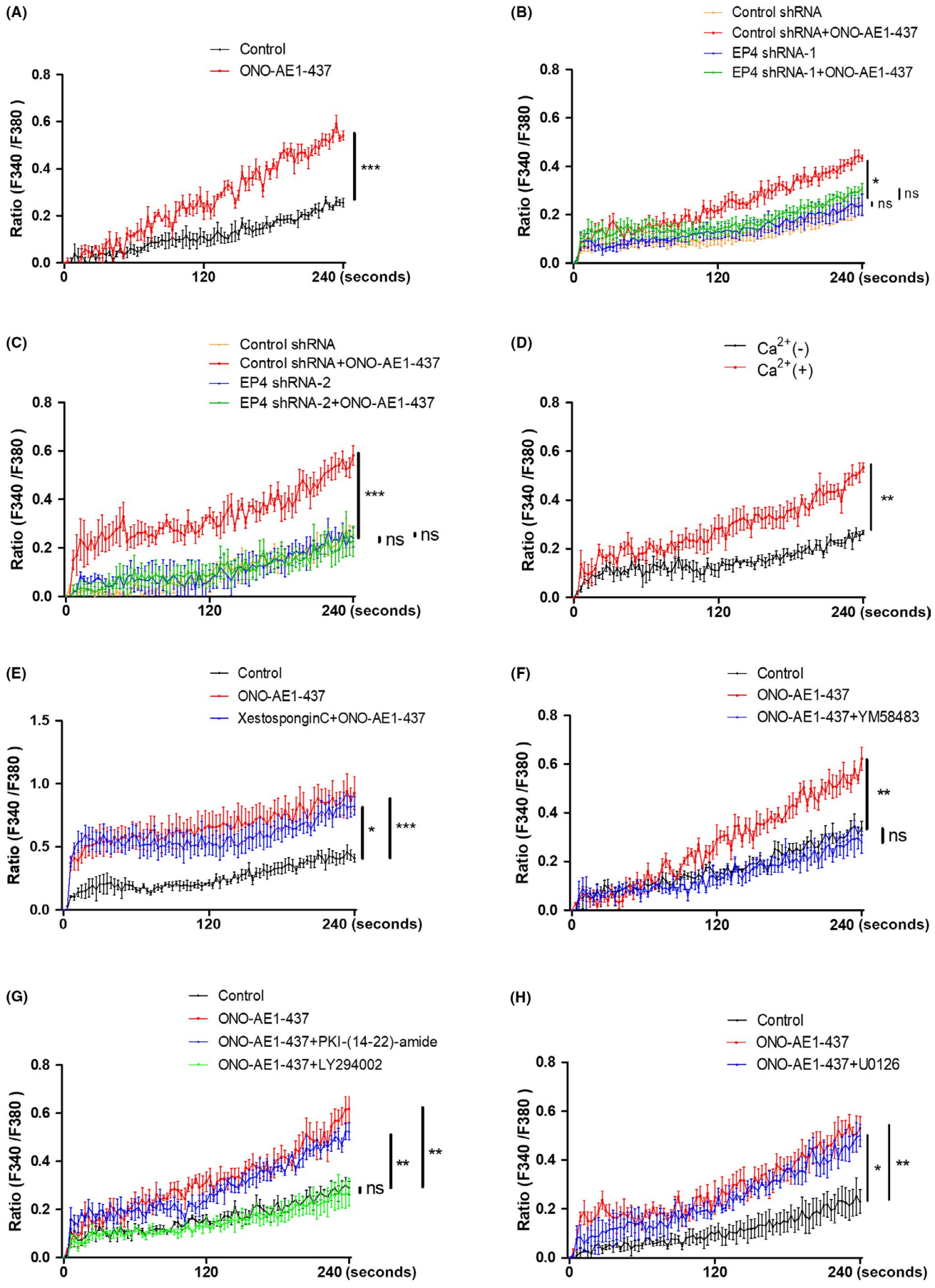
### 3.2 | EP4 knockdown suppressed cell migration in human oral cancer cells

When EP4 was ablated by shRNA (Figure 2A), migration was reduced in both EP4 shRNA-1 and EP4 shRNA-2 cells (Figure 2B). In contrast, proliferation was not reduced in EP4-knockdown oral cancer cells (Figure S1B). Furthermore, we explored the signaling pathway by which EP4 signaling promotes cell migration in HSC-3 cell lines. Because several recent studies have shown that $PGE_2$ promotes cancer cell migration through the EP4-Akt pathway in lung cancer and renal cancer, we hypothesized that the PI3K signaling pathway may be involved in oral cancer.[33,36] However, the PKA inhibitor PKI-(14-22)-amide did not negate EP4 agonist-induced cell migration. In contrast, the PI3K inhibitor LY294002 negated EP4 agonist-induced cell migration (Figure S2). These results suggested that EP4 signaling regulated the migration of oral cancer cells through the PI3K pathway, not through the PKA pathway.

### 3.3 | Inhibition of EP4 suppressed oral cancer cell metastasis in mice

We next examined whether ablation of EP4 reduced cell migration and thus metastasis to distant organs. HSC-3 cells with/without knockdown of EP4 were injected into the tail vein of Balb/c nu/nu mice. Five weeks after injection, colonies in the lungs of mice were visualized by computed tomography (CT) (Figure 3A). CT images showed that the EP4-knockdown group had decreased numbers of metastatic colonies in the lungs of mice compared to the control group. When the lungs were removed and fixed with formalin, the EP4-knockdown group showed decreased numbers of metastatic colonies on the surface of the mouse lung (Figure 3B). Furthermore, lung weights in the control group were heavier than those in the EP4-knockdown group

**FIGURE 4** EP4 signal induced intracellular calcium elevation in oral cancer cells. A, The EP4 agonist significantly induced intracellular calcium elevation in HSC-3 cells ($^{***}P < .001$, n = 4). B, EP4 agonist-induced intracellular calcium elevation was reduced by EP4 knockdown (EP4 shRNA-1) ($^{*}P < .05$, ns; no significant difference, n = 4). C, EP4 agonist-induced intracellular calcium elevation was reduced by EP4 knockdown (EP4 shRNA-2) ($^{***}P < .001$, n = 4). D, Absence of extracellular calcium (calcium-free HEPES buffer) negated EP4 agonist-induced intracellular calcium elevation ($^{**}P < .01$, n = 4). E, Inositol-1,4,5-trisphosphate ($IP_3$) receptor inhibitor (Xestospongin C) did not negate EP4 agonist-induced intracellular calcium elevation ($^{*}P < .05$, $^{***}P < .001$, n = 4). F, Store-operated $Ca^{2+}$ entry (SOCE) inhibitor (YM58483) negated EP4 agonist-induced intracellular calcium elevation ($^{**}P < .01$, n = 4). G, PI3K inhibitor (LY294002) negated EP4 agonist-induced intracellular calcium elevation, not protein kinase (PK)A inhibitor (PKI-(14-22)-amide) ($^{**}P < .01$, n = 4). H, MEK inhibitor (U0126) did not negate EP4 agonist-induced intracellular calcium elevation ($^{*}P < .05$, $^{**}P < .01$, n = 4)


(A)
Control
ONO-AE1-437
Ratio (F340 /F380 )
0.0
0.2
0.4
0.6
0.8
0
120
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(B)
Control shRNA
Control shRNA+ONO-AE1-437
EP4 shRNA-1
EP4 shRNA-1+ONO-AE1-437
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ns
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Ca2+(-)
Ca2+(+)
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(E)
Control
ONO-AE1-437
XestosponginC+ONO-AE1-437
1.0
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Control
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ONO-AE1-437
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(Figure 3C). Similar findings were found in pathological specimens. The nodules indicated lung metastasis. The cells were round and polygonal with clear cytoplasm. Nuclei were enlarged and oval (Figure 3D). These data suggest that inhibition of endogenous EP4 suppresses oral cancer cell migration by reducing lung metastasis.

### 3.4 | EP4 signaling induced $Ca^{2+}$ influx in human oral cancer cells

Intracellular $Ca^{2+}$, a ubiquitous second messenger, regulates diverse cellular functions, including cell migration.[37] We previously reported that SOCE, which is a major mechanism of import from the extracellular to the intracellular space, contributes to melanoma progression.[13] As mentioned earlier, in the current study, we confirmed that EP4 is associated with migration/metastasis in oral cancer in vitro and in vivo; therefore, we focused on EP4 and $Ca^{2+}$ signaling. We first examined whether EP4 is involved in the regulation of intracellular $Ca^{2+}$. We confirmed that the EP4 agonist notably increased intracellular $Ca^{2+}$ in oral cancer cells (HSC-3) (Figure 4A). Similarly, the EP4 agonist increased intracellular $Ca^{2+}$ in human glioblastoma cells (LN229), human breast cancer cells (MCF-7), and human cardiac fibroblast cells (HCF) (Figures S3A-C). EP4-induced $Ca^{2+}$ elevation was not detected in two types of EP4-knockdown cells (Figure 4B,C). Interestingly, depletion of extracellular $Ca^{2+}$ did not increase EP4 agonist-induced $Ca^{2+}$ elevation (ie, $Ca^{2+}$ influx) (Figure 4D). Moreover, inositol trisphosphate ($IP_3$) receptor inhibitor Xestospongin C did not inhibit EP4 agonist-induced $Ca^{2+}$ elevation (Figure 4E). Conversely, the SOCE inhibitor YM58483 negated EP4-induced $Ca^{2+}$ influx (Figure 4F). Taken together, EP4-induced $Ca^{2+}$ influx occurred from extracellular $Ca^{2+}$ entry, not from the ER. In addition, the PI3K inhibitor LY294002 suppressed EP4-induced $Ca^{2+}$ influx (Figure 4G). In contrast, the PKA inhibitor PKI-(14-22)-amide did not negate the EP4 agonist-induced $Ca^{2+}$ elevation (Figure 4G). Similarly, the MEK inhibitor U0126 did not negate the EP4-induced $Ca^{2+}$ influx, suggesting that $Ca^{2+}$ signaling is located upstream of MEK (Figure 4H). Taken together, these data suggest that EP4 signaling mediates $Ca^{2+}$ influx from the extracellular space through the PI3K pathway, not the PKA pathway.

### 3.5 | EP4 interacted with Orai1 and increased intracellular calcium elevation

Because EP4 agonist-induced $Ca^{2+}$ influx occurred from the extracellular $Ca^{2+}$ space, we hypothesized that the EP4 agonist induced $Ca^{2+}$ influx through Orai1, which is a highly $Ca^{2+}$ selective channel located on the PM, leading to $Ca^{2+}$ influx from the extracellular space to restore the $Ca^{2+}$ concentration in the ER (ie, SOCE).[11] Therefore, we next examined the involvement of Orai1 in the EP4-induced $Ca^{2+}$ influx. We first confirmed that Orai1 and STIM1, which translocated to the plasma membrane as a $Ca^{2+}$ sensor, were expressed in HSC-3 and OSC-19 (Figure 5A). We confirmed that cell proliferation was not reduced in either Orai1 or STIM1-knockdown oral cancer cells (Figure S1B). As mentioned earlier, it has been reported that Orai1 is the SOCE component; therefore, we examined the interaction of EP4 and Orai1 in HSC-3.[11] Immunoprecipitation showed that EP4 was colocalized and formed complexes with Orai1 (Figure 5B; Figure S4). Surprisingly, EP4 also formed complexes with TRPC1 (Figure S5). In contrast, EP4 did not form a complex with STIM1 (data not shown). Taken together, our results indicated that EP4 colocalized and formed complexes with both Orai1 and TRPC1. As far as we know, this is the first report that EP4 was colocalized and forms complexes with Orai1 and TRPC1.

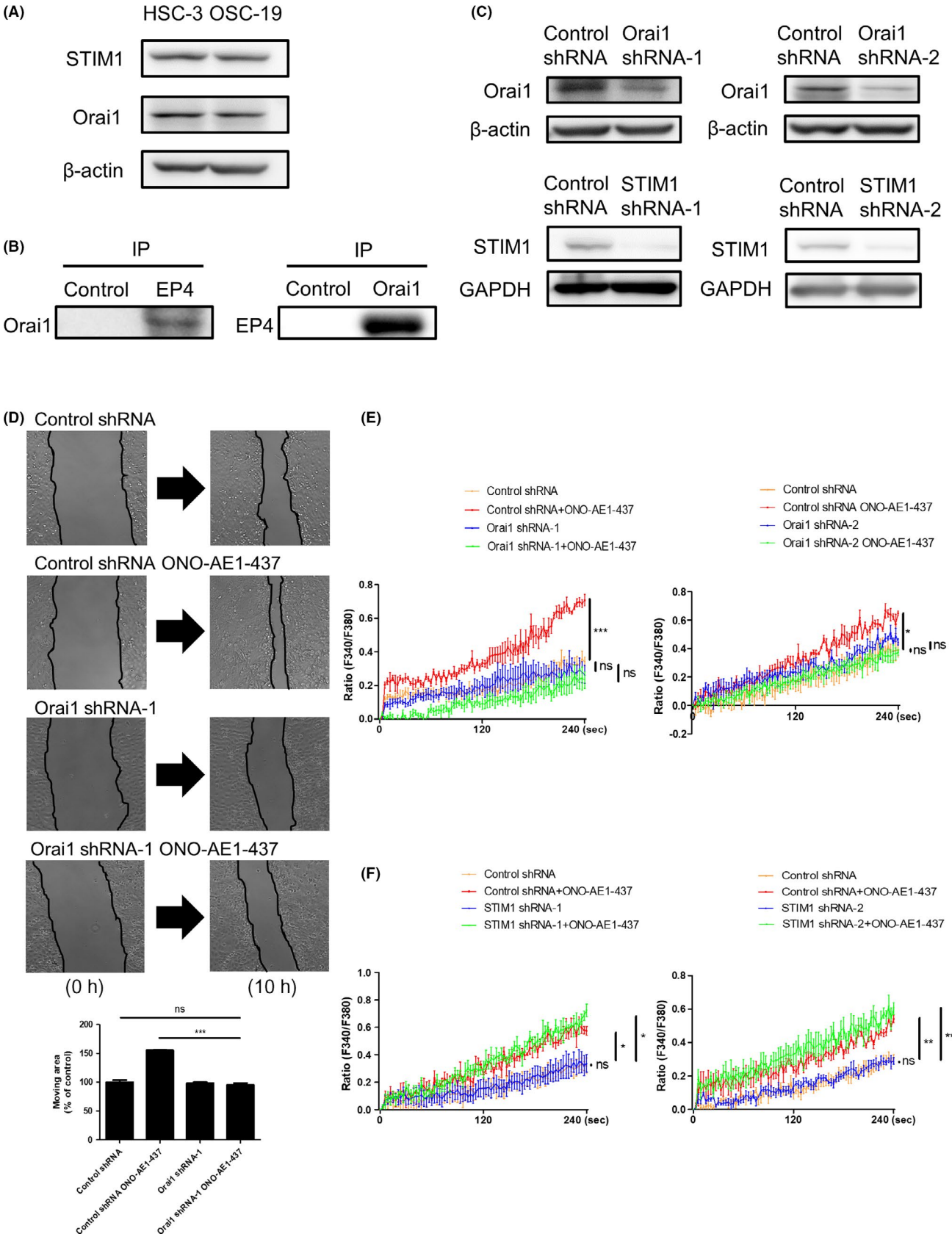
More importantly, when Orai1 was ablated by shRNA in HSC-3 (Figure 5C), the EP4 agonist did not promote cell migration and failed to induce $Ca^{2+}$ influx (Figure 5D,E; Figure S6). In contrast, when STIM1 was also ablated by shRNA in HSC-3 (Figure 5C), the EP4 agonist induced $Ca^{2+}$ influx (Figure 5F). Taken together, these results suggested that Orai1 was necessary for EP4 to induce $Ca^{2+}$ influx from the extracellular space and that Orai1 worked without the interaction of STIM1.

### 3.6 | EP4 signaling phosphorylated ERK through the PI3K pathway

We previously reported that SOCE regulates melanoma cell migration by ERK phosphorylation.[13] $Ca^{2+}$ elevation positively regulates small GTPase Ras signaling, leading to increased ERK phosphorylation.[38] Because ERK is a regulator of cell motility, we hypothesized that the MEK/ERK pathway may be involved in EP4 agonist-induced cell migration.[39] Indeed, the EP4 agonist mostly phosphorylated ERK 6 hours after stimulation (Figure 6A). However, PKI-(14-22)-amide did not negate the EP4 agonist-induced phosphorylation of ERK, yet LY294002 negated it, suggesting that EP4 phosphorylated ERK through the PI3K pathway and that PI3K is located upstream of ERK (Figure 6B). Furthermore, when Orai1 was ablated by shRNA in HSC-3, the EP4 agonist failed to phosphorylate ERK compared to the control (Figure 6C,D). These results suggested that Orai1 was necessary for EP4 agonist-induced ERK phosphorylation.

**FIGURE 5** EP4 was colocalized, forming complexes with Orai1 and inducing intracellular calcium elevation. A, Western blotting was carried out to confirm whether STIM1 and Orai1 were expressed in HSC-3 and OSC-19. B, Immunoprecipitation for EP4 and Orai1 was carried out. C, Western blot analysis showed that Orai1 or stromal interaction molecule 1 (STIM1) was significantly reduced by shRNA transduction with lentivirus in HSC-3 cells (Orai1 shRNA-1 and Orai1 shRNA-2, and STIM1 shRNA-1 and STIM1 shRNA-2). D, Representative pictures and quantification of the scratch assay. Scratch assays confirmed that EP4 agonist-induced cell migration was decreased by ablation of Orai1 in HSC-3 (***$P < .001$, ns; no significant difference, n = 4). E, EP4 agonist-induced intracellular calcium elevation was reduced by Orai1 knockdown (Orai1 shRNA-1 and Orai1 shRNA-2) (*$P < .05$, ***$P < .001$, ns; no significant difference, n = 4). F, EP4 agonist-induced intracellular calcium elevation was not reduced by STIM1 knockdown (STIM1 shRNA-1 and STIM1 shRNA-2) (*$P < .05$, **$P < .01$, n = 4)

We previously reported that ERK signaling can increase calpain activity in melanoma cells.[40] Calpain was found to be a $Ca^{2+}$-dependent enzyme.[41] α-Spectrin is an important cytoskeletal protein that ensures vital cellular properties, including polarity and cell stabilization. α-Spectrin is cleaved by calpain, resulting in increasing proteolytic activities and enhancement of actin-binding



capacities of spectrin.[42] Therefore, we next evaluated whether EP4 agonists activate calpain and α-spectrin. We confirmed that calpain and cleaved α-spectrin, a target enzyme of calpain, were increased by EP4 agonists in a time-dependent way (Figure 6E). These data suggested that EP4 signaling induced $Ca^{2+}$ influx and then activated calpain, resulting in increased cleavage of α-spectrin.

### 3.7 | EP4 signaling increased mRNA and activity of MMP-2 and MMP-9

Matrix metalloproteinase-2 and MMP-9 are elastolytic enzymes. We recently found that CJ-42794, a selective EP4 antagonist, suppressed MMP-2 and MMP-9 activation in mouse abdominal aortic aneurysm (AAA) tissues in vivo.[28] MMP-2 and MM-9 are known enzymes that break the extracellular matrix. It is necessary to break the extracellular matrix around the tumor in migration and invasion of cancer cells.[43,44] The ERK pathway is also associated with MMP-2 activation in oral cancer.[45,46] Indeed, the EP4 agonist increased the mRNA of MMP-2 and MMP-9 after 3 hours (Figure 7A,B). LY294002 negated EP4 agonist-induced MMP-2 mRNA expression (Figure 7C). YM58483 and U0126 also negated it. Similarly, these inhibitors negated EP4 agonist-induced MMP-9 mRNA expression (Figure 7D). Gelatin zymography showed that $PGE_2$ increased the activation of MMP-2 and MMP-9, and the synthesis of pro-MMP-2 and pro-MMP-9. ONO-AE3-208 negated $PGE_2$-induced MMP-2 and MMP-9 expression (Figure 7E). Taken together, we propose the mechanism of migration of EP4 in oral cancer cells (Figure 7F).

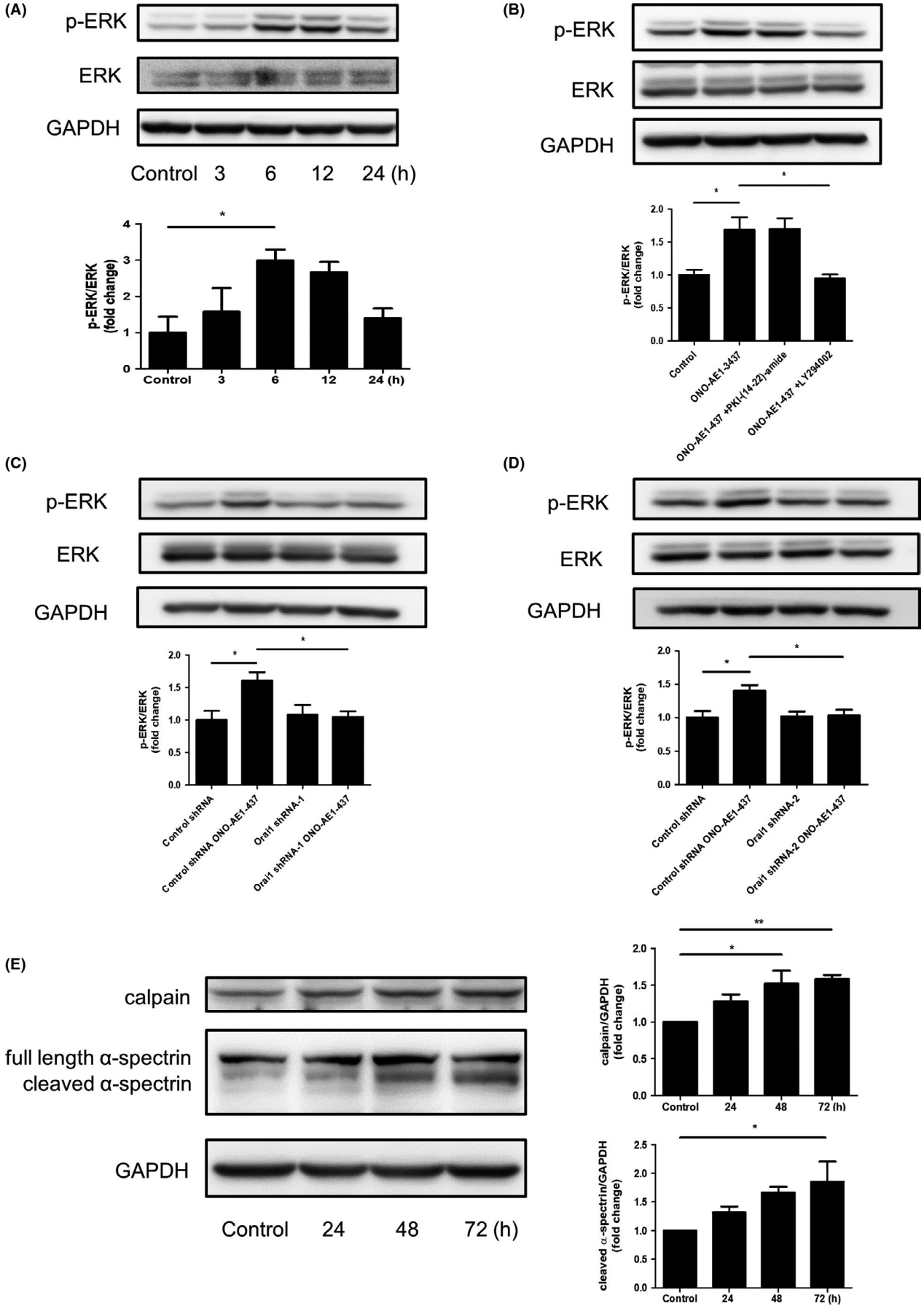
## 4 | DISCUSSION

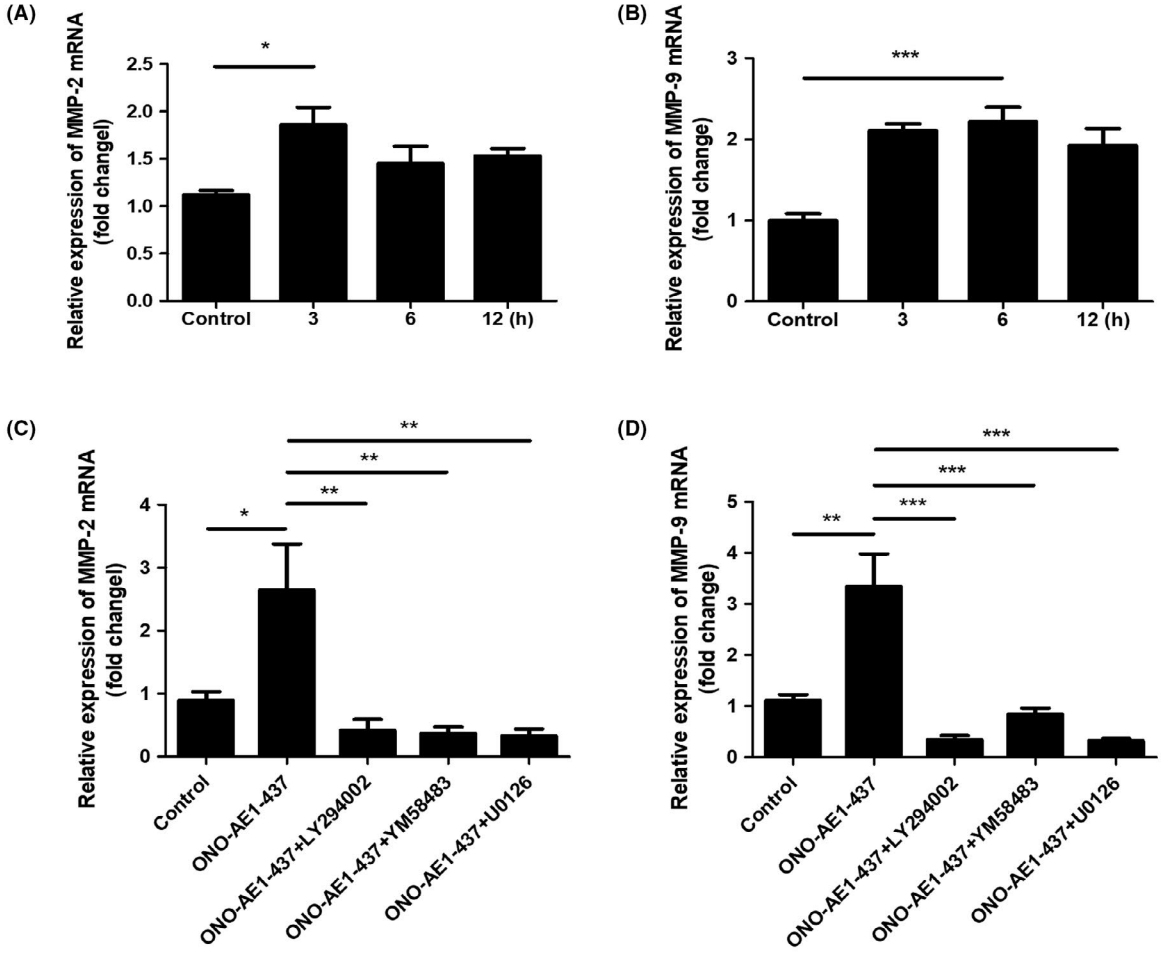
Prostaglandin $E_2$ exerts its diverse effects through four GPCR (EP1-4). In general, EP1 activation is associated with intracellular $Ca^{2+}$ elevation, mediated by phospholipase C (PLC) and $IP_3$.[6] EP2 and EP4 receptors are known to couple to Gs and mediate the activation of PKA through cAMP.[6] Along with PKA, PI3K is also a major downstream target of EP4.[5] We identified that EP4 regulated cell migration through PI3K signaling and ERK signaling in oral cancer cells. Fujino et al noted that $PGE_2$ stimulation of HEK cells stably expressing EP4 receptors caused ERK phosphorylation by a PI3K-dependent mechanism.[29] Ma et al reported that the EP4 receptor mediates ERK activation, resulting in cell migration of breast cancer.[47] Yang et al showed that selective antagonism of EP4 receptor signaling results in profound reduction in lung and colon cancer metastasis.[33] They also reported that the EP4 antagonist ONO-AE3-208 inhibited the phosphorylation of PBK/Akt, the downstream effector of PI3K, in Lewis lung carcinoma cells (3 LL) treated with $PGE_2$. These data suggest that PI3K plays an important role in cancer cell migration associated with EP4 signaling and are consistent with our data. Because the PKA inhibitor did not negate EP4 agonist-induced $Ca^{2+}$ influx and cell migration, our data showed that the PI3K pathway in migration is more dominant than the PKA pathway in oral cancer cells.

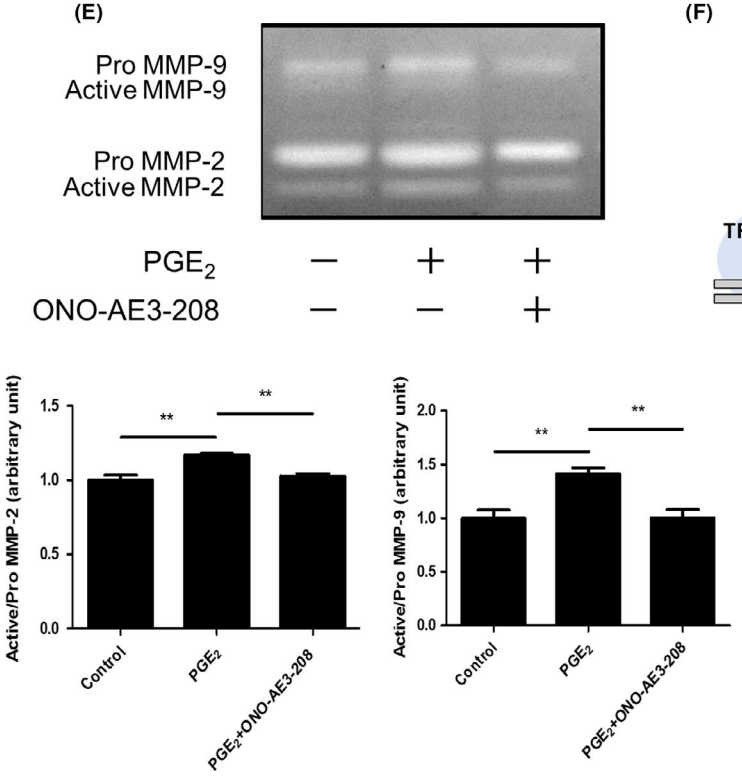
It is well known that stimulation of the EP1 receptor results in Gq-mediated activation of PLC, phosphatidylinositol hydrolysis and intracellular $Ca^{2+}$ elevation.[5] It causes activation of PKC. EP3, through activation of a $G_i$ protein, inhibits adenylyl cyclase, leading to reduction of cAMP and intracellular $Ca^{2+}$ elevation. As mentioned earlier, little information is available regarding $Ca^{2+}$ signaling and the cellular signaling pathway of EP4. At the point of EP4-induced $Ca^{2+}$ elevation, there are some controversial reports. Tamiji and Crawford reported that $PGE_2$ and misoprostol, a PGE analogue, increased intracellular $Ca^{2+}$ and the EP4 antagonist promoted $Ca^{2+}$ elevation in mouse neuroblastoma (Neuro-2a) cells.[8] In contrast, the EP4 agonist ONO-AE1-329 decreased $Ca^{2+}$ influx in human eosinophils.[7] These results are not in accord with our results. We assumed that EP4 agonist-induced $Ca^{2+}$ regulation may be different in different cell types or that there is a difference in the $Ca^{2+}$ response between EP4 agonists and antagonists or selectivities of these reagents. This topic should be further investigated in a future study.

In the current study, we confirmed that EP4 colocalized and formed a complex with Orai1 and TRPC1, not STIM1. It has been reported that Orai1 interacts with proteins such as transient receptor potential channels and STIM1.[21,26,27] Liao et al showed that Orai1 physically interacted with the N and C termini of TRPC3 and TRPC6.[48] They also showed that Orai1 and TRPC proteins colocalized and formed complexes that participate in $Ca^{2+}$ entry with or without activation of store depletion.[49] Ong and Ambudkar showed that Orai1 is a critical component of the active TRPC1 channelosome and that Orai1 determines the activation of TRPC1 following depletion of ER-$Ca^{2+}$ stores.[12] Conversely, in the current study, we showed that knockdown of STIM1 did not negate EP4-agonist-induced $Ca^{2+}$ influx. Furthermore, we confirmed that EP4 formed complexes with both Orai1 and TRPC1 that participate in $Ca^{2+}$ influx. More interestingly, nevertheless, the EP4 receptor is coupled to Gα and Gαi, EP4 induced $Ca^{2+}$ influx from the extracellular space. Regarding the mechanism of SOCE, it is well known that activation of GPCR (Gq) or immunoreceptors such as T-cell, B-cell and Fc receptors results in the production of $IP_3$ that binds to the $IP_3$ receptor located in the membrane of the ER, which is a $Ca^{2+}$-permeable ion channel and

**FIGURE 6** EP4 signaling regulated ERK phosphorylation through Orai1. A, Representative images of ERK phosphorylation are shown. Densitometric analyses of western blot show that phosphorylation of ERK was increased by EP4 agonist after 6 and 12 h (*$P$ < .05, n = 4). B, Densitometric analyses of western blot show that LY294002 negated EP4 agonist-induced phosphorylation of ERK, not PKI-(14-22)-amide (*$P$ < .05, n = 4). C and D, Densitometric analysis of western blots confirmed that ERK phosphorylation induced by EP4 agonists was not caused by Orai1 ablation of HSC-3 (*$P$ < .05, n = 4). E, EP4 agonist ONO-AE1-437 increased the expression of calpain and cleaved α-spectrin in a time-dependent way. Densitometric analyses (bar graph) of western blots show the expression of calpain and cleaved α-spectrin (*$P$ < .05, **$P$ < .01, n = 4)


(A)
p-ERK
ERK
GAPDH
Control 3 6 12 24 (h)
p-ERK/ERK (fold change)
Control 3 6 12 24 (h)
(B)
p-ERK
ERK
GAPDH
p-ERK/ERK (fold change)
Control
ONO-AE1-3437
ONO-AE1-437 +PKI-(14-22)-amide
ONO-AE1-437 +LY294002
(C)
p-ERK
ERK
GAPDH
p-ERK/ERK (fold change)
Control shRNA
Control shRNA ONO-AE1-437
Orai1 shRNA-1
Orai1 shRNA-1 ONO-AE1-437
(D)
p-ERK
ERK
GAPDH
p-ERK/ERK (fold change)
Control shRNA
Control shRNA ONO-AE1-437
Orai1 shRNA-2
Orai1 shRNA-2 ONO-AE1-437
(E)
calpain
full length α-spectrin
cleaved α-spectrin
GAPDH
Control 24 48 72 (h)
calpain/GAPDH (fold change)
Control 24 48 72 (h)
cleaved α-spectrin/GAPDH (fold change)
Control 24 48 72 (h)


(A)
Relative expression of MMP-2 mRNA (fold change)
Control
3
6
12 (h)
(B)
Relative expression of MMP-9 mRNA (fold change)
Control
3
6
12 (h)
(C)
Relative expression of MMP-2 mRNA (fold change)
Control
ONO-AE1-437
ONO-AE1-437+LY294002
ONO-AE1-437+YM58483
ONO-AE1-437+U0126
(D)
Relative expression of MMP-9 mRNA (fold change)
Control
ONO-AE1-437
ONO-AE1-437+LY294002
ONO-AE1-437+YM58483
ONO-AE1-437+U0126


(E)
Pro MMP-9
Active MMP-9
Pro MMP-2
Active MMP-2
PGE2
ONO-AE3-208
Active/Pro MMP-2 (arbitrary unit)
Control
PGE2
PGE2+ONO-AE3-208
Active/Pro MMP-9 (arbitrary unit)
Control
PGE2
PGE2+ONO-AE3-208

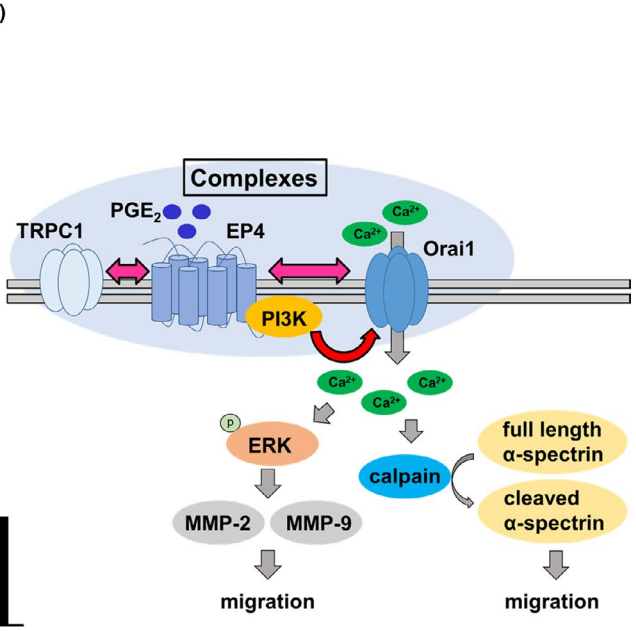

(F)
Complexes
PGE2
TRPC1
EP4
Orai1
Ca2+
PI3K
ERK
calpain
full length α-spectrin
cleaved α-spectrin
MMP-2
MMP-9
migration
migration

**FIGURE 7** EP4 signaling increased MMP-2 and MMP-9 gene expression and activation. A and B, Quantitative RT-PCR shows that the EP4 agonist increased mRNA of MMP-2 and MMP-9 over 3 and 6 h (*$P$ < .05, ***$P$ < .001, n = 4). C and D, Quantitative RT-PCR shows that LY294002, YM58483 and U0126 negated EP4 agonist-induced MMP-2 (left) and MMP-9 activation (right) (*$P$ < .05, **$P$ < .01, ***$P$ < .001, n = 4). E, Gelatin zymography shows that $PGE_2$ significantly increased MMP-2 and MMP-9 activity, and the synthesis of pro-MMP-2 and pro-MMP-9 after 24 h (**$P$ < .01, n = 4). EP4 antagonist ONO-AE3-208 negated $PGE_2$-induced MMP-2 and MMP-9 activation. F, Proposed mechanism of EP4 signaling-mediated cell migration in oral cancer. EP4 stimulated by prostaglandin E $(PGE)_2$ activated PI3K and then induced $Ca^{2+}$ influx from the extracellular space through Orai1, resulting in ERK phosphorylation and the promotion of cell migration in oral cancer cells. EP4 stimulated Orai1, which formed a complex through PI3K. $Ca^{2+}$ then flowed into the cell from outside the cell through Orai1. The secondary messenger $Ca^{2+}$ phosphorylated ERK and activated MMP. Meanwhile, $Ca^{2+}$ increased the expression of calpain, which is a $Ca^{2+}$-dependent enzyme, and increased the cleavage of α-spectrin, a target enzyme of calpain. As a result, EP4 promoted cell migration in oral cancer cells. TRPC1, transient receptor potential canonical 1

mediates the release of $Ca^{2+}$ from the ER.[50] The resulting decrease in $Ca^{2+}$ concentration in the ER is sensed by the EF-hand motif of STIM. STIM then translocate to the PM, where they interact with Orai $Ca^{2+}$-channel subunits, leading to $Ca^{2+}$ influx from the extracellular space to restore the $Ca^{2+}$ concentration in ER.[11] Therefore, we are surprised that EP4, which is coupled to Gα and Gαi, activates Orai1 through PI3K (not PLC) resulting in the induction of $Ca^{2+}$ influx from the extracellular space without the interaction of STIM1 (ie, without activation of store depletion). We previously reported that the $PGE_2$-EP4-c-Src-PLCγ-signaling pathway most likely promoted lysosomal degradation of lysyl oxidase (LOX) in the ductus arteriosus (DA) smooth muscle cells (SMC).[51] Considering the results of the pharmacological inhibition study by $IP_3$ inhibitor in the present study, although there is a possibility that PLC is associated with this mechanism, PI3K is a dominant regulator in EP4-Orai1-$Ca^{2+}$ signaling.

Ghigo et al reviewed that PI3K are master regulators of voltage-gated L-type $Ca^{2+}$ channel (LTCC) activity.[52] PI3Kα promotes Akt (protein kinase B)-mediated phosphorylation of the LTCC subunit in cardiomyocytes. In that review, they suggested that PI3K can promote $Ca^{2+}$ influx through an unidentified PIP3 (PtdIns(3,4,5P3)-sensitive channel. PIP3 produced by PI3K activates Cdc42 and Rac1, leading to elevated cell migration.[53] In the present study, we propose the possibility that EP4 is one of the activated GPCR that induces PIP3-sensitive $Ca^{2+}$ entry and that Orai is a candidate channel for PIP-sensitive $Ca^{2+}$ entry.

Taken together, we showed that EP4 was colocalized and formed complexes with Orai1 and TRPC1, activated PI3K and induced $Ca^{2+}$ influx from the extracellular space and regulated the concentration of intracellular $Ca^{2+}$ through Orai1 without the interaction of STIM1 (ie, without activation of store depletion) resulting in cell migration. However, further investigation may be necessary to evaluate how EP4, Orai1 and TRPC1 interact.

Yang et al reported that COX-2 expression was not statistically associated with advanced tumor stage but correlated with a high risk of lymph node metastasis and advanced TNM stage in patients with head and neck cancer.[54] In addition, Ogawa et al reported that EP4 in human clinicopathological examination of oral squamous cell carcinoma was significantly more highly expressed than in normal oral tissue.[55] Therefore, we assumed that $PGE_2$/EP4 signaling was highly upregulated and promoted tumor progression in oral cancer cells.

As far as we know, the novelty of the present study is that GPCR-like EP4, which is coupled to Gα and Gαi, induced $Ca^{2+}$ influx from the extracellular space through Orai1 without the interaction of STIM1 (ie, without activation of store depletion). As far as we know, this is the first report that shows such a new mechanism not only in oral cancer cells but also in other cells, including normal cells. Therefore, our findings showed EP4-Orai1-$Ca^{2+}$ signaling as a novel EP4 downstream pathway. Our results suggest that EP4 and Orai1 are promising candidates as targets for oral cancer therapy.

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## CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

## ORCID

*Masanari Umemura* https://orcid.org/0000-0002-1812-5720

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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