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Transcription factor EB (TFEB) influences invasion and migration in oral squamous cell carcinomas

Running title: TFEB in oral cancer

Keywords: TFEB, oral squamous cell carcinoma, invasion

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Abstract

Objective:
Transcription factor EB (TFEB) is a master regulator of lysosomal biogenesis and plays an important role in various cancers. However, the function of TFEB in oral squamous cell carcinomas has not been examined. The aim of this study was to elucidate the role of TFEB in oral squamous cell carcinomas.

Materials and Methods:
Expression levels of TFEB were examined in six different human oral squamous carcinoma cells: HSC2, HSC3, HSC4, SAS, OSC20, and SCC25. Knockdown of TFEB using small interfering RNA in HSC2 and HSC4 cells was performed. Cell morphology was observed by immunofluorescence microscopy. Cell proliferation, invasion, and adhesion were analysed.

Results:
Expression levels of TFEB were high in HSC2, moderate in HSC4 and SCC25, and low in HSC3 and OSC20 cells. Knockdown of TFEB did not affect proliferation of HSC2 and HSC4 cells, but did induce enlargement of lysosomes and endosomes in HSC4 cells. TFEB silencing reduced invasion and migration of these HSC cell squamous carcinoma cells; however, increased cell adhesion was also observed.

Conclusions:
TFEB knockdown reduces invasion and migration of cancer cells, likely through lysosomal regulation. Taken together, TFEB influences cell invasion and migration of oral squamous cell carcinomas.
Introduction

Transcription factor EB (TFEB) is a basic helix-loop-helix leucine zipper transcription factor that belongs to the MiTF/TFE family (Martina et al., 2014). TFEB functions as a master regulator of lysosomal biogenesis (Sardiello et al., 2009, Settembre et al., 2013). Overexpression of TFEB causes induction of various lysosome genes such as lysosomal membrane proteins, lysosomal enzymes, and autophagy-related proteins (Sardiello et al., 2009, Settembre & Ballabio, 2011). Mechanistically, TFEB binds to the promoter region of various lysosomal genes termed as the Coordinated Lysosomal Expression and Regulation (CLEAR) elements to modulate the expression and levels of lysosomal proteins and autophagy-related proteins (Sardiello et al., 2009, Settembre et al., 2013).

Several lines of evidence indicate that TFEB enhance oncogenic properties in certain types of cancer cells. In renal cell carcinomas, TFEB has been discovered as a fusion gene (MALAT1-TFEB) (Kauffman et al., 2014), suggesting that TFEB is an important factor for carcinogenesis, since the fusion of two different genes is the most important determinant for mutation in cancer. TFEB expression is increased in some cancer cells, such as pancreatic ductal adenocarcinoma (Klein et al., 2016) and breast cancer (Giatromanolaki et al., 2017). In ovarian cancer, knockdown of TFEB using small interfering RNA (siRNA) reduces the expression levels of many genes involved in drug resistance (Zhitomirsky & Assaraf, 2015). Similarly, studies with both overexpression and knockdown with siRNA of TFEB indicate that TFEB decreases the sensitivity of doxorubicin in cancer cells through autophagy mediated mechanisms. (Fang et al., 2017). However, the role of TFEB in invasion, migration, and adhesion of cancer cells remains unclear.
Recently, our research group has shown that the lysosomal cysteine protease cathepsin K is implicated in invasion, migration, and adhesion of oral squamous cell carcinomas (Yamashita et al., 2017). Overexpression of cathepsin K inhibited proliferation of carcinomas, while it enhanced invasion, migration, and adhesion of carcinomas (Yamashita et al., 2017). Conversely, pharmacological repression of cathepsin K using odanacatib, a specific inhibitor of cathepsin K, led to the opposite effects in the same cell lines (Yamashita et al., 2017). Given that the lysosomal enzyme cathepsin K is an important factor for invasion, migration, and adhesion of carcinomas, it is reasonable to speculate that the master regulator of lysosomal biogenesis, TFEB, is also implicated in invasion, migration, and adhesion of oral squamous cell carcinomas. However, no studies have been conducted to examine the role of TFEB in oral squamous cell carcinomas.

In this study, we investigated the possibility for TFEB to regulate cell invasion, migration, and cell adhesion by genetic knockdown methodology.
Materials and methods

Reagents

Dulbecco’s Modified Eagle’s Medium and Ham’s F-12 nutrient mixture were obtained from WAKO (Osaka, Japan). Anti-β-actin antibody was purchased from Sigma-Aldrich. Rat monoclonal anti-mouse lysosome-associated membrane protein 1 (LAMP1) was from Southern Biotech, rabbit monoclonal anti-Early Endosome Antigen 1 (EEA1) was from Medical & Biological Laboratories MBL (Nagoya, Japan), rabbit monoclonal anti-GM130 were purchased from Cell Signaling.

Cell lines

Human oral squamous carcinoma cell lines HSC2, HSC3, HSC4, SAS, OSC20, and SCC25 were prepared as previously described (Yamada et al., 2010)

Small interfering RNA (siRNA) for TFEB knockdown

Knockdown experiments of human oral squamous carcinoma cell lines using control or TFEB siRNA were conducted as described previously (Yoneshima et al., 2016, Yamaguchi et al., 2017). The MISSION lentiviral based esiRNA(EHU059261-20UG) from SIGMA-ALDRICH (St.Louis, MO, USA) was used for silencing. esiRNA(EHUEGFP-20UG) was used for control. The squamous carcinoma cells (5 × 10⁴ cells/ml) were plated in 6-well plates, and were further cultured in antibiotic-free media for 1 day. Subsequently, cells were transfected with 1.152 µg of siRNA using Lipofectamine RNAiMAX™ transfection reagent (Invitrogen, Carlsbad, CA, USA). The cells were further incubated for 3 days. Subsequently, the total RNA was isolated for RT-PCR.
Overexpression of TFEB

Human TFEB cDNA clone (MGC:40490, IMAGE:5180066) was purchased from ATCC. HSC3 cells \( (5 \times 10^5) \) were transfected with an empty vector (pcDNA 3.1 or p3XFLAG-Myc-CMV™-24 (Sigma-Aldrich) or TFEB containing vector using TurboFectin™8.0 (pouring 6 µl by per 1 µg of vector) (ORIGENE).

Invasion and migration assays

The migration assay was conducted using 8-µm pore size Transwell® chambers (Corning). Briefly, cells \( (5 \times 10^4) \) were pre-incubated in a serum-free culture medium and applied to the upper chamber. Medium containing 20% foetal bovine serum (FBS) for use as a chemoattractant was added to the lower chamber, and the cells were incubated for 48 h or 72 h at 37 °C. The remaining cells in the upper chamber were removed with a cell scraper. The filters were then fixed with 100% methanol for 10 min, and then stained with Diff-Quik™ (Sysmex) for 5 min. The moving cells were counted in ten random microscopic fields per well. Invasion assay was performed by the same method using BioCoat Matrigel® invasion chambers (Corning).

Cell adhesion assay

Cells were added to a 96-well plate at a density of \( 5\times10^5 \) cells per well and incubated for a period of time described previously (Yamashita et al., 2017). The plate was washed and the bound cells were fixed with 4% paraformaldehyde dissolved in PBS (phosphate-buffered saline) for 10 min. The fixed cells were stained with 5 mg/ml crystal violet and then washed three times with PBS. The stained plate was analysed.
using a microplate reader at 540 nm.

**Immunofluorescence microscopy**

Immunofluorescence microscopy was performed as previously described (Shimada-Sugawara et al., 2015). Briefly, cells were grown on glass coverslips and fixed with 4.0% paraformaldehyde dissolved in PBS for 30 min at 25 °C. The fixed cells were permeabilized with PBS containing 0.2% Tween 20 for 10 min. Thereafter, the cells were treated with 0.2% gelatin in PBS for 1 h to prevent nonspecific interactions, and subsequently incubated overnight at 4 °C with primary antibodies. Further, the cells were washed and incubated with secondary antibodies. The samples were imaged by a laser scanning confocal imaging system (LSM 510, LSM 710, and LSM 780 META combined system Carl Zeiss, AG, Jena, Germany).

**Western blot analysis**

Cells were washed with PBS, and lysed by boiling in 50 mM Tris-HCl (pH 8.0) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM sodium orthovanadate, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and a proteinase inhibitor cocktail. A fixed amount of protein (5 µg) was loaded for each sample and resolved by SDS-PAGE. Subsequently, the protein was electroblotted onto a polyvinylidene difluoride membrane. The membrane was blocked with 3% skimmed milk dissolved in 20 mM Tris-HCl (pH 7.5), containing 150 mM NaCl, and 0.1% Tween 20, at 25 °C for 1 h. The samples were incubated with various antibodies overnight at 4 °C, then further incubated with HRP-conjugated secondary antibodies, and finally treated with ECL-Prime (GE Healthcare Life Sciences, Amersham Place,
UK). The membranes were imaged by LAS4000 mini (Fuji Photo Film, Tokyo).

**Quantitative real-time PCR**

The real-time PCR was performed as reported previously (Yamashita et al., 2017).

The primer sets were

**Human TFEB**

Forward: 5’-TCA ATA CCC CCG TCC ACT TC-3’
Reverse: 5’-5’-CGA CTG CTG CAG ATG GTA GG-3’

**β-actin:**

Forward: 5’-ACCCAGATCATGTTGAGAC-3’
Reverse: 5’-GTCAGGATCTTCATGAGGTAGTG-3’

**Statistical analyses**

The data shown in this work is a mean of 3 independent experiments with standard deviation included. The Tukey-Kramer method was used to compare differences between values. Analysis of variance (ANOVA) was conducted to indicate significant differences (*P < 0.05 or **P < 0.01). Alternatively, two-way ANOVA was also employed.

**Results**

**TFEB is differentially expressed in respective squamous cell carcinomas**

To determine mRNA and protein expression levels of human TFEB in oral squamous cell carcinomas, we conducted quantitative real-time PCR and western blot analyses in HSC2, HSC3, HSC4, SAS, OSC20, and SCC25 cells. The mRNA levels of TFEB in HSC2, HSC4, SAS, and SCC25 were comparatively high, while those in
OSC20 were moderate (Figure 1A). However, TFEB mRNA levels in HSC3 were low (Figure 1A). Western blotting of TFEB indicated that the expression levels of TFEB in HSC2 were high, those in HSC4, SAS and SCC25 were moderate, and those in HSC3 and OSC20 were low (Figure 1B). Thus, there was a rough correlation between mRNA and protein levels of TFEB expression in these cells. Therefore, HSC2 and HSC4 were selected as TFEB-expressing cell lines.

Transfection for TFEB-knockdown or TFEB-overexpression in oral squamous cell carcinomas

To explore the potential roles of TFEB in carcinomas, we constructed TFEB-knockdown HSC2 and HSC4 cells by siRNA transfection. The protein and mRNA expression levels of TFEB in HSC2 and HSC4 cells were confirmed by western blotting and real-time PCR analyses (Figure 2 A and B). We further transfected HSC3 cells with a vector-containing TFEB-GFP for TFEB overexpression, or GFP only as the control. Unexpectedly, however, we did not obtain TFEB-overexpressing squamous cell carcinomas (Data not shown). Therefore, it is likely that the overexpression of TFEB undergoes degradation in squamous cell carcinomas by an unknown mechanism.

TFEB depletion does not affect the proliferation of squamous cell carcinomas

To test whether TFEB affects the proliferation of squamous cell carcinomas, we examined proliferation of the control and TFEB-knockdown HSC2 and HSC4 cells (Figure 3). After incubation for 24 or 48 h, the cell numbers between control and TFEB-knockdown HSC2 and HSC4 cells were comparable (Figure 3). Thus, TFEB knockdown had little to no effect on the proliferation of HSC2 and HSC4 cells (Figure
3).

**TFEB knockdown induces an enlargement of lysosomes and endosomes in squamous cell carcinomas**

Since TFEB is a master regulator of lysosomal biogenesis, we examined morphological changes concerning endosomes/lysosomes (LAMP1), early endosomes (EEA1) and Golgi complex (GM130). Confocal microscopic analysis showed that the size of LAMP-1-positive late endosomes/lysosomes or EEA1-positive early endosomes in TFEB-knockdown HSC4 cells was slightly larger than that in control cells (Figure 4 A and B). However, apparent morphological changes were not observed in GM130-positive Golgi complex between control and TFEB-knockdown HSC4 cells (Figure 4A). Consistent with morphological observation, the calculated vesicle size and total vesicle size of LAMP1 and EEA1 in TFEB-knockdown HSC4 cells were significantly larger than those of control HSC4 cells (Figure 4A and B). Thus, TFEB knockdown induces an enlargement of lysosomes and endosomes in squamous cell carcinomas.

**TFEB depletion reduces the invasion and migration of squamous cell carcinomas**

Next, we examined the invasion ability of control and TFEB-knockdown HSC cells using Matrigel invasion chambers. After incubation for 48 h, we counted the number of cells that had invaded through the Matrigel. In the case of the TFEB-knockdown HSC2 cells, this value was not significant when compared to the control cells (Figure 5A). This is probably due to the high variability of the experiments. The number of invaded TFEB-knockdown HSC4 cells was significantly lower than that
of the control cells (Figure 5B). Moreover, the invasion numbers for TFEB-knockdown HSC2 and HSC4 cells were both lower than those of the control cells after assaying following 72 h of incubation (Figure 5C and D). These results suggest that TFEB depletion impair invasion of squamous cell carcinomas.

The invasion ability of cells is known to integrate migration and degradation abilities. We, therefore, tested the migration of TFEB-knockdown and control cells. Following 48 h of incubation in Transwell chambers, TFEB knockdown slightly, but not significantly, diminished the numbers of both HSC2 and HSC4 cells in response to the culture serum, compared with the control cells (Figure 6A and B). However, after 72 h of incubation, TFEB depletion significantly reduced the number of migrated HSC2 and HSC4 cells (Figure 6C and D). These results indicate that TFEB depletion decreases invasion and migration abilities of squamous cell carcinomas.

**TFEB silencing increases adhesion of squamous carcinomas**

We further studied TFEB influence on squamous cell carcinomas by an adhesion assay. There were no differences in the cell density between the control and HSC-knockdown cells at incubation time of 30 and 60 min (Figure 5A). However, after 90 min of incubation, a higher density of bound cells was found in both HSC2 and HSC4-knockdown cells than in the control cells (Figure 7A and B). These results indicate that TFEB silencing increases adhesion of squamous cell carcinomas.

**Discussion**

In this study, we investigated the effects of TFEB on proliferation, invasion, migration and adhesion of oral squamous cell carcinomas. Although TFEB was
differentially expressed in six types of squamous cell carcinomas, TFEB knockdown did not affect proliferation but instead induced an enlargement of lysosomes and endosomes in squamous cell carcinomas. TFEB silencing impaired invasion and migration abilities; however, TFEB depletion increases adhesion of squamous cell carcinomas. These results indicate that TFEB influences cell invasion and migration in oral squamous cell carcinomas.

There was a discrepancy between TFEB mRNA and protein expression levels in our study. This could be due to post-translational modifications, protein-protein interactions, or proteasome degradation. In fact, it is known that the localization and expression of TFEB are controlled by several kinases (mTOR, PKCβ, and ERK2), other interacting proteins (mTOR, 14-3-3 protein), and the ubiquitin-proteasome pathway (Martina et al., 2014, Nabar & Kehrl, 2017, Napolitano & Ballabio, 2016). Therefore, the discrepancy in TFEB expression can be explained by many factors.

The present results are partially, but not completely, consistent with the results of our recent study regarding the lysosomal protease cathepsin K in oral squamous cell carcinomas (Yamashita et al., 2017). In that study, overexpression of cathepsin K found to enhance the invasion, migration, and adhesion abilities of squamous cell carcinomas. Conversely, suppression of cathepsin K using odanacatib, a specific inhibitor of cathepsin K, resulted in opposite effects in carcinomas. Indeed, TFEB knockdown reduced the invasion and migration of squamous carcinomas. By contrast, TFEB depletion increased adhesion of squamous cell carcinomas. The mechanisms by which the effects of TFEB on the invasion, migration, and adhesion abilities of cancer cells are currently unknown. However, considering that TFEB is a master regulator of lysosomal biogenesis, we hypothesized that defects in TFEB expression may lead to a reduction in
the expression of adhesion and chemotactic receptors. Consistent with this hypothesis, our previous study indicated that macrophages deficient in the endo-lysosomal protease, cathepsin E, exhibit a decrease in the expression of chemokine and adhesion receptors, resulting in impaired chemotaxis and cell attachment (Tsukuba et al., 2009).

TFEB-mediated migration, but not proliferation, is likely to be a common characteristic of several cancer cells. Previous studies using lung cancer cells have shown that knockdown of TFEB by siRNA transfection in A549 and H1299 cells has no effects on proliferation, but diminishes migration abilities of these cells in the context of wound healing rates in the scratch assay (Giatromanolaki et al., 2015). These authors, however, did not examine the effects of TFEB on the invasion and adhesion of lung cancer cells. Although we performed migration experiments using Transwell chambers in this present study, TFEB knockdown was found to commonly impair migration in both studies across different cancer cell lines. Furthermore, our present study also indicates that TFEB is responsible for the invasion by squamous cell carcinomas. Given that the invasion ability integrates the migration ability plus degradation ability of matrix proteins, TFEB is also involved in the degradation of matrix proteins. The impaired degradation in TFEB-knockdown cells is probably due to down-regulation of lysosomal hydrolases, whose expression levels are regulated by TFEB. Therefore, involvement of TFEB in invasion may be a common characteristic among cancer cells.

Morphologically, TFEB knockdown induced an enlargement of lysosomes and endosomes in squamous carcinomas. In general, inhibition of lysosomes by pharmacological inhibitors or genetic prevention causes an enlargement of lysosomes and endosomes (Kuzu et al., 2017, Endo et al., 2015). This phenotype is known as “vacuolation”. It should be noted that the vacuolation by lysosomal inhibition affects
peripheral organelles. Therefore, we also examined early endosomes and the Golgi complex. As a result, TFEB knockdown induced an enlargement of lysosomes and endosomes in squamous cell carcinomas. Interestingly, activation of TFEB also causes enlargement of lysosomes. Indeed, previous studies have shown that TFEB overexpression in Hela and HEK293 cells causes an enlargement of lysosomes (Sardiello et al., 2009). In contrast to the inhibition, it is likely that TFEB activation remains intact of peripheral organelles.

In conclusion, TFEB knockdown impairs invasion in squamous cell carcinomas, which is possibly due to the inhibition of cell migration and degradation of matrix proteins by lysosomes. Taken together, TFEB is involved in the invasion, migration and adhesion of oral squamous cell carcinomas.

Acknowledgments

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NFATc1 activation. *Cellular and molecular life sciences : CMLS.*


**Figure Legends**

**Figure 1.** Expression of TFEB in various human oral squamous cell carcinomas

(A) Reverse transcriptional real-time PCR for mRNA levels of TFEB. Relative expression levels were normalized to β-actin. The data are shown as mean ± S.D. of data from 3 independent trials. (B) Equal amounts of protein from cell lysates were resolved by sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE), followed by western blotting using anti-human TFEB antibody and anti-β-actin antibody as the loading control. Representative data from 3 independent experiments are shown.

**Figure 2.** TFEB knockdown and overexpression in oral squamous cell carcinomas.

HSC2 and HSC4 cells were transfected with control or human TFEB specific siRNA
(10 pmol) for 24 h, and then incubated for 24 h. (A) Equal amounts of protein from cell lysates were resolved by sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE), followed by western blotting using anti-human TFEB antibody and anti-β-actin antibody as the loading control. Representative data from 3 independent experiments are shown. (B) Knockdown efficacy was determined by quantitative reverse transcription PCR for mRNA levels of TFEB. The data are represented from 3 independent experiments. **P < 0.01 for control group.

**Figure 3.** Proliferation of control and TFEB-knockdown HSC cells

Cells were incubated on a filter for 24 or 48 h. Subsequently, the filters were fixed with methanol and stained with Giemsa solution. (A) Photographs of the controls and TFEB-knockdown HSC2 and HSC4 cells after 48 h of incubation. (B) After 24 or 48 h of incubation, the cell count was determined with a light microscope. The data are indicated as mean ± SD for 3 independent experiments.

**Figure 4.** Immunofluorescence microscopy of control and TFEB-knockdown HSC4 cells

Cells on glass coverslip were fixed, and permeabilised with 0.1% Triton X-100, and then treated with anti-LAMP1 (lysosome marker), anti-GM130 (Golgi complex marker) or anti-EEA1 (early-endosome marker) antibodies, respectively. The samples were incubated with Alexa 488 (green) or 594 (red)-conjugated secondary antibodies. The fluorescence images were analysed using a confocal microscope. Number of the different organelles was quantified from images obtained by confocal microscopy by IMARIS 6.0 (Bitplane, Zurich, Switzerland). Data obtained from three independent
experiments are presented as mean ± standard deviation. Particle volume was measured in units of micrometres cubed (µm³) using IMARIS. The representative data are shown from 3 independent experiments. **P < 0.01 for control group.

Figure 5. Effects of TFEB on invasion of oral squamous cell carcinomas.

Invasion ability was evaluated using Matrigel invasion chambers. The 24-well chambers were cultured at 37 °C for 48 or 72 h. The cells that invaded from the upper to the lower well were fixed, and stained with May-Giemsa solution. The number of cells was counted using a light microscope. (A) and (C) refer to control and TFEB-knockdown HSC2 cells, respectively. (B) and (D) refer to control and TFEB-knockdown HSC4 cells, respectively. (A), (B): after incubation of 48h. (C), (D): after incubation of 72h. The data are indicated as mean ± SD for 3 independent experiments* P < 0.05, **P < 0.01 for control group.

Figure 6. Effects of TFEB on migration of oral squamous cell carcinomas

Cell migration was evaluated using Transwell chemotaxis chamber. The 24-well chambers were cultured at 37 °C for 48 or 72 h. The cells that migrated from the upper to the lower well were fixed, and stained with May-Giemsa solution. The number of cells was counted by a light microscope. (A) and (C) refer to control and TFEB-knockdown HSC2 cells, respectively. (B) and (D) refer to control and TFEB-knockdown HSC4 cells, respectively. (A), (B): after incubation of 48h. (C), (D): after incubation of 72h. The data are indicated as mean ± SD for 3 independent experiments* P < 0.05, **P < 0.01 for control group.
Figure 7. Effects of TFEB on adhesion of oral squamous cell carcinomas

Cancer cells were incubated at 37 °C for 30, 60, or 90 min. After washing, adhered cells were fixed, and then stained with trypan blue. The stained plate was analysed using a microplate reader at 540 nm. (A): control and TFEB-knockdown HSC2 cells, (B): control and TFEB-knockdown HSC4 cells. The data are indicated as mean ± SD for 3 independent experiments* $P < 0.05$, for control group.
Fig. 1

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Figure 2

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Fig. 3

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