Original Article

Prostaglandin E$_2$ receptor EP4 regulates cell migration through Orai1

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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 only in 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.

1 INTRODUCTION

Oral cancer is the sixth most common cancer. Oral cavity cancer comprises 52% of head and neck cancer cases, and 90% of these are squamous cell carcinoma. 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...
survival rate is 63% in the USA. 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. 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. COX-2 produces prostaglandin E2 (PGE2). PGE2 is one of the major products generated by the actions of COX on arachidonic acid. It has been established that PGE2 is an important mediator of fever, pain and inflammation. PGE2 functions mainly through G protein-coupled PGE receptors designated EP1, EP2, EP3 and EP4.

The EP4 receptor is one of four receptor subtypes for PGE2. Stimulation of EP4 receptors usually increases intracellular cyclic AMP (cAMP) levels and activates protein kinase A (PKA) through stimulatory G protein (Gs). We previously reviewed that PGE2 and EP4 regulate cell migration or metastasis of colorectal cancer, lung cancer, breast cancer, prostate cancer, ovarian cancer and renal cancer. However, little information is available regarding the relationship between EP4 and Ca2+ signaling in oral cancer.

In contrast, there are few reports on the relationships between EP4 and Ca2+ signaling in human eosinophil and mouse neuroblastoma (Neuro-2a) cells. Therefore, despite intracellular Ca2+ 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 Ca2+: therefore, we focused on the relationship between EP4 and Ca2+ signaling in oral cancer.

Store-operated Ca2+ entry (SOCE) is a major mechanism of Ca2+ import from the extracellular to the intracellular space. SOCE occurs in both nonexcitable and excitable cells. Physiologically, binding of agonists to G protein-coupled receptor (GPCR) (Gs) 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 (IP3) and membrane-bound diacylglycerol (DAG). Activation of IP3 receptors on the endoplasmic reticulum (ER) causes a rapid and transient release of Ca2+ from the ER store. The resulting decrease of Ca2+ 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 Ca2+ selective channels located on the plasma membrane (PM), leading to Ca2+ influx from the extracellular space to restore the Ca2+ concentration in ER. SOCE plays important roles in gene expression, proliferation, migration, fluid secretion, mast cell degranulation, platelet aggregation and T-cell activation. We previously showed that SOCE contributes to melanoma progression. However, little information is available regarding the relationship between EP4 signaling and Ca2+ signaling in both normal cells and cancer cells.

Therefore, in the present study, we evaluated the mechanism of EP4 and Ca2+ 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 Ca2+ 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 Gαs and Gαi.

2 MATERIALS AND METHODS

2.1 Reagents

LY294002 was purchased from Cell Signaling. Prostaglandin E2 (PGE2) 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.

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). 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. The human cardiac fibroblast adult cell line (No. 6330) (HCF) was purchased from ScienCell Research Laboratories.

2.3 Cell viability assay

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

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.\textsuperscript{13,21}

\section*{2.5 Reverse transcriptase-polymerase chain reaction}

Isolation of total RNA, generation of cDNA and RT-PCR analysis were carried out as previously described.\textsuperscript{22,23} Sequences of the specific primers were as follows: EP4 (forward, 5′-CCGGCGGTGATGTTCTTT-3′; reverse, 5′-CCCACCATACCATCTCCTTCCAGGAGCC-3′), MMP-2 (forward, 5′-CGACCACAGCAGTTCT-3′; reverse, 5′-GTCAGGAGATGGTTCT-3′), MMP-9 (forward, 5′-TGATCGCTATGTTACACTCG-3′; reverse, 5′-GCCAGGAGAAGTTTCTTCCAGGAGCC-3′), and GAPDH (forward, 5′-CCCACATACCATCTCCTTCCAGGAGCC-3′; reverse, 5′-GCCAGGAGATGGTTCTTCCAGGAGCC-3′).

\section*{2.6 Western blotting}

Western blot analyses were carried out as previously described.\textsuperscript{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.

\textbf{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\textsubscript{2} (PGE\textsubscript{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 (\(P < .05\), \(n = 4\)).
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.\textsuperscript{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 shRNA-2) lentivirus were harvested and injected (2 × 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).\textsuperscript{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.\textsuperscript{13,25} HSC-3 cells were incubated with 2 µM 1-(6-amino-2-[5-carboxy-2-oxazolyl]-5-benzofuranyl oxy)-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.\textsuperscript{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 < .01, 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 PGE2 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 PGE2 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 PGE2-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).

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 (IP3) receptor inhibitor (Xestospongin C) did not negate EP4 agonist-induced intracellular calcium elevation (*P < .05, ***P < .001, n = 4). F. Store-operated Ca2+ entry (SOCE) inhibitor (YM58483) negated EP4 agonist-induced intracellular calcium elevation (**P < .01, n = 4). G. 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.

3.2 | 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 PGE2 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.
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 (i.e., Ca\(^{2+}\) influx) (Figure 4D). Moreover, inositol trisphosphate (IP\(_3\)) receptor inhibitor Xestospongion 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.

3.4 EP4 signaling induced Ca\(^{2+}\) influx in human oral cancer cells

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 (i.e., 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 5G). 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.\(^ {38}\) 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 < .01; ***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. Calpain was found to be a \( \text{Ca}^{2+} \)-dependent enzyme. \( \alpha \)-Spectrin is an important cytoskeletal protein that ensures vital cellular properties, including polarity and cell stabilization. \( \alpha \)-Spectrin is cleaved by calpain, resulting in increasing proteolytic activities and enhancement of actin-binding.

(A) HSC-3 OSC-19

| STIM1 | Orai1 | \( \beta \)-actin |
|-------|-------|------------------|
|       |       |                  |

(B) IP

| Control | EP4  |
|---------|------|
| Orai1   | EP4  |

(C) Control Orai1 shRNA-1

| Orai1 | \( \beta \)-actin |
|-------|------------------|
|       |                  |

Control STIM1 shRNA-1

| STIM1 | GAPDH |
|-------|-------|
|       |       |

(D) Control shRNA

Control shRNA ONO-AE1-437

Orai1 shRNA-1

Orai1 shRNA-1 ONO-AE1-437

(0 h) (10 h)

(E) Control shRNA

Control shRNA+ONO-AE1-437

Orai1 shRNA-1

Orai1 shRNA-1+ONO-AE1-437

(F) Control shRNA

Control shRNA+ONO-AE1-437

STIM1 shRNA-1

STIM1 shRNA-1+ONO-AE1-437
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). YMS8483 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$_3$. EP2 and EP4 receptors are known to couple to Gs and mediate the activation of PKA through cAMP. Along with PKA, PI3K is also a major downstream target of EP4. 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.32 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 adenyl 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.5 In contrast, the EP4 agonist ONO-AE1-329 decreased Ca$^{2+}$ influx in human eosinophils.2 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 Gs and Gai, 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).]
mediates the release of Ca\textsuperscript{2+} from the ER.\textsuperscript{30} The resulting decrease in Ca\textsuperscript{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\textsuperscript{2+}-channel subunits, leading to Ca\textsuperscript{2+} influx from the extracellular space to restore the Ca\textsuperscript{2+} concentration in ER.\textsuperscript{31} Therefore, we are surprised that EP4, which is coupled to G\textsubscript{α} and G\textsubscript{i}, activates Orai1 through PI3K (not PLC) resulting in the induction of Ca\textsuperscript{2+} influx from the extracellular space without the interaction of STIM1 (ie, without activation of store depletion). We previously reported that the PGE\textsubscript{2}-EP4-c-Src-PLC\textgamma-signaling pathway most likely promoted lysosomal degradation of lysyl oxidase (LOX) in the ductus arteriosus (DA) smooth muscle cells (SMC).\textsuperscript{51} Considering the results of the pharmacological inhibition study by IP\textgamma 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\textsuperscript{2+} signaling.

Ghigo et al reviewed that PI3K is master regulators of voltage-gated L-type Ca\textsuperscript{2+} channel (LTCC) activity.\textsuperscript{52} PI3K\textalpha promotes Akt (protein kinase B)-mediated phosphorylation of the LTCC subunit in cardiomyocytes. In that review, they suggested that PI3K can promote Ca\textsuperscript{2+} influx through an unidentified PIP3 (Ptdlns(3,4,5P3)-sensitive channel. PIP3 produced by PI3K activates Cdc42 and Rac1, leading to elevated cell migration.\textsuperscript{52} In the present study, we propose that EP4 is one of the activated GPCR that induces PIP3-sensitive Ca\textsuperscript{2+} entry and that Orai1 is a candidate channel for PIP-sensitive Ca\textsuperscript{2+} entry.

Taken together, we showed that EP4 was colocalized and formed complexes with Orai1 and TRPC1, activated PI3K and induced Ca\textsuperscript{2+} influx from the extracellular space and regulated the concentration of intracellular Ca\textsuperscript{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.\textsuperscript{34} 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.\textsuperscript{53} Therefore, we assumed that PGE\textsubscript{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 G\textsubscript{α} and G\textsubscript{i}, induced Ca\textsuperscript{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\textsuperscript{2+} signaling as a novel EP4 downstream pathway. Our results suggest that EP4 and Orai1 are promising candidates for targets of oral cancer therapy.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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

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