Regulation of estrogen receptor α function in oral squamous cell carcinoma cells by FAK signaling

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Abstract

Estrogen receptor α (ER α) is a DNA-binding transcription factor that plays an important role in the regulation of cell growth. Previous studies indicated that the expression of ER α in cell lines and tumors derived from oral squamous cell carcinoma (OSCC). The aim of this study was to examine the activity and function of ER α in OSCC cells and the mechanism underlying ER α activation. Immunohistochemical analyses in benign ($n = 11$) and malignant ($n = 21$) lesions of the oral cavity showed that ER α immunoreactivity was observed in 43% (9/21) of malignant lesions, whereas none of benign lesions showed ER α immunoreactivity. The ER α expression was also found in three OSCC cell lines and its transcriptional activity was correlated with cell growth. Addition of estradiol stimulated cell growth, whereas treatment of tamoxifen or knockdown of ER α expression caused reduced cell growth. Interestingly, the expression and activity of focal adhesion kinase (FAK) were associated with the phosphorylation of ER α at serine 118 in OSCC cells. Elevated expression of FAK in the slow-growing SCC25 cells caused increases in ER α phosphorylation, transcriptional activity, and cell growth rate, whereas knockdown of FAK expression in the rapid-growing OECM-1 cells led to reduced ER α phosphorylation and activity and retarded cell growth. Inhibition of the activity of protein kinase B (AKT), but not ERK, abolished FAK-promoted ER α phosphorylation. These results suggest that OSCC cells expressed functional ER α , whose activity can be enhanced by FAK/AKT signaling, and this was critical for promoting cell growth. Thus, FAK and ER α can serve as the therapeutic targets for the treatment of OSCC.

Key Words

- ▶ oral squamous cell carcinoma
- ▶ estrogen receptor α
- ▶ focal adhesion kinase

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Introduction

Estrogen receptors (ERs), including ER α and ER β are nuclear receptors that function as transcription factors to active gene expression for regulating cell proliferation and survival. Upon ligand binding, ERs undergo phosphorylation and conformational changes that lead to the dissociation of heat shock proteins and subsequent

receptor dimerization. The receptor dimers then undergo nuclear translocation and bind to estrogen-responsive elements (EREs) present in the promoter of estrogen-responsive genes. Once bound to EREs, ERs recruit coactivators or corepressors to form a complex that regulates the transcription of estrogen-responsive genes

and thus modulates a variety of physiological functions. Instead of binding to DNA, ligand-activated ERs can function as coactivators to regulate gene transcription by interacting with certain transcription factors, such as activating protein 1 and specificity protein 1 (Osborne & Schiff 2005, Thomas & Gustafsson 2011). Alternatively, ER functions can be regulated by intracellular signaling via phosphorylation in the absence of ligands. For example, phosphorylation of ER α plays critical roles in the regulation of ER α transcriptional activity, stability, and DNA binding (Weigel & Moore 2007, Murphy *et al.* 2011). Although ER α can be phosphorylated at multiple sites, serine 118 (Ser118) is one of the most reported phosphorylation sites with clinical significance (Murphy *et al.* 2009). Phosphorylation of ER α at Ser118 promotes the recruitment of coregulators to ER α -regulated genes and modulates estrogen-induced gene expression (Duplessis *et al.* 2011).

Accumulated evidence demonstrates the oncogenic roles of ERs in hormone-regulated cancers, such as breast, prostate, ovarian, or endometrial cancers; however, the role of ERs in oral squamous cell carcinoma (OSCC) is less understood. Previous studies have shown that the expression of ER α and ER β in OSCC tumors and cell lines, and treatment with tamoxifen significantly, inhibits cell proliferation and invasion (Ishida *et al.* 2007, Kim *et al.* 2007, Ku & Crowe 2007, Lukits *et al.* 2007). The expression of ER α and ER β is also found in head and neck squamous cell carcinoma (HNSCC) tumors, but the nuclear levels of ER α , but not ER β , in tumor lesions are significantly higher than that in paired adjacent mucosa. Importantly, patients with high nuclear ER α expression in tumors have a diminished survival rate compared with patients with low nuclear ER α (Egloff *et al.* 2009). These results suggest that HNSCC cells express ER α , the activities of which are associated with tumor development. However, the activation status of ER α and the regulatory mechanism of ER α activation in OSCC cells are mostly unknown.

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase and is ubiquitously expressed in all tissues and cell types. It plays important roles in the mediation of adhesion-dependent and growth factor-dependent signals through the activation of the MEK/ERK and the PI3K/AKT pathways, regulating a variety of cellular functions including proliferation, survival, and motility. In contrast, deregulated FAK signaling causes uncontrolled proliferation and enhanced migration, promoting the malignant transformation of tumor cells (Zhao & Guan 2009). A large number of studies have shown an increased expression of FAK (RB1CC1) mRNA and/or

protein in different human cancers (Golubovskaya *et al.* 2009). In OSCC, the elevated expression and phosphorylation of FAK have been observed (Kornberg 1998, Schneider *et al.* 2002, Aronsohn *et al.* 2003). Overexpression of FAK in low-invasive HNSCC cells leads to an elevated growth rate and an enhanced invasive capability (Schneider *et al.* 2002). Despite intensive studies on FAK functions in different cancers, the oncogenic role of FAK in OSCC cells is not fully understood.

The aim of this study was to analyze the expression of ER α in OSCC tumors and cell lines and to examine whether activated FAK signaling is the underlying mechanism responsible for ER α activation in OSCC cells, causing enhanced cell growth.

Materials and methods

Reagents and antibodies

Cell culture media and supplements were purchased from Invitrogen. The primary antibodies used for immunoblotting were epidermal growth factor receptor (EGFR), FAK, ERK, AKT, ER α , β -actin (Santa Cruz Biotechnology), p-Tyr1173 EGFR, ER α , ER β , p53 (Epitomics, Burlingame, CA, USA), p-Ser118 ER α , p-Thr202/Tyr204 ERK, p-Ser473 AKT, p-Tyr397 FAK (Cell Signaling, Danvers, MA, USA), GAPDH, and lamin B1 (GeneTex, Irvine, CA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology. MEK inhibitor U0126 and PI3K inhibitor LY294002 were obtained from Calbiochem (Darmstadt, Germany). All chemicals were purchased from Sigma.

Cell culture

Human tongue SCC cell lines (SCC4 and SCC25) and an ER-positive breast cancer cell line (MCF-7) were obtained from the Biosource Collection and Research Center (BCRC, Hsinchu, Taiwan) which routinely authenticates these lines by short tandem repeat (STR) analysis. A human gingival SCC cell line (OECM-1; Meng *et al.* 1998) was obtained from Dr K W Chang (National Yang-Ming University, Taipei, Taiwan). The STR profiling of OECM-1 cells was carried out at the BCRC. All experiments were carried out by each cell line at passages below 30. While the SCC4 and SCC25 cells were maintained in DMEM/F12 medium, the OECM-1 and MCF-7 cells were cultured in RPMI 1640 and DMEM medium respectively. Each medium was supplemented with 5% fetal bovine serum (FBS), 2 mM glutamine, and 0.05 mg/ml of gentamicin.

Luciferase reporter assay

An aliquot of 1×10^4 cells/well were plated into 24-well plates. After a 48 h incubation, the cells were co-transfected with 1 μ g of pGL2-ERE-luc plasmids (Addgene, Cambridge, MA, USA) and 0.2 μ g of Renilla luciferase expressing vector using a TransLT1-transfection reagent (Mirus, Madison, WI, USA). After an additional 48 h incubation, the cells were lysed, and the ratio of the two luciferase activities was analyzed using the Dual-luciferase Reporter Assay Kit (Promega) and a 2020n luminometer (Turner BioSystems, Sunnyvale, CA, USA).

Lentiviral infection

For stably expressing FAK proteins in SCC25 cells, 293FT cells (Invitrogen) were co-transfected with the pCDH-CMV-MCS-EF1-Puro (pCDH) vector (SBI, Mountain View, CA, USA) containing the WT FAK cDNA plus packaging plasmids, using TransLT1-transfection reagent (Mirus). The control cells were co-transfected with the pCDH vector alone plus packaging plasmids. For gene knockdown experiments, lentiviral vectors carrying shRNAs against the *PTK2*, *ESR1*, or *ESR2* genes were obtained from the RNAi Core at Academic Sicina (Taipei, Taiwan). For the preparation of the shRNA-containing lentiviruses, 293FT cells were co-transfected with the lentiviral expression vector, containing the gene-targeted shRNA and with packaging plasmids using the PolyJet Transfection Reagent (SignaGen Lab., Ijamsville, MD, USA). The cells expressing shRNA against the gene encoding firefly luciferases or β -galactosidases served as a control (shCt). After a 72 h incubation, the culture medium containing lentiviruses was harvested. For infection, cells grown in six-well plates were incubated with viruses in the presence of 8 μ g/ml of polybrene and were centrifuged at 840 g for 1 h at 37 °C. The infected cells were then grown for 2–4 days in the absence or presence of 4 μ g/ml puromycin. The expression level of ER α , ER β , or FAK in these infected cells was detected by western blot analysis.

Cell growth assay

To compare the growth of the different types of OSCC cells and to determine the effects of tamoxifen on cell growth, an aliquot of 1×10^4 cells were seeded into 24-well plates. After a 48 h incubation, one set of attached cells was harvested and counted as day 0. The remaining cells were fed with fresh medium and harvested for counting on days 2 and/or 4. To examine the effect of estradiol (E₂) on cell

growth, the SCC4 and SCC25 cells were cultured for 48 h in a steroid-reduced (SR) medium, i.e. phenol red-free DMEM/F12 medium supplemented with 5% charcoal/dextran-treated FBS. The cells were then fed with fresh SR medium, containing 10 nM E₂. After 4 days of culture, the cells were harvested and the cell number was counted. Similarly, to determine the impacts of elevated FAK expression on cell growth, an aliquot of 1×10^4 of FAK-overexpressed SCC25 cells was seeded on a 24-well plate and maintained in SR medium for 48 h. Then, one set of attached cells was harvested and counted as day 0, while the remaining cells were fed with fresh SR medium, and the cell number were counted on day 4. For shRNA knockdown experiments, viral-infected cells were seeded into 24-well plates. Forty-eight hours after incubation, one set of attached cells was harvested and counted as day 0, while the remaining cells were fed with fresh medium. The cells were harvested on day 3 or 4 for counting.

Cell lysis and immunoblotting

The cells were harvested, and the cell lysates were prepared as described previously (Yuan et al. 2007). For immunoblotting, an aliquot of total cell lysates in an SDS-PAGE sample buffer was separated by electrophoresis and then transferred to a nitrocellulose membrane. After blocking with 5% nonfat milk or BSA in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature (RT), the membrane was incubated with primary antibodies for either 3 h at RT or overnight at 4 °C. After rinsing to remove the excess antibodies, the membrane was incubated with HRP-conjugated secondary antibodies for 1 h at RT. The specific protein was then detected by an ECL Reagent Kit (Amersham).

Nuclear/cytoplasmic fractionation

The cells were pelleted at 1000 g for 5 min and washed with ice-cold PBS. The cells were then resuspended in ice-cold buffer A, i.e. 20 mM HEPES (pH 7.0), 10 mM KCl, 2 mM MgCl₂, 0.5% NP-40, and 10 mM NaF, in the presence of protease inhibitor cocktail. After keeping them on ice for 10 min, the cells were broken to release nuclei using a pre-chilled Dounce homogenizer (20 strokes with a tight pestle). Dounced cell lysates were then centrifuged at 1000 g for 5 min at 4 °C, while the supernatant was collected as the cytoplasmic fraction. The nuclear pellet was washed three times with buffer A and then resuspended in buffer B, i.e. 150 mM NaCl, 1 mM EDTA, 20 mM Tris-Cl (pH 8.0), 0.5% NP-40, and 10 mM

NaF, followed by vigorously vortexing for 30–60 s. The supernatant containing nuclear proteins was collected by centrifuging at 16 000 *g* for 10 min at 4 °C.

Tissue microarray and immunohistochemistry

Tissue specimens including 11 benign and 21 malignant lesions of the oral cavity were collected from patients who underwent surgical resection at Mennonite Christian Hospital (Hualien, Taiwan). This procedure was approved by the Institutional Review Board of the Buddhist Tzu Chi General Hospital (#IRB101-115), and written informed consents were obtained from all participants. Tissue specimens were incorporated into a tissue microarray. The sections were deparaffinized in xylene, rehydrated with graded ethanol, and then boiled in 1 mM EDTA (pH 8.0) for 5 min in an autoclave. After three washes with 3% H₂O₂, the sections were blocked with a protein block serum (Dako, Glostrup, Denmark) for 5 min. Subsequently, the sections were incubated with an anti-ER α MAB (clone EP1; Dako) at 1:100 dilution for 30 min, and the signal was then amplified using an Envision kit (Dako) according to manufacturer's protocol. All sections were counterstained with hematoxylin for 2.5 min. The expression of ER α was evaluated using a light microscope, and the scores were divided into three grades: negative (0), low (+1), and high (+2/+3). Tumor cores with >10% of cells staining +1 or greater were defined as positive.

Statistical analysis

Significance for group comparisons was assessed by Student's two-tailed *t*-test or a one-way ANOVA followed by Tukey's or Dunnett's *post hoc* comparison. The χ^2 test was used for comparison between categorical variables. A *P* value <0.05 was considered significant. All statistical analyses were performed using Prism (GraphPad Software, La Jolla, CA, USA).

Results

Expression of ER α in benign and malignant lesions of the oral cavity

We initially examined the expression of ER α in benign and malignant lesions of the oral cavity by immunohistochemistry. Representative images of ER α immunostaining for each lesion are shown in Fig. 1. The immunoreactivity of ER α in the malignant tissue (Fig. 1B) was higher than that in the benign lesion (Fig. 1A). Unexpectedly, the

Figure 1

Expression of ER α in benign and malignant lesions of the oral cavity. (A) Negative immunostaining of ER α in normal oral mucosa. (B) The positive immunostaining of cytoplasmic ER α in an OSCC tissue. Magnification, 400 $\times$. The scale bar represents 50 μ m.

OSCC tissue shows cytoplasmic ER α staining. Table 1 summarizes the intensity data of the ER α immunostaining in the series of 11 benign and 21 malignant lesions of the oral cavity. In benign lesions, we failed to detect any ER α staining. In contrast, ER α immunoreactivity was found in nine (9/21, 43%) of the malignant tissues. The results of a χ^2 test indicate the significant difference in the intensity of ER α staining between benign and malignant lesions of the oral cavity (*P*=0.013).

OSCC cells express activated ER α correlated with their growth

To investigate the oncogenic function of ER α in OSCC cells, we examined the expression of ER α in OSCC cell lines. As shown in Fig. 2A, three OSCC cell lines expressed differential levels of ER α proteins. OECM-1 cells expressed

Table 1 ER α expression in benign and malignant lesions of the oral cavity

Tissue types	n	ER α -negative (%)	ER α -positive (%)	P value
Benign	11	11 (100%)	0 (0%)	0.013
Malignant	21	12 (57%)	9 (43%)	

a higher level of ER α compared with SCC4 and SCC25 cells; however, the expression level of ER α in OECM-1 cells was much lower than that in the ER-positive breast cancer MCF-7 cells. We further analyzed the transcriptional activity of ER α in OSCC cells by the luciferase reporter assay. We found that the ER α activity in OECM-1 cells was significantly higher than that in SCC4 or SCC25 cells ($P < 0.001$; Fig. 2B). The results of subcellular fractionation analyses further revealed that OECM-1 cells contained a higher level of nuclear ER α compared with a low or undetectable level of nuclear ER α in SCC4 and SCC25 cells (Fig. 2C). Importantly, the expression and activity of ER α in OSCC cells were correlated with their growth rates. On day 4, the ratio of growth in OECM-1 cells was approximately four times higher than that of SCC4 or SCC25 cells (Fig. 2D). These data suggest that OSCC cells expressed ER α proteins and the differential expression and activity of ER α were correlated with their growth.

Modulation of ER α activity or expression regulates the growth of OSCC cells

To examine whether ER α is functionally involved in the regulation of the growth of OSCC cells, we initially analyzed the growth-stimulating effects of E₂ on the slow-growing SCC4 and SCC25 cells, because both cells expressed inactive ER α with low transcriptional activities. Under a hormone-deprived condition, both cells were cultured in the presence or absence of 10 nM E₂ and the growth rate was then analyzed. Compared with the vehicle-treated cells, treatment with E₂ led to increased cell growth of ~ 1.7 times (Fig. 3A). In contrast, tamoxifen, an ER antagonist, significantly inhibited the growth of OECM-1 cells ($P < 0.001$). As shown in Fig. 3B, treating OECM-1 cells with 1 or 2 μ M tamoxifen resulted in ~ 40 or 80% suppression of cell growth respectively. Because E₂ and tamoxifen can modulate both ER α and ER β activities, we further clarified ER α function in promoting cell growth by performing shRNA-mediated gene knockdown. To show the impacts of ER α knockdown on the cell growth, the fast-growing OECM-1 cells which exhibited a high level of ER α were used for the experiments. As shown in

Figure 2

The expression and activity of ER α in OSCC cell lines. (A) The expression levels of ER α in three OSCC cell lines were analyzed by western blot analyses. The ER α -positive MCF-7 cells served as positive controls. The levels of β -actin are shown as internal controls. (B) The transcriptional activity of ER α in OSCC cells. Data are presented as the mean $\pm$ s.d. from three independent experiments. *** $P < 0.001$ and ### $P < 0.001$ vs the ratios of ERE-driven luciferase activity in SCC4 and SCC25 cells respectively. (C) The nuclear (N) and the cytoplasmic (C) expression levels of ER α in OSCC cells. The levels of lamin B1 and GAPDH in different fractions served as nuclear and cytoplasmic markers respectively. (D) The growth of OSCC cell lines. Data were expressed as the mean $\pm$ s.d. from three independent experiments. Each set of experiments was conducted in duplicate. * $P < 0.05$ and ** $P < 0.01$ vs the growth ratio of SCC4 cells, whereas # $P < 0.05$ vs the growth ratio of SCC25 cells on the corresponding day.

Figure 3

Effects of altered ER α activity or expression on the growth of OSCC cells. (A) SCC4 or SCC25 cells were cultured in a steroid-deprived medium for 48 h and then were fed with fresh medium containing 10 nM estradiol (E₂). After 4 days of culture, cells were harvested and the cell number was counted. (B) OECM-1 cells were cultured in the regular culture medium for 48 h and then were treated with 1 or 2 μ M tamoxifen (Tam). Attached cells were harvested, and the cell number was counted on day 4. The cells treated with an equal volume of solvent (ethanol) served as a vehicle control. (C) OECM-1 cells infected with viruses carrying the control or *ESR1*-targeted shRNA (i.e. shCt or shER α). After 2 days of viral infection, cells were harvested and total lysates were prepared for western blot analyses (upper panel). The infected cells were also seeded into 24-well plates. After 3 days of culture, the morphological changes (middle panel) were photographed under the light microscope (magnification, 100 $\times$), and the cell growth rate (lower panel) was determined as described in 'Materials and methods' section. All the data were expressed as the mean $\pm$ s.d. from three independent experiments. Each set of experiments was conducted in duplicate. * P < 0.05, ** P < 0.01, and *** P < 0.001 vs the ratios of vehicle-treated cells or shCt control cells.

Fig. 3C (upper panel), ER α -depleted OECM-1 cells expressed an undetectable level of ER α and a slightly decreased level of ER β compared with that in the shCt control cells. After 3 days of culture, ER α -knockdown OECM-1 cells showed the morphological feature of cell shrinkage (Fig. 3C, middle panel) and exhibited a slow growth rate, $\sim$ 80% lower than that of shCt cells (Fig. 3C, lower panel). These results suggest that OSCC cells expressed functional ER α , whose expression or activity was important for promoting their growth.

FAK activity correlates with ER α expression and Ser118 phosphorylation in OSCC cells

To determine the molecular mechanism(s) of how ER α was activated in OSCC cells, the expression or phosphorylation levels of EGFR and FAK, two important tyrosine kinases in the malignancy of HNSCC, were analyzed in three OSCC cell lines. As shown in Fig. 4A, OECM-1 cells had lower expression and phosphorylation levels of EGFR, but exhibited higher FAK expression and phosphorylation, compared with SCC4 and SCC25 cells. Importantly, FAK expression and phosphorylation did not correlate with the expression or activity of ERKs and p53 proteins, but were positively correlated with the activities of protein kinase B (PKB/AKT). Compared with the relatively low level of AKT phosphorylation in SCC4 or SCC25 cells, OECM-1 cells exhibited activated AKT, which was associated with high levels of ER α expression and phosphorylation at Ser118 (Fig. 4A). We further examined the impact of inhibiting FAK activity on ER α Ser118 phosphorylation by treating OECM-1 cells with PF-431496, a FAK inhibitor (Buckbinder *et al.* 2007). Compared with the vehicle control, treatment of 1 or 5 μ M PF-431496 caused decreases in the phosphorylation of FAK, AKT, and ER α in a dose-dependent manner. However, PF-431396 had no inhibitory effect on ERK phosphorylation (Fig. 4B).

FAK regulates ER α phosphorylation and activity and the growth of OSCC cells

We next examined the impact of elevated FAK expression on ER α phosphorylation, transcriptional activity, and cell growth. To reduce the hormonal effect on ER α phosphorylation and activity, FAK-overexpressed SCC25 cells were cultured in a hormone-deprived condition. Compared with the vector control, FAK-overexpressed cells exhibited higher phosphorylation levels of FAK itself, ERK, AKT, and ER α (Fig. 5A). Importantly, elevated FAK expression also led to increases in ER α transcriptional

Figure 4

Correlation between FAK and ER α expression and phosphorylation in OSCC cells. (A) Total lysates were used for immunoblotting with antibodies against the EGFR, FAK, p53, ERK, AKT, and ER α proteins, and the levels of phospho-EGFR at Tyr1173 (p-EGFR), phospho-FAK at Tyr397 (p-FAK), phospho-ERK at Thr202/Tyr204 (p-ERK), phospho-AKT at Ser473 (p-AKT), and phospho-ER α at Ser118 (p-ER α) are given. As a loading control, the level of β -actin was examined. (B) OECM-1 cells were treated with 1 or 5 μ M FAK inhibitor PF-431396 for 24 h. The cells treated with an equal volume of solvent (DMSO) served as a vehicle control. The cell lysates were prepared for immunoblotting with antibodies against the protein and phosphorylation levels of FAK, ERK, AKT, and ER α proteins.

activity and cell growth in a ligand-independent manner (Fig. 5B and C). In contrast, the knockdown of FAK expression in OECM-1 cells resulted in reduced phosphorylation of ERK, AKT, and ER α that was correlated with decreased ER α activity, $\sim$ 50% less than that in the shCt control cells (Fig. 5D and E). In parallel with decreased ER α phosphorylation and activity, FAK-depleted cells exhibited a slow growth rate, about 60% lower than that in the shCt control cells (Fig. 5F). To further examine the role of ERK or AKT in mediating FAK-promoted ER α Ser118 phosphorylation, FAK-overexpressed SCC25 cells were treated with U0126 or LY294002, a MEK or a PI3K inhibitor, respectively, under a SR condition. As shown in Fig. 5G, treatment with U0126 led to the inhibition of ERK phosphorylation but had no effect on ER α phosphorylation. However, treatment with LY294002 caused the inhibition of AKT phosphorylation itself, and led to decreases in ER α phosphorylation in a dosage-dependent manner. These results collectively suggest that FAK

signaling via AKT activation regulated ER α Ser118 phosphorylation, promoting ER α activation and cell growth.

Discussion

The oncogenic role of ER α is well established in hormone-related cancers. In OSCC, evidence from cell-based or clinical studies indicates the expression of ER α ; however, the functional role of ER α in OSCC malignancy and the mechanism underlying ER α activation in OSCC cells are not fully elucidated. In this study, we confirmed the expression of ER α in OSCC tumors and cell lines as reported in previous studies. Importantly, our main finding was that OSCC cells expressed functional ER α whose Ser118 phosphorylation and transcriptional activity were enhanced by FAK/AKT signaling, leading to promoted cell growth. To our knowledge, this is the first report to demonstrate the molecular mechanism of ligand-independent ER α activation in OSCC cells.

The functional properties of ER α are associated with its expression and activity. In agreement with a previous report (Colella *et al.* 2011), our data show that OSCC tumors expressed a high level of ER α compared with the benign tissues of the oral cavity. Furthermore, three OSCC cell lines expressed functional ER α and its activity was correlated with the cell growth. This notion was supported by results from E₂ or tamoxifen treatment. However, studies also suggest the functional involvement of ER β in OSCC cell proliferation and invasion (Ishida *et al.* 2007, Kim *et al.* 2007, Nelson *et al.* 2007). Ishida *et al.* (2007) further shows that knockdown of ER β expression in an OSCC cell line, SCCNK, leads to a more prominent effect on growth inhibition than that of knocking down ER α . Differing from the observation, our data show that knocking down ER α expression in OECM-1 cells resulted in retarded cell growth, and no growth inhibition was observed in ER β -knockdown cells (Supplementary Fig. 1, see section on supplementary data given at the end of this article). Although the contradictory results might be due to cell-type specificity, our data are consistent with the observations in other cancer cells that ER α , but not ER β , plays a predominant role in the promotion of cell growth and survival (Thomas & Gustafsson 2011). While a few reports propose the proliferative and anti-apoptotic roles of ER β in cancer cells, the tumor suppressor properties of ER β have been demonstrated by the fact that overexpression of ER β results in anti-proliferative responses, whereas knockdown of ER β promotes the proliferative properties in different cancer cell types (Treeck *et al.* 2007,

Figure 5

FAK regulates ER α phosphorylation and activity and the growth of OSCC cells. (A) SCC25 cells stably expressing the vector or the WT FAK were cultured in a steroid-deprived medium for 48 h, and the cell lysates were used for immunoblotting with antibodies against the expression or phosphorylation of FAK, ERK, AKT, and ER α proteins. The luciferase reporter assay (B) and cell growth assay (C) of the vector- or FAK-expressed SCC25 cells were carried out as described in 'Materials and methods' section. OECM-1 cells were infected with viruses carrying control or *PTK2*-targeted shRNA, i.e. shCt, or shFAK respectively. After an additional

72-h incubation, cells were harvested for western blot analyses (D), the luciferase reporter assay (E), or the cell growth assay (F). All the data were expressed as the mean $\pm$ s.d. from three independent experiments. Each set of experiments was conducted in duplicate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs the ratios of vector or shCt control cells. (G) FAK-overexpressed SCC25 cells (SCC25-FAK) were treated with 2.5 or 5 μ M U0126 or LY294002 for 16 h under a steroid-deprived condition. The cells treated with an equal volume of solvent (DMSO) served as a control. Total lysates were used for western blot analyses.

2010, Paruthiyil *et al.* 2011, Bossard *et al.* 2012). Moreover, a study of 24 OSCC specimens shows that the frequency of ER α expression (50%) is higher than that of ER β expression (33%) (Lukits *et al.* 2007). In a separate study of HNSCC tumors, the nuclear level of ER α , but not ER β , is significantly higher in the tumor lesions than in paired adjacent normal mucosa (Egloff *et al.* 2009). Thus, our data support the functional significance of ER α in OSCC cells.

FAK is a key regulator to mediate integrin signaling. A line of evidence indicates that integrin signaling can regulate ER α expression and activity (Novaro *et al.* 2003). Our data further suggest that FAK can modulate ER α phosphorylation at Ser118. Although FAK can interact with ER α (Planas-Silva *et al.* 2006), it cannot directly phosphorylate ER α . Therefore, FAK may regulate Ser118 phosphorylation through the downstream serine/threonine kinases. It is of interest to note that two key protein kinases, ERK and AKT, which function as the downstream molecules of FAK signaling, are greatly involved in ER α phosphorylation in a ligand-dependent or ligand-independent manner. Studies in clinical specimens suggest the positive correlation between the phosphorylation levels of Ser118 and Ser167 of ER α , ERK, and AKT (Jiang *et al.* 2007, Yamashita *et al.* 2008). Evidence from *in vitro* studies revealed that activated ERK phosphorylates Ser118, whereas AKT is predominantly responsible for Ser167 phosphorylation (Kato *et al.* 1995, Campbell *et al.* 2001). However, most studies *in vitro*

regarding the relationship between the phosphorylation of ERK, AKT, and ER α were carried out in breast tumors or cell lines, especially MCF-7 cells. In OSCC cells, our data clearly show that the expression and activity of FAK were associated with the phosphorylation levels of AKT and ER α at Ser118. Although a correlation between FAK and ERK was observed, inhibition of FAK activity by PF-431396 had no effect on ERK activity. Similarly, treatment with PF-431396 in B cells can abolish anti-immunoglobulin-induced FAK phosphorylation but has no inhibitory effect on ERK activity (Tse *et al.* 2009). Our data further show that LY294002 can abolish FAK-promoted Ser118 phosphorylation, whereas U0126 failed to show any inhibitory effect on the phosphorylation level of Ser118. A similar observation in HER2 (ERBB2)-overexpressed MCF-7 cells is that U0126 has no effect on HER2-activated Ser118 phosphorylation, although the cells contain highly activated ERK (Weitsman *et al.* 2006). Thus, our data support the notion that in OSCC cells, FAK signaling via AKT activation promoted ER α phosphorylation at Ser118 in a ligand-independent manner, leading to ER α activation and enhanced cell growth.

In addition to Ser118 phosphorylation, other phosphorylation sites of ER α may involve the FAK-regulated ER α activation. Studies in breast cancer cells show that activated ribosomal protein S6 kinase 1 (S6K1) or c-Src can phosphorylate ER α at Ser167 or Tyr537, respectively, which causes ER α activation (Arnold *et al.* 1995,

Yamnik *et al.* 2009). As FAK can modulate the activities of S6K1 and c-Src (Schlaepfer & Hunter 1997, Crossland *et al.* 2013), whether both kinases are involved in FAK-regulated ER α activation in OSCC cells deserves further study. Our results also indicate the role of ER α in FAK-regulated cell growth. However, a line of evidence shows that FAK facilitates cell survival through enhanced degradation of WT p53 proteins (Lim *et al.* 2008). In our study, three OSCC cell lines expressed an undetectable level or mutated form of p53 proteins (Sakai & Tsuchida 1992, Lin *et al.* 2004), and knockdown of FAK expression in OECM-1 cells had no effect on the expression of p53 (Supplementary Fig. 2, see section on supplementary data given at the end of this article). Thus, our data suggest that the regulatory effect of FAK signaling on the growth of OSCC cells was not mediated by p53 degradation.

Our data have shown that E₂ and tamoxifen can modulate the growth of OSCC cells; however, it is of interest to know whether E₂ or tamoxifen can regulate FAK activation. Previous studies have shown the effects of E₂ or tamoxifen on FAK activation in different cell types, but the results are inconsistent. In breast cancer cells, treatment of E₂ leads to a decrease in FAK phosphorylation in MCF-7 cells but causes increased phosphorylation in T47D cells (Bartholomew *et al.* 1998, Sanchez *et al.* 2010). In ovarian cancer PA-1 and HeyA8 cells, treatment of E₂ or propylpyrazole triol, an ER α agonist, enhances FAK phosphorylation (Hung *et al.* 2014). In endometrial cancer RL95-2 and Ishikawa cells, both E₂ and tamoxifen treatment can promote FAK phosphorylation (Tsai *et al.* 2013). Importantly, FAK expression and phosphorylation in OSCC cells can be inhibited by treatment of tamoxifen but are not affected by E₂ stimulation (Ishida *et al.* 2007). Although the impact of E₂ or tamoxifen on FAK activation was not examined in our study, whether FAK is involved in E₂- or tamoxifen-regulated growth in OSCC cells deserves further investigation.

The aberrant expression or activation of FAK and ER α proteins are risk factors for promoting the malignant progression of various cancers. In the head and neck region, the increased expression of FAK or ER α is found in malignant tissues, which is significantly correlated with reduced progression-free survival in HNSCC patients (Egloff *et al.* 2009, de Vicente *et al.* 2013). Thus, FAK and ER α can serve as potential targets for the treatment of HNSCC. Indeed, tamoxifen combined with chemotherapy agents has been applied to treat HNSCC patients in a clinical trial with phase II completed (NCT00002608; <http://www.clinicaltrials.gov>). In addition, PF-00562271, a selective FAK inhibitor, is undergoing a phase I trial for

HNSCC treatment (NCT00666926). Although there is no report to show the correlation between ER α and FAK expression in OSCC tissues, and no evidence to prove the therapeutic efficacy of tamoxifen or FAK inhibitors in OSCC patients, Kim *et al.* (2007) first showed that tamoxifen has a cytotoxic effect on OSCC cell lines, and the effect can be enhanced by combination with cisplatin. Our data further show that tamoxifen or PF-431396 significantly inhibited the growth of OECM-1 or FAK-overexpressed SCC25 cells. The combination of both inhibitors had a synergistic effect on the growth inhibition compared with each treatment alone, despite the lack of statistical significance (Supplementary Fig. 3).

In summary, we have demonstrated that the expression of ER α in OSCC tumors and cell lines, and the Ser118 phosphorylation and activity of ER α in OSCC cells were enhanced by FAK/AKT signaling, leading to increased cell growth. Thus, our data strongly suggest that FAK and ER α can serve as therapeutic targets for the treatment of OSCC patients.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-14-0102>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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