Ephrin A4-ephrin receptor A10 signaling promotes cell migration and spheroid formation by upregulating NANOG expression in oral squamous cell carcinoma cells

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Ephrin type-A receptor 10 (EPHA10) has been implicated as a potential target for breast and prostate cancer therapy. However, its involvement in oral squamous cell carcinoma (OSCC) remains unclear. We demonstrated that EPHA10 supports in vivo tumor growth and lymphatic metastasis of OSCC cells. OSCC cell migration, epithelial mesenchymal transition (EMT), and sphere formation were found to be regulated by EPHA10, and EPHA10 was found to drive expression of some EMT- and stemness-associated transcription factors. Among EPHA10 ligands, exogenous ephrin A4 (EFNA4) induced the most OSCC cell migration and sphere formation, as well as up-regulation of SNAIL, NANOG, and OCT4. These effects were abolished by extracellular signal-regulated kinase (ERK) inhibition and NANOG knockdown. Also, EPHA10 was required for EFNA4-induced cell migration, sphere formation, and expression of NANOG and OCT4 mRNA. Our microarray dataset revealed that EFNA4 mRNA expression was associated with expression of NANOG and OCT4 mRNA, and OSCC patients showing high co-expression of EFNA4 with NANOG or OCT4 mRNA demonstrated poor recurrence-free survival rates. Targeting forward signaling of the EFNA4-EPHA10 axis may be a promising therapeutic approach for oral malignancies, and the combination of EFNA4 mRNA and downstream gene expression may be a useful prognostic biomarker for OSCC.

Abbreviations
CFSE Carboxyfluorescein succinimidyl ester
CSCs Cancer stem-like cells
EFNA3 Ephrin A3
EFNA4 Ephrin A4
EFNA5 Ephrin A5
EMT Epithelial–mesenchymal transition
EPHA2 Ephrin type-A receptor 2
EPHA10 Ephrin type-A receptor 10
ERK Extracellular signal-regulated kinase
FACS Fluorescence activated cell sorting
IHC Immunohistochemistry
HNSCC Head and neck squamous cell carcinoma

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Head and neck squamous cell carcinoma (HNSCC) includes a group of tumors arising in the oral cavity, oropharynx, and larynx. Oral squamous cell carcinoma (OSCC), a subset of HNSCC, accounts for over 90% of all malignancies in the oral cavity. Most deaths from OSCC involve local recurrence at the primary site and regional recurrence at sites of peripheral lymph node metastasis. The overall 5-year survival rate of OSCC patients remains low. Understanding the pathophysiology of oral tumorigenesis is important for identifying effective therapeutic targets.

Epithelial–mesenchymal transition (EMT) is a process in which cells lose their epithelial-specific characteristics, including cell polarity and cellular adhesion, and acquire migratory and invasive capabilities. Although EMT is essential to normal development, EMT in tumors is associated with increased aggressiveness and poor prognosis, emphasizing EMT as a mechanistic role for tumor progression and metastasis. Decreased E-cadherin and increased vimentin/N-cadherin expression are conventional EMT markers. Additionally, the transcription factors Snail, Slug, Twist and zinc finger E-box-binding homeobox 1/2 (ZEB1/2) have been shown to modulate EMT by reducing epithelial marker expression. Cancer stem-like cells (CSCs) are converted from non-CSCs by activation of EMT programming. CSCs are a group of highly tumorigenic cells that can self-renew and differentiate into heterogeneous progeny. CSCs have been identified in an array of solid and hematological cancer types. Transcription factors such as Kruppel-like factor 4 (KLF4), sex determining region Y-box 2 (SOX2), octamer-binding transcription factor 4 (OCT4), and NANOG are required for maintenance of stemness in CSCs. CSCs play significant roles in tumor metastasis and recurrence, which are common causes of the high morbidity of oral cancer. However, little is known about the regulatory mechanisms of EMT and CSCs in OSCC.

The Eph receptor (Eph) and its ligands, ephrins, regulate development and tissue homeostasis. Ephrins are attached to the cell membrane by either a glycosylphosphatidylinositol anchor (A-type) or by a membrane-spanning protein domain (B-type). Their receptors are also divided into A and B classes based on gene sequence similarity and the binding of ephrin A or B ligands. Ephrin-Eph receptor binding leads to bidirectional signaling via transcellular interaction. In contrast, cis-interactions between molecules on the same cell attenuate Eph receptor signaling, possibly by inhibiting the formation of Eph receptor clusters. Eph receptor “forward” signaling depends on the tyrosine kinase domain, which is responsible for autophosphorylation, phosphorylation of other proteins, and associations with various effector proteins. The Eph receptor and ephrins are also able to mediate cell signaling in the absence of Eph receptor kinase activity. Ephrin-A receptor 10 (EPHA10) and ephrin B receptor 6 have critical functions under normal and pathological conditions, but lack the amino acid residues required for kinase activity. “Reverse” signaling in ephrin-expressing cells has been explored less than Eph receptor forward signaling. There is a scarcity of knowledge of the function and mechanisms that mediate ephrin type A signaling, specifically, as members of this subclass of molecules lack a cytoplasmic signaling domain. The biological functions induced by Eph receptor activation are diverse and cell-type specific. They regulate a variety of physiological and developmental processes, and have been implicated in both anti- and pro-tumorigenic activities in different cancer types.

Eph receptors are associated with the development of mesenchymal-like characteristics and inhibition of epithelial-like phenotypes. Recently, EPHA10 was shown to be overexpressed in breast cancer tissues, and the levels of EPHA10 mRNA and protein were significantly correlated with lymph node metastasis, cancer stage, and tumor progression. Targeting EPHA10 with anti-EPHA10 monoclonal antibodies significantly suppressed tumor growth in xenograft mouse models of triple-negative breast cancers. However, the underlying mechanisms of EPHA10-mediated tumorigenesis, EMT, and CSC induction in OSCC remain unclear. Therefore, we investigated the role of EPHA10 in regulating tumor growth and lymph node metastasis of OSCC cells and demonstrated that forward signaling mediated by the EFNA4-EPHA10 axis modulates cell migration and sphere formation, as well as up-regulation of the transcription factors NANOG and OCT4 via ERK activation. Additionally, EFNA4 mRNA expression was positively correlated with that of NANOG or OCT4, and high co-expression of EFNA4 with NANOG or OCT4 was associated with poor recurrence-free survival in OSCC patients.

Results

EPHA10 knockdown reduced tumorigenesis and lymph node metastasis of OSCC cells. To assess EPHA10 expression in different cancer types, we searched for data on EPHA10 expression in datasets from the Oncomine database. We surveyed 4 datasets from breast, esophageal, and lung cancers and found significantly higher expression of EPHA10 in cancer versus normal tissue. In contrast, 7 datasets showed markedly lower EPHA10 expression in colorectal, brain, and central nervous system cancers compared to site-matched normal tissue. However, EPHA10 expression was not significantly different when comparing data from cancer and normal tissue in available HNSCC datasets. To investigate EPHA10 protein expression, we examined OSCC cells, a subset of HNSCC, and found variable levels of EPHA10 protein, ranging from 0.69- to 2.12-fold, in the dysplastic oral keratinocyte (DOK) and OSCC cell lines compared with human oral keratinocytes (HOKs). We also found slightly higher expression of EPHA10 in LN1-1 cells, an OSCC cell line (in vivo OEC-M1 cell derivation) with high activities of tumor growth and lymphatic metastasis, compared to parental OEC-M1 cells (Fig. 1B).

To evaluate the effects of EPHA10 on OSCC tumor growth and lymphatic metastasis, we ablated EPHA10 expression in LN1-1 cells using lentiviral shRNA. We confirmed EPHA10 knockdown in LN1-1 cells by quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

H&E: Hematoxylin and eosin
OCT4: Octamer-binding transcription factor 4
OSCC: Oral squamous cell carcinoma
PI: Propidium iodide
qRT-PCR: Quantitative reverse transcription-polymerase chain reaction
Figure 1. EPHA10 is required for tumorigenesis and metastasis of OSCC cells. (A) Expression of EPHA10 in 20 types of cancer versus corresponding normal tissues using the Oncomine database with the threshold of fold change $\geq 2$, $p \leq 10^{-4}$, and gene rank $\geq$ top 10%. Red and blue, respectively, indicate the numbers of datasets with statistically significant increases and decreases in EPHA10 gene expression. (B) EPHA10 expression in human oral keratinocytes (HOK), immortalized dysplastic oral keratinocytes (DOK), and 7 OSCC cell lines was examined by western blotting. Protein levels were normalized to an internal control (α-tubulin). Relative ratios were determined by dividing the EPHA10 protein level in each cell type by that in HOK cells. (C) EPHA10 protein levels in LN1-1 cells expressing EPHA10-specific shRNA and vector control (pLKO-GFP) were determined by western blot. Protein levels were normalized to α-tubulin. Relative ratios were determined by dividing the EPHA10 protein level in each expression variant by that in the pLKO-GFP vector-expressing cells. (D) EPHA10 protein levels in LN1-1 cells expressing pLKO-GFP (green line), sh3 (dark green line), and sh5 (pink line) were determined by fluorescence activated cell sorting (FACS). Relative EPHA10 expression was determined by dividing the fluorescent intensity in each expression variant by that in the pLKO-GFP vector-expressing cells. (E) Tumor weights and volumes in mice orthotopically injected with LN1-1 pLKO-GFP (n = 9) or EPHA10 sh3 cells (n = 8). (F) Ki-67 expression by immunohistochemistry (IHC) in LN1-1 pLKO-GFP (n = 9) and EPHA10 sh3 tumors (n = 7). Left: Representative fields of IHC stained sections. Scale bars, 20 μm. Right: The percentages of Ki-67-positive cells per field were calculated for each group. Error bars represent SE; * $p < 0.05$; ** $p < 0.001$. 
immunoblot and flow cytometry (Fig. 1C,D). We performed oral cavity inoculation of immunodeficient nude mice with EPHA10 knockdown and control cells. Tumors developed in all animals regardless of EPHA10 level (Table 1). The average tumor weight was 0.2278 ± 0.03017 g (n = 9) and 0.0475 ± 0.01191 g (n = 8) in mice receiving control cells and EPHA10 knockdown LN1-1 cells, respectively (Fig. 1E). Similarly, EPHA10 knockdown led to significantly lower tumor volume compared to controls (Figs. 1E, S1A). There were consistently, fewer Ki-67-positive cells in EPHA10 sh3 orthotopic tumors (16.63 ± 2.247%, n = 7) compared to controls (27.46 ± 3.597%, n = 9; Fig. 1F). Additionally, 67% (n = 9) of the animals injected with control cells developed lymph node metastases compared to none of the EPHA10 sh3 injected animals (0%, n = 8), as assessed by examination of H&E-stained cervical lymph nodes for evidence of tumor formation (Fig. S1B, Table 1). Quantification of LYVE-1-positive areas demonstrated reduced intratumoral lymphatics in tumors of mice injected with EPHA10 sh3 (0.9656 ± 0.139 vessels/field per specimen, n = 8) compared to those injected with parental LN1-1 cells (2.225 ± 0.454 vessels/field per specimen, n = 9; Fig. S1C). These results indicate that EPHA10 is important for OSCC tumor growth and lymph node metastasis.

Manipulation of EPHA10 levels altered OSCC cell properties. Knockdown of EPHA10 in LN1-1 cells showed no difference in cell proliferation compared with cells expressing the vector control (Fig. S1D). In contrast, knockdown of EPHA10 diminished the migration potential of LN1-1 cells, as indicated via transwell assay, compared to corresponding controls (Fig. S1E). LN1-1 cells expressing EPHA10 sh5 were used to confirm the inhibitory effects of EPHA10 knockdown on cell migration (Fig. 2A). Phase contrast microscopy revealed obvious morphological differences in EPHA10 knockdown versus control cells; EPHA10 knockdown cells had a predominately polygonal shape, while the control cells showed fibroblastic morphology (Fig. 2B). In addition, immunoblot analysis showed that EPHA10 knockdown led to differential expression of epithelial proteins, such as α-catenin, E-cadherin, and β-catenin, as well as the mesenchymal protein vimentin (Fig. 2C). Sphere formation is an efficient way to assess the properties of CSCs in vitro, and cells with EPHA10 knockdown showed reduced sphere formation compared to controls (Fig. 2D). Likewise, mRNA expression of EMT- and stemness-associated transcription factors, including TWIST, SLUG, SNAIL, OCT4, and NANOG, was diminished in EPHA10 knockdown cells (Fig. 2E). These results suggest that EPHA10 knockdown alters OSCC cell properties, such as EMT, migration, and capacity for sphere formation, as well as expression of EMT- and stemness-associated transcription factors.

Additionally, OEC-M1 cells with low EPHA10 expression were infected with a retroviral vector encoding human EPHA10 (PB-EPHA10 cells) or an empty vector (PB cells), and ectopic EPHA10 expression was confirmed by immunoblot (Fig. S2A). Ectopic expression of EPHA10 did not affect OEC-M1 cell growth, division, death (Fig. S2B–D), or E-cadherin expression; however, vimentin expression was reduced (Fig. S2A). Unexpectedly, ectopic EPHA10 expression was associated with diminished cell migration and sphere formation (Fig. S2E,F). Furthermore, ectopic EPHA10 expression significantly decreased the expression of SNAIL, SLUG, OCT4, and NANOG mRNA. Our data demonstrated that EPHA10 knockdown and ectopic EPHA10 expression results in a similar phenotype in OSCC cells.

EFNA4 promoted cell migration, spheroid formation, and induction of EMT- and stemness-related transcription factors. The most high-affinity ligands for EPHA10 are ephrin-A3 (EFNA3), A4 (EFNA4), and A5 (EFNA5)26. We examined the expression of EFNA3, EFNA4, and EFNA5 in OEC-M1 and LN1-1 cells via the GSE62326 dataset and found that EFNA3 and EFNA5 expression was undetectable in both cell types. Lower EFNA4 mRNA and protein expression was found in OEC-M1 cells compared to LN1-1 cells using microarray analysis and western blotting, respectively (Fig. S3A), therefore OEC-M1 cells were used to investigate the effects of EFNA10 ligands on cellular functions. When exogenous EPHA10 ligands capable of binding to the receptors and labelled with IgG-Fc tags were added to cultured cells, neither EFNA3-Fc nor EFNA5-Fc affected OEC-M1 cell migration (Fig. S3B,C). Exogenous EFNA3-Fc enhanced tumorsphere size distribution, while exogenous EFNA3-Fc and EFNA5-Fc had no effects on sphere formation activity compared to controls (Fig. S3D,E). Our data suggest that exogenous EFNA3 and EFNA5 have no marked effects on migration or sphere-forming activities of OEC-M1 cells.

Exogenous EFNA4-Fc did not have a significant effect on cell growth or division (Fig. 3A,B), but there was a small effect on cell death in OEC-M1 cells treated with low dose EFNA4 (Fig. 3C). Exogenous EFNA4 enhanced OEC-M1 cell migration in a dose-dependent manner (Fig. 3D), an effect that was also observed with other OSCC cells, including SAS and TW2.6 (Fig. S4A). Exogenous EFNA4-Fc significantly increased sphere-forming activity in OEC-M1 cells (Fig. 3E), and qRT-PCR analysis showed that the expression of TWIST, SNAIL, OCT4, and NANOG mRNA was significantly elevated after addition of EFNA4-Fc to OEC-M1 cells (Fig. 3F).

| Groups          | Tumors/no of mice | Tumorigenesis (%) | Metastasis/no of mice | Metastasis (%) |
|-----------------|-------------------|-------------------|-----------------------|----------------|
| LN1-1 pLKO-GFP  | 9/9               | 100               | 6/9                   | 67             |
| LN1-1 EPHA10 sh3| 8/8               | 100               | 0/8                   | 0              |

Table 1. Tumor formation and spontaneous lymph node metastases in mice injected with oral squamous cell carcinoma (OSCC) cells expressing EPHA10 shRNA or the vector control.
ERK inhibition abolished EFNA4-induced cellular functions. To further characterize EFNA4-induced forward signaling, we treated OEC-M1 cells with EFNA4-Fc, and the levels of phosphorylated forms of focal adhesion kinase (FAK), protein kinase B (PKB/AKT), and extracellular signal-regulated kinase (ERK), as well as non-phospho-β-catenin and integrin-linked kinase (ILK) were assayed at different time points by western blotting (Figs. S5, 4A). EFNA4-Fc induced activation/phosphorylation of only ERK in OEC-M1 cells (Fig. 4A). The blockage of ERK activation by PD98059, an inhibitor of mitogen-activated protein kinase (MAPK) kinase, inhibited EFNA4-induced cell migration (Fig. 4B), spheroid formation (Fig. 4C), and expression of SNAIL, OCT4, and NANOG mRNA (Fig. 4D). Confirmation of the effects of PD98059 on EFNA4-induced NANOG expression is shown in Fig. 4E. Our results indicated that exogenous EFNA4-induced functional changes were dependent on MAPK/ERK activation. To verify the role of NANOG in EFNA4-induced migration and sphere formation, NANOG expression in OEC-M1 cells was abolished using lentiviral shRNA and confirmed by qRT-
Figure 3. Exogenous EFNA4-Fc enhanced cell migration and sphere formation of OEC-M1 cells. (A) Representative growth curves of OEC-M1 cells treated with 0.1 or 0.5 μg/ml EFNA4-Fc (n = 4) or 0.5 μg/ml IgG control (n = 4) based on MTS assay data. (B) Left: Representative CFSE cell division assay data from OEC-M1 cells treated with 0.1 or 0.5 μg/ml EFNA4-Fc (n = 2) or 0.5 μg/ml IgG control (n = 2). The interval bar indicates cell division signal on day 3. Right: The percentage of the cell population within the interval bar limits. (C) Left: Representative cell death analysis via PI/Annexin V double staining of OEC-M1 cells treated with 0.1 or 0.5 μg/ml EFNA4-Fc (n = 2) or 0.5 μg/ml IgG control (n = 2). Right: The percentage of cell death, including quadrants Q1, Q2, and Q3. (D) Left: Representative images of migrated cells. Scale bars, 100 μm. Right: Relative migration activity was determined by normalizing the mean number of migrated cells per field of EFNA4-Fc treated cells (n = 10) to that of the IgG-treated cells (n = 10). (E) Left: Representative images of tumorspheres. Scale bars, 100 μm. Right: Sphere formation in OEC-M1 cells treated with 0.1 or 0.5 μg/ml EFNA4-Fc or 0.5 μg/ml IgG control in sphere culture. Relative sphere formation activity in EFNA4-treated cells (n = 2) was determined by normalizing the mean number of spheres per well to that of the IgG-treated cells (n = 2). (F) Relative levels of TWIST, SNAIL, SLUG, OCT4, NANOG, and SOX2 mRNA in OEC-M1 cells treated with 0.1 or 0.5 μg/ml EFNA4-Fc or 0.5 μg/ml IgG as determined by qRT-PCR. The amplifications were first normalized to β-actin (internal control). The relative mRNA expression in EFNA4-treated OEC-M1 cells (n = 3) was then normalized to that in IgG-treated cells (n = 3). Bars represent SE. *p < 0.05; **p < 0.01; ***p < 0.001.
SNAIL expression of NANOG cancer xenograft mouse models. However, the underlying mechanisms of EPHA10-mediated tumorigenesis gene regulation in a given cell type. Antibodies targeting EPHA10 significantly inhibited tumor growth in breast effect of EPHA10 was tissue-specific and likely dependent on the milieu of binding partners and related target signal among cells (Figs. 2, S2).

relative level of EFNA4 for trans-interaction between EFNA4-EPHA10, thereby inhibiting the EPHA10 forward we found that EPHA10 mRNA expression varied among different cancer types (Fig. 1A), indicating that the expression of EFNA4 in OSCC, we analyzed EFNA4 expression in publicly available cDNA microarray datasets (Table S1). Two datasets from the Oncomine database demonstrated significant increases in EFNA4 mRNA in OSCC compared to normal tissues ($p < 0.0001$ and $p < 0.05$; Fig. 6A, a and b, respectively). Expression of EFNA4 mRNA was also significantly increased in 40 OSCC specimens compared to nontumor controls ($p < 0.0001$; Fig. 6A, c). $EFNA4$ mRNA expression was significantly higher in grade 2 OSCC than in grade 1 tumors ($p < 0.05$, Fig. 6A, d) and $EFNA4$ mRNA was significantly increased in OSCC with angiolymphatic invasion compared to tissue without invasion ($p < 0.01$, Fig. 6A, e). We assessed EFNA4 protein level in both tumor tissue and corresponding noncancerous epithelia in OSCC samples by immunohistochemical staining (IHC; Fig. 6B). High EFNA4 expression (intensity grade 2 or 3) was observed in 72.2% (13/18) of tumors, compared with only 11.8% of noncancerous tissues showing high EFNA4 expression ($p < 0.001$, Fig. 6C). Moreover, 75% of OSCC specimens demonstrated higher EFNA4 expression in tumor regions than in noncancerous regions (Fig. 6D).

To investigate the clinical significance of EFNA4-EPHA10 signaling and expression of NANOG and OCT4 mRNA, we further investigated the cDNA expression dataset GSE37991 (gene expression profiling of OSCC) based on its complete clinical information. We found that the expression of $EFNA4$, $NANOG$, and $OCT4$, but not $EPHA10$, mRNA was significantly increased in OSCC tissues compared to adjacent nontumor samples (Fig. 6D). Also, tumor/nontumor (T/N) ratios of $EFNA4$, $NANOG$, $OCT4$, and $EPHA10$ mRNA expression had averages of 1.39, 1.25, 2.37, and 1.03, respectively. Interestingly, a positive correlation existed between $EFNA4$ and $NANOG$ mRNA expression in OSCC samples (compared to paired nontumor tissues) using Pearson correlation (Fig. 6E). Similarly, significant correlations in mRNA expression also occurred between $EFNA4$ and $OCT4$ and between $NANOG$ and $OCT4$ (Fig. 6E). Furthermore, patients with higher $EFNA4$/NANOG or $EFNA4$/OCT4 mRNA expression levels had worse recurrence-free survival compared to patients with lower $EFNA4$/NANOG or $EFNA4$/OCT4 mRNA expression ($p = 0.027$, Fig. 6F). The clinical data confirmed our in vitro findings that $EFNA4$ mRNA expression correlated with expression of $NANOG$ or $OCT4$ mRNA.

Discussion

EPHA10 is associated with tumor progression and metastasis in breast cancer, and is a promising therapeutic target in triple-negative breast cancer and prostate cancer. Based on the publicly available clinical datasets, we found that $EPHA10$ mRNA expression varied among different cancer types (Fig. 1A), indicating that the effect of EPHA10 was tissue-specific and likely dependent on the milieu of binding partners and related target gene regulation in a given cell type. Antibodies targeting EPHA10 significantly inhibited tumor growth in breast cancer xenograft mouse models. However, the underlying mechanisms of EPHA10-mediated tumorogenesis are undefined. In this study, we first confirmed the essential role of EPHA10 in tumorogenesis and metastasis of OSCC cells with in vivo models (Figs. 1E, F, S1A,B,C, and Table 1), suggesting that EPHA10 signaling may be a useful therapeutic target in OSCC.

EPHA10 knockdown suppressed in vivo tumor growth and in vitro sphere formation (Figs. 1E, F, S1A, 2D), but did not affect cell proliferation (Fig. S1D). This discrepancy may be due to the complexity of spatial distribution and cell contact in tumor spheres, as well as interplay between tumor cells and tumor-associated stromal cells, suggesting a critical role for EPHA10 in complex cell–cell interactions and the tumor-associated microenvironment. Yin et al. found that EphA receptors and co-expressed ephrin-A ligands directly interact in cis via their binding domains, and that this interaction does not seem to induce intracellular signals, but inhibits the trans interaction. Similarly, our data imply that ectopic expression of EPHA10 could dramatically enhance the possibility of cis interactions with binding ligands, such as EFNA4, and that these interactions could reduce the relative level of EFNA4 for trans-interaction between EFNA4-EPHA10, thereby inhibiting the EPHA10 forward signal among cells (Figs. 2, S2).
EFNA3 is a tumor suppressor of malignant peripheral nerve sheath tumors\textsuperscript{35}, while EFNA5 overexpression is associated with prostate tumorigenesis\textsuperscript{35}. No significant effects of exogenous EFNA3 or EFNA5 on OSCC cell migration or spheroid formation were observed in our study (Fig. S3). In datasets of oral cancer gene expression, EFNA4 is upregulated in OSCC tissues compared with nontumor tissues and is correlated with later stage and angiolymphatic invasion of OSCC (Fig. 6A). Similarly, EFNA4 expression was elevated in breast cancer, ovarian cancer, and hepatocellular carcinoma\textsuperscript{34}, and may play a role in cell fate determination of mammary epithelial cells\textsuperscript{35}. EFNA4 may be a promising target for identification of tumor-initiating cells in triple-negative breast cancer and ovarian cancer\textsuperscript{34}. EFNA4-mediated bidirectional signaling is not well-characterized and many questions remain. Originally, our data suggested that EFNA4-Fc, like membrane-bound EFNA4, is involved in regulating TWIST, SNAIL, OCT4, and NANOG mRNA expression, as well as inducing cell migration and spheroid formation in OSCC cells (Fig. 3). Yin et al. illustrated that Stat/Snail signaling modulated the co-expression of OCT4 and NANOG\textsuperscript{36}. We found that EFNA4 induced expression of SNAIL, NANOG, OCT4 mRNA via ERK activation (Fig. 4), however, Stat3 activation was not addressed in this study. Additionally, our data demonstrated the inhibitory effects of NANOG knockdown on EFNA4-induced cell migration and sphere formation (Fig. 4). Similarly, Yin et al. reported that co-expression of OCT4 and NANOG induced development of CSC characteristics and enhanced EMT in hepatocellular carcinoma, and Huang et al. showed that ERK-NANOG signaling promoted CSC phenotypes and EMT in HNSCC\textsuperscript{36,37}.

Overexpression of EPHA2 is related to malignancy and tumor angiogenesis of tongue squamous cell carcinoma\textsuperscript{38}. EPHA2 also contributes to human glioma stem cell formation and stemness marker SOX2 expression\textsuperscript{39}. However, the roles of EPHA10 in development of cancer stemness remain unknown. Our data showed that knockdown of EPHA10, but not EPHA2, influences exogenous EFNA4-induced spheroid formation (Fig. 5C,F), while both EFNA4-EPHA2 and EFNA4-EPHA10 forward signaling axes regulate migration of OSCC cells (Fig. 5B,E). EPHA2 activation and EPHA2 overexpression displayed opposite roles in regulation of cell migration and invasion; EPHA2 activation by ephrin A1 suppressed chemotactic migration, whereas overexpression of EPHA2 enhanced migration in a ligand-independent manner\textsuperscript{40}. EFNA4-EPHA2 signaling in chronic lymphocytic leukemia cells significantly reduced their adhesion and impaired cell trafficking and chemotaxis\textsuperscript{41}. Any association between Eph receptor-ligand signaling and migration may depend on tumor subtype, microenvironmental context, and other parameters. Interestingly, we found that EFNA4-EPHA2 forward signaling affects cell migration but not spheroid formation, indicating a difference in receptor-specific functions and signaling between EPHA2 and EPHA10. Downstream proteins that interact with kinase-deficient EPHA10 and the mechanisms involved in EPHA10 phosphorylation require further investigation to clarify the differences between EPHA10 and EPHA2 signaling\textsuperscript{42}. In OSCC cells, EPHA10 plays a critical role in linking external stimuli (EFNA4 or other ligands) to the internal signal transduction that leads to cancer cell migration and spheroid formation. When considering ephrin ligands and their receptors as potential therapeutic targets, excessive toxicity associated with pan-ephrin receptor inhibition must be considered.
As previously shown in other tumor types, we demonstrated upregulated expression of EFNA4 in OSCC tissues compared to nontumor tissues (Fig. 6B,C). Interestingly, we found that EFNA4 was expressed in both tumor cells and tumor-infiltrating immune cells (unpublished data), suggesting that EFNA4 could be expressed by oral cancer cells and their associated stromal cells. Further investigation is needed to determine whether contact between tumor cells and infiltrating immune cells contributes to oral tumorigenesis. We provided data that characterizes EFNA4-EPHA10 forward signaling in OSCC cells using exogenous Eph receptor ligands, however, characterization of reverse signaling in EFNA4-expressing cells requires additional research. In this study, we demonstrated correlation of EFNA4, NANOG, and OCT4 mRNA expression in clinical OSCC specimens (Fig. 6E), and showed that the co-expression of EFNA4 with NANOG or OCT4 was associated with poor prognosis in OSCC patients (Fig. 6F). These data were consistent with the finding that the expression of NANOG and OCT4 mRNA was enhanced by induction of EFNA4-EPHA10 forward signaling in OSCC cells (Fig. 5G). Our data did not show correlation of EPHA10 expression with OSCC clinical outcome and differential expression of EPHA10 in the GSE37991 dataset (Fig. 6D). However, our data indicated inhibition of trans-interaction by ectopic EPHA10 expression (Fig. S2), suggesting that upregulation of both EPHA10 and EFNA4 in OSCC tissues increased cis-interactions, and that upregulation of EFNA4 in cancer tissues was enough to induce the downstream effects of EFNA4-EPHA10 forward signaling.

Collectively, EPHA10 supports tumor growth and lymph node metastasis of OSCC cells in vivo. Signaling by EPHA10 and its ligand, EFNA4, promotes OSCC cell migration and tumor spheroid formation through induction of NANOG mRNA expression via ERK activation (Fig. 6G). Finally, our findings are supported by clinical data showing that patients with high co-expression of EFNA4/NANOG or EFNA4/OCT4 mRNA had worse recurrence-free survival than those with low co-expression (Fig. 6F), supporting a significant role for EPHA10 and EFNA4 in OSCC development and progression, as well as the significance of these signaling molecules as potential therapeutic targets.

Methods
Gene expression data mining. Oncomine (www.oncomine.org), an online web-based cancer database for RNA and DNA sequences, was used to facilitate data-mining of the expression of gene transcripts in 20 types of cancer. Expression of EPHA10 or EFNA4 mRNA in cancer samples was compared with expression in samples of normal tissue using the Student’s t-test.

Cell lines and reagents. Human oral keratinocytes (HOK) were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and grown in an oral keratinocyte medium (OKM) according to the manufacturer’s protocols. OSCC cell lines, including the OC3 line established from an OSCC specimen, the CGHNC9 line established from an oral cancer specimen, the TW2.6 line established from a buccal carcinoma, the OEC-M1 line established from an oral epitheliod carcinoma, the SAS line established from a poorly differentiated tongue squamous cell carcinoma, the DOK line established from a human dysplastic oral mucosa, the HSC3 line established from tongue carcinoma with cervical metastasis, and the LN1-1 line developed via in vivo selection from OEC-M1-derived tumor, were obtained and kept within 20 passages in each experiment.

Immunoblot assay. Immunoblot assays were conducted as previously described. The primary antibodies used were anti-EPHA10 (ab75955, Abcam, Cambridge, UK), anti-EFNA4 (MAB369, R&D Systems), anti-EPHA2 (sc-924, Santa Cruz, Santa Cruz, CA, USA), anti-β-catenin (610253, BD Biosciences, Franklin Lakes, NJ, USA), anti-E-cadherin (610182, BD Biosciences), anti-a-catenin (610193, BD Biosciences), anti-vimentin...
Figure 6. Co-expression of EFNA4 with NANOG and OCT4 mRNA in OSCC. (A) Increased EFNA4 mRNA expression in OSCC tissues compared to normal oral tissues or non-tumor areas via clinical dataset analysis (a–c). Increased EFNA4 mRNA expression in OSCC of higher tumor grade or angiolymphatic invasion via clinical dataset analysis (d–e). The relative EFNA4 mRNA expression is represented by log, median-centered intensity in datasets a–d. The average tumor/non-tumor (T/N) ratio of EFNA4 mRNA is shown in dataset e. (B) Immunohistochemical analysis of EFNA4 in human OSCC samples. No expression or weak EFNA4 expression in the nontumor epithelium (left panel) and strong EFNA4 staining in the OSCC areas is visible at 200 x (scale bar, 200 μm) and 400 x (scale bar, 50 μm) magnifications. (C) Left: Scoring of EFNA4 staining intensity in 17 noncancerous epithelium samples (light grey bars) and 18 tumor samples (dark grey bars). Expression levels are scored as: 0, none; 1, weak; 2, moderate; 3, strong. Right: Comparison of the EFNA4 staining intensity between tumor areas (T) and noncancerous epithelium (N) based on each histological section. (D) EFNA4, NANOG, OCT4, and EPHA10 mRNA expression in OSCC tissues (n = 40) and corresponding nontumor (NT) tissues (n = 40). Data were obtained from clinical dataset GSE37991. The expression is represented by log, median-centered intensity. (E) Correlations between the T/N ratios of EFNA4, EPHA10, NANOG, and OCT4 mRNA using the GSE37991 dataset and Pearson correlation analysis. Pearson’s correlation coefficient (r) between two variants is shown in the center of the box at the intersect of each pair (n = 40). (F) Recurrence-free survival analysis with EFNA4, NANOG, and OCT4 mRNA expression as classification criteria using dataset GSE37991. Patients were stratified into low (EFNA4low/NANOGlow or EFNA4low/OCT4low, n = 23) and high (EFNA4high/NANOGhigh or EFNA4high/OCT4high, n = 17) groups using the median expression level of each mRNA as the cutoff. (G) The role of ephrin A4 (EFNA4)-ephrin receptor A10 (EPHA10) forward signaling in promoting OSCC tumorigenesis and metastasis. EFNA4 from adjacent tumor cells or stromal cells binds to EPHA10 on OSCC cells and induces extracellular signal-regulated kinase (ERK) activation. ERK activation drives progressive effects, including cell migration and spheroid formation, and up-regulation of NANOG expression. NANOG is required for EFNA4-induced cell migration and sphere formation (indicated as dark blue dashed arrows). Bars represent SE; *p < 0.05; **p < 0.01.

Fluorescence activated cell sorting (FACS). FACS analysis was performed on a cytometer (FACSCalibur, BD) and analyzed using FlowJo 7.6 software (FlowJo, Ashland, OR, USA). Cells were stained with anti-EPHA10 (Abcam, ab75955) and a secondary antibody conjugated with DyLight 488 (611-141-002, Rockland, Limerick, PA, USA). The shift in peak fluorescence intensity was measured and the geometric mean of fluorescence was calculated.
Cell death analysis by Annexin V/propidium iodide (PI) assay. The cell death assay was performed using the Annexin V Apoptosis Detection Kit (BD Biosciences), according to the manufacturer’s instructions. The samples were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and PI on ice and analyzed by flow cytometry within one hour. The experiments were performed at least twice.

Migration assay. Cell migration analyses were conducted using a transwell assay as previously described52. Relative migration activity was determined by normalizing the mean number of cells that had migrated per field (n = 10) in the experimental condition to that of control cells. The experiments were performed at least twice.

Orthotopic inoculation in nude mice. The procedures for orthotopic inoculation were previously described53. A total of 5 × 10^5 cells in 50 μL sterile DPBS was injected into the buccal mucosa of two batches of 5–6-week-old male nude mice (BALB/cAnN.Cg-Foxn1nu/CrlNarl; n = 4–5 per batch) and mice were sacrificed at 28–31 days post-inoculation for the first and second batches, respectively. Tumor size was determined by measuring the tumor dimensions and calculating volume (mm^3) using the formula 1/2 × (length) × (width)^2. The orthotopic tumors were weighted and then processed by the Pathology Core Lab (National Health Research Institutes, Taiwan).

Immunohistochemical analysis. IHC analysis was conducted as previously described50. The primary antibodies used were anti-Ki-67 (NCL-Ki67p, Leica Biosystems, Buffalo Grove, IL, USA), anti-LYVE-1 (07-358, Upstate Biotechnology, Lake Placid, NY, USA) and anti-human EFNA4 (MAB369, 1:50, R&D Systems). Sections were counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MO, USA). The percentage of positive Ki-67 nuclei was determined using ImmunoRatio software and dividing the total intensity of positive nuclei by that of all nuclei in the field53. For LYVE-1 staining, the data were expressed as mean number of LYVE-1 positive vessels per microscopic field in each specimen. To determine EFNA4 expression, formalin-fixed and paraffin-embedded OSCC tissues containing both tumor and adjacent nontumor epithelium were obtained from the Department of Pathology at National Cheng Kung University Hospital (HR-97-100 and EC1040406-E). Expression of EFNA4 in each tissue section was scored as 0, 1, 2, or 3 (0 = negative, 1 = weak, 2 = intermediate, 3 = strong) based on staining intensity.

Cell morphology. Imaging of actively proliferating cells was achieved using an inverted microscope with a phase-contrast lens, as previously described53.

Sphere formation. Cells (1 × 10^5) in DMEM/F12 medium (Sigma-Aldrich) with 20 ng/ml of basic fibroblast growth factor (bFGF, Abcam), 10 ng/ml of epidermal growth factor (EGF; Thermo Fisher Scientific, Waltham, MA, USA), and B27 supplement (Thermo Fisher Scientific), were cultured in Corning Costar Ultra-Low Attachment 6-Well Plates (CLS3471, Merck, Darmstadt, Germany) for 14 days. The number and size of tumorspheres were quantitatively assessed using ImageJ. Relative sphere formation activity was determined by normalizing the mean number of spheres per well (n = 2) in the experimental condition compared to that of control cells. The experiments were performed at least twice.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The qRT-PCR was performed as previously described54. The primer sequences used are listed below. TWIST-F: 5′-ACGCTGCCC TGGGACA A-3′; TWIST-R: 5′-AGACCCTTGGTAGAGGAAGTCTGA-3′; SNAIL-F: 5′-GTCAGATGAGGACACAG TGGAAAG-3′; SNAIL-R: 5′-CAAGGAAAGACGTGAAAGTGAAGGAAAG-3′; SLUG-F: 5′-AGACCTGGTGT CTGCAAGGA-3′; SLUG-R: 5′-GACCTGGTGTGCAAATGCTGCTG-3′; OCT4-F: 5′-GAGAACCGAGTGGAGGC AAC-3′; OCT4-R: 5′-CTGATCTGGTCAGTTGCG-3′; NANOG-F: 5′-CCGAAAACCGAGAATGAAATCTGA GA-3′; NANOG-R: 5′-TGAGGCGCTTCTGCGTCAACA-3′; SOX2-F: 5′-CGTTCTATGGAGGGCAGTGAAGGAGG-3′; SOX2-R: 5′-TCATGACGGCCTTTGTTTCC-3′; β-actin-F: 5′-TGGATCAGCAAGCAGGATATGAGGACTGGAGG-3′; β-actin-R: 5′-GCATTGCAGTTGACAGCAT-3′. Each amplification was run in triplicate.

Statistical analysis. Analysis was conducted with GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA). The AVOVA test and Student’s t-test were used to assess statistical differences between groups. The paired t-test was used to determine differences in gene expression between tumor samples and adjacent nontumor samples. The Pearson correlation was used to evaluate the linear relation between two variants. The log rank test was used to evaluate differences in survival between stratified groups. The R base heatmap function (R Performance Analytics Package, https://www.rdocumentation.org/packages/PerformanceAnalytics/versions/2.0.4) was used to calculate the correlation between gene expression patterns and produce the correlation matrix. For all comparisons, p < 0.05 was considered statistically significant.

Ethics approval and consent to participate. All animal studies followed the guidelines for the Care and Use of Laboratory Animals of National Health Research Institutes, Taiwan. The protocols were approved by the Institutional Animal Care and Use Committee of National Health Research Institutes (Protocol No. NHRI-IACUC-106001-A). Use of tissue sections and oral cancer cells was approved by IRB (EC1040406-E, National Health Research Institutes). Informed consent was obtained from all subjects or, if subjects were under 18, from
a parent and/or legal guardian. All methods were carried out in accordance with relevant guidelines and regulations.

Data availability
The data, generated and/or analyzed, that are included in this article are available from the corresponding author upon reasonable request.

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Author contributions
Y.-L.C., Y.-C.Y., S.-H.W., and H.-T.H. conducted molecular, biochemical, and cellular biology experiments; J.-R.H. and J.-Y.C. contributed to collection of OSCC specimens; Y.-L.C. and C.-W.J. performed data analysis and manuscript writing; C.-W.J. and C.-H.C. performed the survival analysis; Y.-W.C. was responsible for experimental design, data analysis, and manuscript preparation; all authors contributed to manuscript editing.

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Competing interests
The authors declare no competing interests.

Additional information
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