RESEARCH ARTICLE

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Oral Squamous Cell Carcinoma-Derived ANGPTL3 Induces Cancer Associated Fibroblastic Phenotypes in Surrounding Fibroblasts

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Abstract

Objective: Angiopoietin-like proteins (ANGPTLs) have emerged as both important regulator of lipid and glucose metabolism as well as insulin sensitivity. In particular, ANGPTL3 activity is one of the most important factors in cancer growth and invasion. Although ANGPTL3 have been studied in OSCC, but the role of ANGPTL3 between OSCC and CAFs has yet to be clearly defined. Thus, this study aimed to investigate the roles of ANGPTL3 in the differentiation of CAFs. **Methods:** For our study, we used hTERT-hNOFs to replace CAFs by coculturing them with oral squamous cell carcinoma (OSCC) cells. We did a microarray dataset analysis to investigate what factors secreted from OSCC cells can induce cancer associated fibroblastic phenotype in surrounding fibroblasts. The secreted factors were confirmed by RT-PCR, real-time PCR, and Western blot. **Result:** ANGPTL3 has the most secreted factor derived from various oral cancer cells. To investigate the role of ANGPTL3 in CAFs, we treated rhANGPTL3 in hTERT-hNOFs. The fibroblasts showed an increase of tumor-promoting cytokines (IL-6 and IL-8) and myofibroblastic markers, such as α-SMA and FAP. **Conclusion:** In conclusion, our study reports the first evidence that ANGPTL3 plays a crucial role in tumor microenvironments by inducing CAF. Therefore, targeting ANGPTL3 may be promising treatment strategy for CAF-targeted therapy in CAF-rich tumors.

Keywords: Oral squamous cell carcinoma (OSCC)- cancer associated fibroblast (CAF)- tumor microenvironment

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Introduction

Carcinomas are malignant neoplasms that originate from epithelial cells and are surrounded by specialized stroma that coordinate with cancer cells to control disease progression (Sahai et al., 2020). Cancer associated fibroblasts (CAFs) have been acknowledged as important modifiers of carcinogenesis in tumor microenvironment (Wang et al., 2017; Fozzatti and Cheng, 2020). They can be mediated through either direct heterotypic cell-cell contacts (Mao et al., 2021) or diffusible molecules, such as inflammatory mediators, cytokines or chemokines (Xing et al., 2010; Adekoya and Richardson, 2020). Most of these molecules are primarily proliferative and participate in promoting carcinogenesis (Greten and Grivennikov, 2019). Transforming growth factor-beta (TGF-β), Interleukin(IL)-6 and IL-8, secreted from CAFs, which are typically secreted by CAFs, form the tumor-promoting microenvironment in oral squamous cell carcinoma (OSCC) progression, including proliferation, angiogenesis, and invasion (Ayob and Ramasamy, 2018). Thus, the interaction between CAFs and OSCC cells is essential for cancer progression (Bae et al., 2014; Fiori et al., 2019).

Angiopoietin-like proteins (ANGPTLs) have emerged as an important regulator of lipid and glucose metabolism as well as insulin sensitivity (Hassan, 2017). It is well known that ANGPTL3 plays a crucial role in regulating triglyceride and cholesterol mainly via reversible inhibition of lipoprotein lipase activity and vascular endothelial growth factors (Hassan, 2017; Carbone et al., 2018). In particular, ANGPTL3 activity is one of the most important factors in cancer growth and invasion through signaling pathways, such as mitogen-activated protein kinase (MAPK) signaling cascades (Yu et al., 2011). Overexpression of ANGPTL3 has been observed in OSCC, hepatocarcinoma, and ovarian cancer (Koyama et al., 2015; Carbone et al., 2018). The potential role of ANGPTL3 has been demonstrated in OSCC, as evidenced by the fact that ANGPTL3 knockdown can be occurred in cell cycle arrests at G1 phase by upregulating cyclin-dependent kinase inhibitors and thus reduced

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cell proliferation and growth (Koyama et al., 2015). Although ANGPTL3 have been studied in OSCC, the role of ANGPTL3 between OSCC and CAFs has yet to be clearly defined.

In this study, we examined secreted factors derived from OSCC cells. Among them, ANGPTL3 has the most secreted factors derived from oral cancer cells. Thus it is involved in fibroblastic differentiation, such as myofibroblastic phenotypes. Our findings are the first to demonstrated a role of ANGPTL3 in the differentiation of CAFs, and thus showing that it may have potential in targeting tumor-promoting microenvironments.

Materials and Methods

Reagents and antibodies

Antibodies for α-smooth muscle actin (α-SMA; #M0851, 1:100, mouse monoclonal, Dako, Glostrup, Denmark), fibroblast activation protein (FAP) (#ab28244, 1:500, abcam, Cambridge, UK), and β-actin (#BS6007M, 1:5000, Bioworld Technology, St. Louis, MN) were used. Horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Recombinant human ANGPTL3 (rhANGPTL3; #3829-AN, R&D systems, Minneapolis, MN, USA) was used.

Cell cultures

Previously described immortalized human gingival fibroblasts by hTERT-transfection (hTERT-hNOFs) were used (Illeperuma et al., 2015). The hTERT-hNOFs were cultured in a F medium, which is made up of Dulbecco's modified Eagle medium (Gibco BRL, NY, USA) and Ham's F12 (Gibco BRL) mixed in a 3:1 ratio and added with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Among the OSCC cells, we maintained HSC2 cells in a F medium. The other OSCC cells (YD cell lines) were cultured in a F medium, added with 1×10^{-10} M cholera toxin, 0.4 mg/mL hydrocortisone, 5 μg/mL insulin, 5 μg/mL transferrin, and 2×10^{-11} M triiodothyronine (T3). Normal human epidermal keratinocytes (HEK) were obtained from Yonsei University College of Dentistry. The cells had been ethically approved by the Institutional Review Board (IRB) of the Yonsei Dental Hospital (IRB-2-2009-0002) (Illeperuma et al., 2015). HEK cells were grown in a KGM medium (#CC-3107, Lonza, Walkersville, MD, USA). For co-culturing with OSCC cells with fibroblasts, we used the transwell inserts containing 0.4 μm pore size filters. OSCC cell and fibroblasts were seeded in upper and lower chambers, respectively. All cells were cultured in an incubator at 37°C containing 5% CO_2, and the growth medium was changed every 3 days.

RNA extraction and Real time PCR, RT-PCR

Total RNA was extracted using the RNeasy plus mini kit (Qiagen, Hilden, Germany), and then cDNA was synthesized using RT&GO-MasterMix (MP Biomedicals, CA, USA) according to the manufacturers' protocols. The information of primer sequences is shown in Table 1. The reaction mixture was subjected to 30 amplification cycles 40 s at 94°C, 58 s at 48°C and 40 s at 72°C. The products were loaded in 1-1.5% agarose gel using StaySafe Nucleic Acid Gel Stain (Real Biotech Corporation, Taipei, Taiwan). Real-time PCR were performed with SYBR Green I Master (Roche Applied Science, Mannheim, Germany) and analyzed with LightCycler 480 Software (Roche Applied Science). Both RT-PCR and real-time PCR results were normalized to GAPDH.

Microarray dataset analysis

Two microarray datasets were used for this study. These datasets were acquired from a previously published public database (Jung et al., 2010)(Gene Expression Omnibus, GEO [GSE18532]; GSM461591 and GSM461592). The sample types of datasets are RNA of YD10B OSCC cells cocultured with CAF and NOF, and monocultured YD10B OSCC cells. We selected 835 overlapping genes from the three datasets. All three datasets were tested on the OpArray Human 35K platform. Overlapping genes (n = 711) were selected for greater than a 1.5-fold increase (n = 708) and less than a 0.5-fold decrease (n = 3).

Protein extraction and western blotting

The hTERT-hNOFs (3×10^6) were cultured in a P medium added with 0.2% FBS with or without 50 ng/mL and 100 ng/mL of rhANGPTL3 in 100-mm dishes. In brief, cells were lysated with a lysis buffer (Cell Signaling Technology) and then harvested. After lysing, protein lysates were incubated for 30 min on ice. The lysates were centrifuged at maximum speed (>15,000 rpm) for 10 min. The lysates were boiled for 5 min in a sodium dodecyl sulfate (SDS) sample buffer and separated in 10% SDS-PAGE. The proteins were transferred membranes and then blocked in 5% milk in phosphate-buffered saline solution (PBS) with Tween 20 for 1 hour. The membranes were immunoblotted with appropriate primary antibodies at 4°C overnight and then applied with horseradish peroxidase-conjugated anti-mouse (#7076S) or anti–rabbit (#7074S) secondary antibodies (Cell Signaling Technology). Protein detection was used by chemiluminescence (GenDEPOT, Barker, TX, USA).

Statistical analysis

Statistical analyses were carried out using SPSS version 20 (SPSS Inc., Chicago, IL, USA). All experiment was performed more than three times. The results were reported as mean ± standard deviation (SD). The differences between control and experimental groups were analyzed using Mann-Whitney U tests. A value of $p < 0.05$ was considered statistically significant.

Results

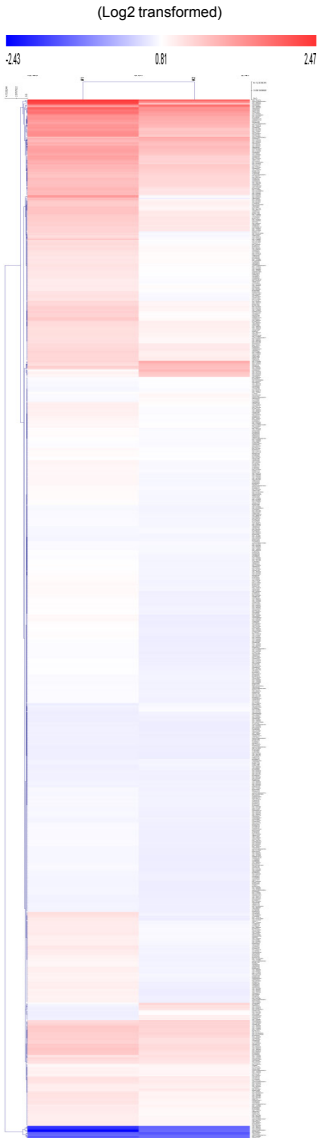
Screening CAF-stimulating factors released from OSCC cells

To understand what factors secreted from OSCC cells can induce surrounding fibroblasts, we did a microarray dataset analysis as a preliminary study. The previously published datasets were utilized (Jung et al., 2010). We extracted sample dataset types from the RNA of YD10B OSCC cells cocultured with CAF, and then we compared

Table 1. Primer Sequence Used for RT-PCR and Real-Time PCR.

Genes	Sense	Antisense
IL-6	ATGAACTCCTTCTCCACAAG	GAAGAGCCCTCAGGCTGGAC
IL-8	AGACAGCAGAGCACACAAGC	TTGGGGTGGAAAGGTTTGGAG
α-SMA	GGCCGAGATCTCACTGACTA	AGTGGCCATCTCATTTTCAA
Gro-α/CXCL1	TGTGAAGGCAGGGGAATGTA	TTAAGCCCCTTTGTTCTAAGCC
ANGPTL3	TCTCCAGAGCCAAAATCAAG	AAGACCATGTCCCAACTGAA
IL16	GCTGTGCCTTCCATCTTCTA	AGCTGAGTCTTCGTTGGATG
WNT2B	TTGGAGTGGTAGCCATAAGC	CTTGTTGAACGCTGACTGTG
TGF-α	TTCGCTCTGGGTATTGTGTT	GACCCAGAATGGCAGACAC
p16	TTCCTGGACACGCTGGT	CAATCGGGGATGTCTGA
p21	GACTTTGTCACCGAGACACC	GTCCACATGGTCTTCCTCTG
GAPDH	GAAGGTGAAGGTCGGAGT	GAAGATGGTGATGGGATTTC

them to YD10B OSCC cells cocultured with NOF and monocultured YD10B OSCC cells. As results, 708 overlapping genes were upregulated (≥1.5-fold increase) in cocultured OSCC cells, compared with monocultured and cocultured OSCC cells with NOF (Figure 1). Among them, we identified the soluble factors, including growth factors, cytokines, and chemokines. Four soluble factors (ANGPTL3, WNT2B; wingless-type MMTV integration site family member 2B, IL-16, TGF-α; transforming growth factor-alpha) were identified as candidates (Table 2) that could induce CAFs surrounding OSCC in a paracrine manner.



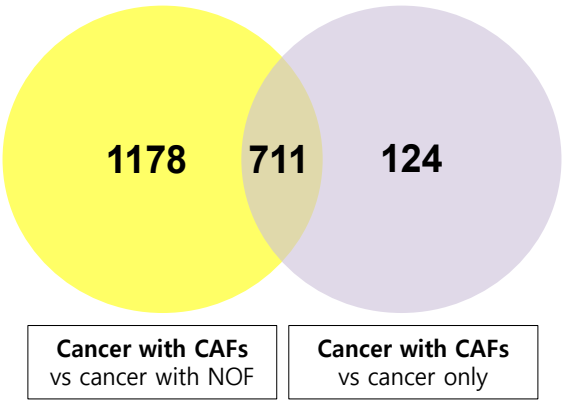


Figure 1. Microarray dataset analysis in OSCC cells cocultured with CAFs. RNA profiling of YD10B OSCC cells cocultured with CAFs. Heat-map shows the relative RNA expression levels of 711 genes (>1.5 fold-increase and <0.5 fold-decrease) overlapping in microarray datasets (log2 transformation). The venn diagram shows 708 upregulated genes and 3 downregulated genes out of a total of 711 genes.

Overexpression of ANGPTL3 and IL-16 mRNA in OSCC cells grown with fibroblasts

Quantitative real-time PCR was performed to identify good concordance with previous microarray data. ANGPTL3 (25.3, 31.2 fold and 23.0, 23.9 fold) and IL-16 (3.61, 3.09 fold and 3.53, 8.23 fold) exhibit significantly upregulated multifold changes in monocultured and cocultured OSCC cells (YD10B and YD38) with hTERT-hNOFs, compare to monocultured and cocultured HEK cells with hTERT-hNOFs (Figure 2). To confirm that two candidates were indeed derived from oral cancer cells, we re-performed the quantitative real-time PCR in a variety of oral cancer cells. ANGPTL3 was increased in all oral cancer cells (Figure 3A), whereas IL-16 was upregulated in most oral cancer cells, excepting HSC2 cells (Figure

Table 2. Growth Factors, Cytokines, and Chemokines in OSCC Cells Co-Cultured with CAFs

Gene symbol	Updated gene name	Cancer with CAFs (Intensity) vs Cancer with NOFs	Cancer with CAFs (Intensity) vs Cancer only (mono)	
			#1	#2
ANGPTL3	Angiopoietin-like 3	2.27	2.65	2.02
WNT2B	Wingless-type MMTV integration site family, member 2B	2.51	2.23	1.78
IL16	Interleukin 16 (lymphocyte chemoattractant factor)	2.03	1.83	1.66
TGF-α	Transforming growth factor, alpha	1.71	1.81	1.67

3B). All oral cancer cells, including OSCC cells (YD9, YD32, HSC2) and tongue mucoepidermoid cancer cells (YD15) secrete ANGPTL3, which is most secreted factor.

ANGPTL3 induces CAF-like phenotypes in stromal fibroblasts

To examine if ANGPTL3 can induce CAF-like phenotype in hTERT-hNOFs, we first identified tumor-promoting cytokines and general markers of CAFs. After treatment with 100 ng/mL rhANGPTL3, hTERT-hNOFs increased Gro-α/CXCL1 and IL-6, but there was no tendency to express IL-8 mRNA (Figure 4A). Furthermore, mRNA expression of α-SMA was increased by treatment with 100 ng/mL of ANGPTL3 (Figure 4A) and the CAF markers α-SMA and FAP were increased by treatment with rhANGPTL3 (Figure 4B). Collectively, ANGPTL3 released from oral cancer cells can induce a CAF-like phenotypes in stromal fibroblasts, as an increase in tumor-promoting cytokines and CAF markers.

Discussion

A cancer cell's interaction with its microenvironment significantly influences its formation and progression

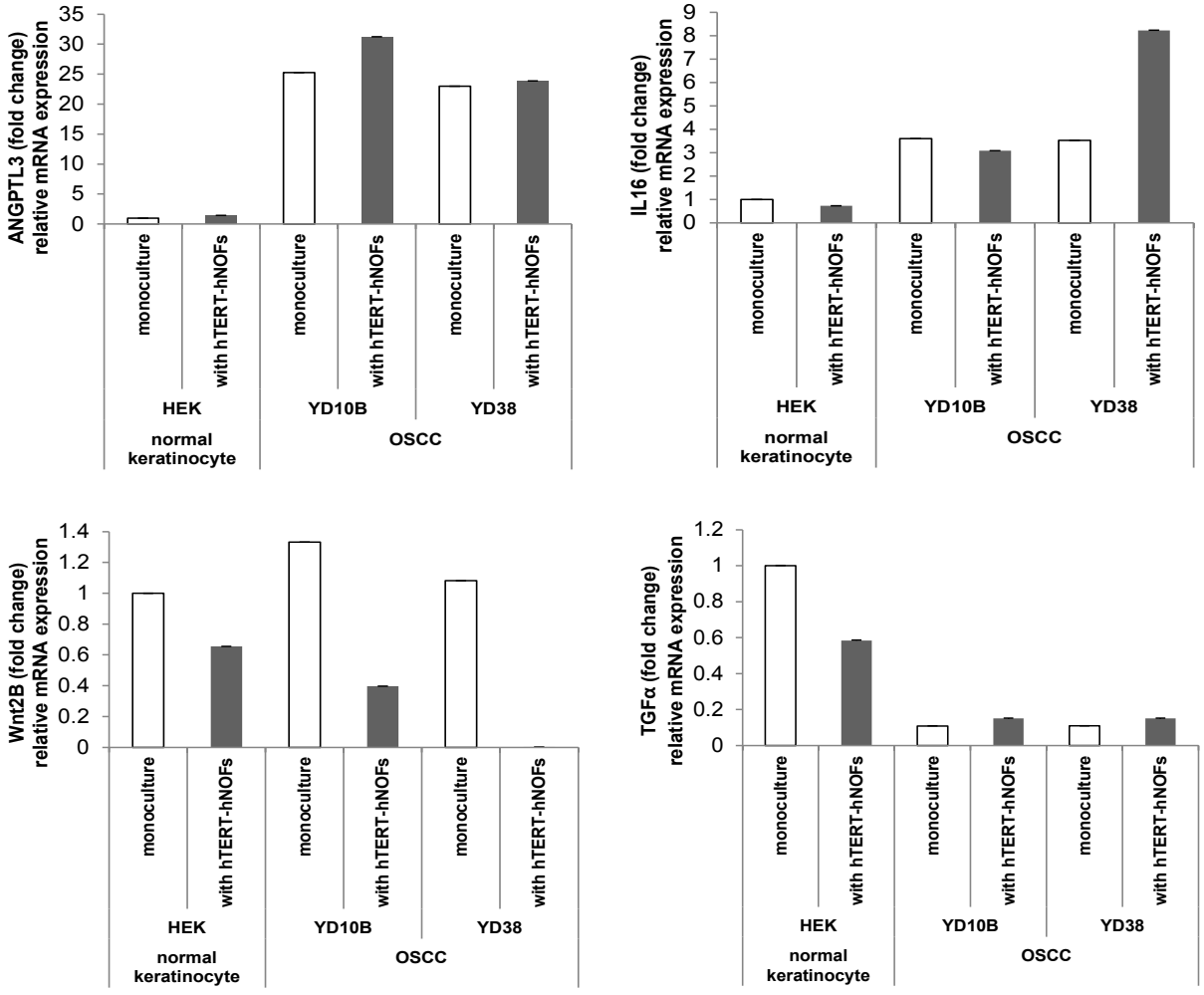


Figure 2. The Secreted Candidates Derived from the OSCC Cells. Candidates for microarray datasets analysis (4 soluble factors such as ANGPTL3, WNT2B, IL-16 and TGF-α) were confirmed by real-time PCR. Coculture was performed for 24 hours. ANGPTL3 and IL-16 had elevated expression in monocultured and cocultured OSCC cells (YD10B and YD38) with hTERT-hNOFs, compare to monocultured and cocultured OSCC cells with HEK.

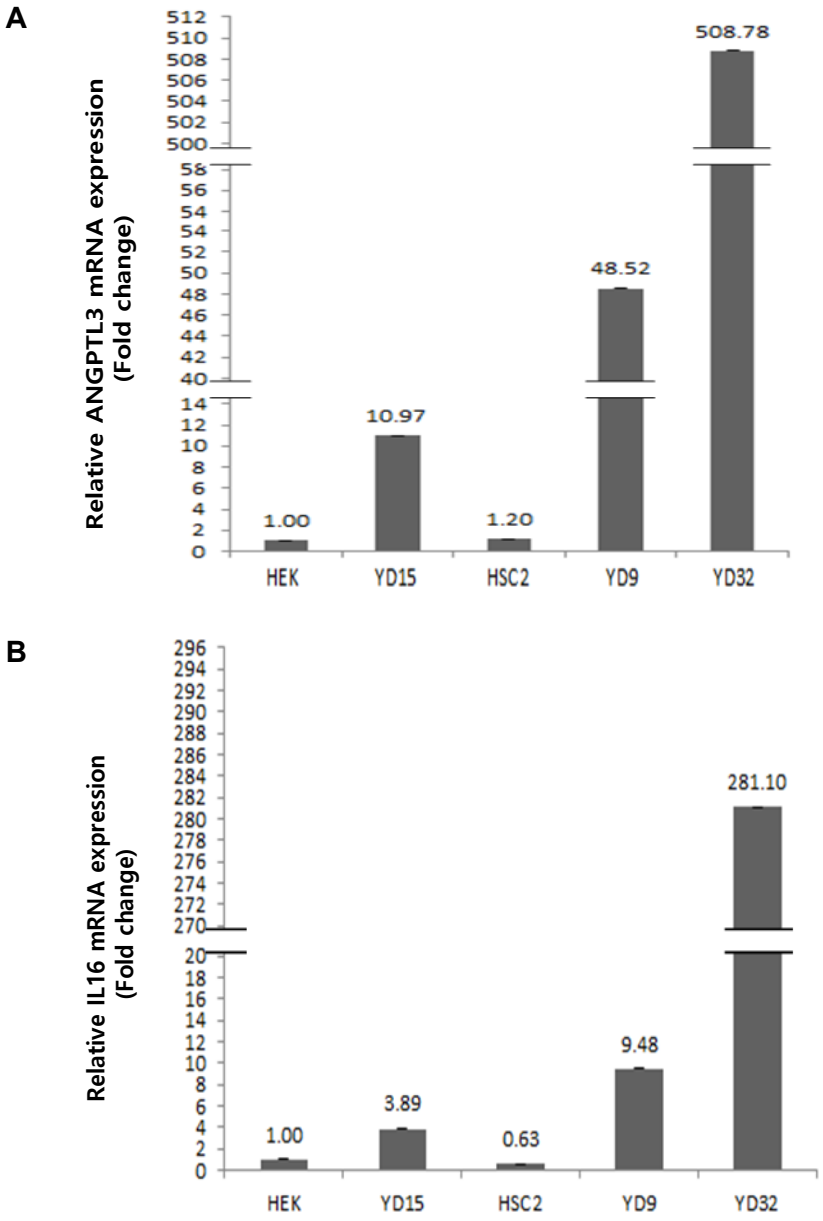


Figure 3. ANGPTL3 and IL-16 were Derived from a Variety of Oral Cancer Cells. The mRNA expression of (A) ANGPTL3 and (B) IL-16 was confirmed in such oral cancer cells as mucoepidermoid carcinoma cell (YD15) and OSCC cells (HSC2, YD9 and YD32), using SYBR Green, normalized with GAPDH mRNA and compared to HEK cells (*$p < 0.05$ by Mann-Whitney U test).

(Emon et al., 2018). Some of the most abundant components in the tumor microenvironment are the CAFs that are activated and reprogrammed in response to secreted factors and cytokines that tumor cell produce (Fozzatti and Cheng, 2020). These can remodel the extracellular matrix (ECM) structure and then guide cellular invasion (Winkler et al., 2020). Multiple mechanisms can contribute to CAF activation. Among them, TGF-β, epidermal growth factors (EGF), and platelet-derived growth factors (PDGF) are known to promote their activation. In addition, tumor derived extracellular vesicles and various inflammatory modulators, such as IL-1, IL-6 and nuclear factor-kappa B (NF-κB), can facilitate differentiation into CAFs through autocrine- and paracrine loops (Erez et al., 2010; Sanz-Moreno et al., 2011; Linares et al., 2020; Bao et al., 2021). Direct contact between cancer cells and normal fibroblasts (Strell et al., 2019), as well as physical changes in the ECM (Calvo et al., 2013; Avery et al., 2018), also contributed in CAF activation. Although CAFs have been studied, more studies are required to clarify their interaction with OSCC. Therefore, this study aims to investigate OSCC-derived secreted factors in order to confirm whether they induce conversion of normal fibroblast into CAFs.

Our study used hTERT-hNOFs as a replacement for CAFs because they exhibit CAF-like characteristics when cocultured with OSCC cells (Kim et al., 2019). To assess what factors induce these characteristics, we analyzed GEO datasets. We identified ANGPTL3 as one of the most significantly upregulated genes in the microarray dataset of overlapping genes. ANGPTLs are secreted glycoproteins structurally similar to the angiopoietins. To date, ANGPTL members have been identified, from

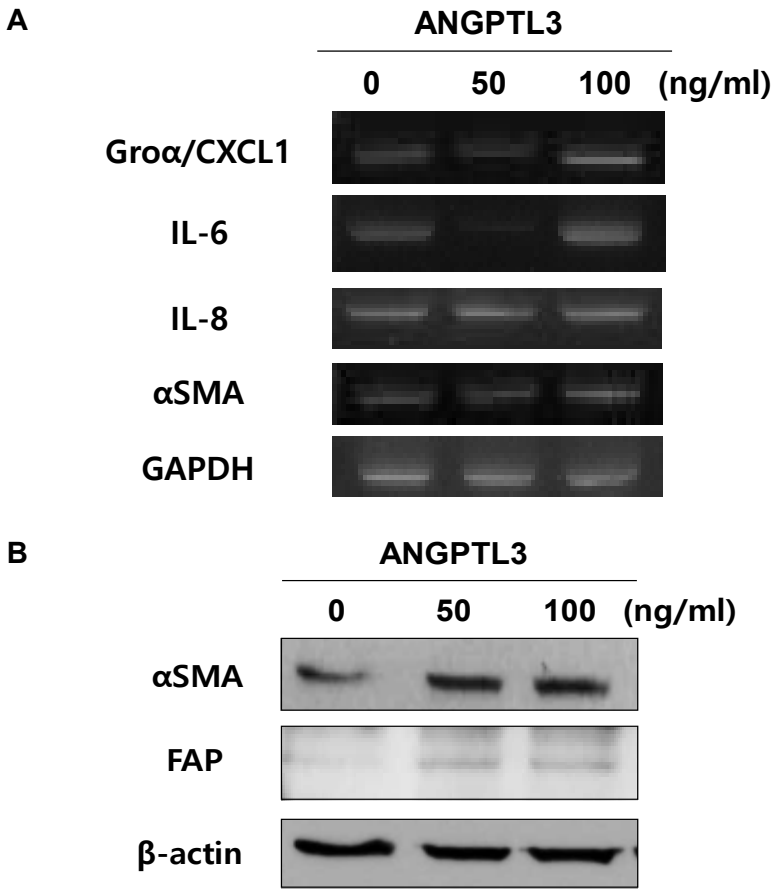


Figure 4. ANGPTL3 Induces a Myofibroblastic Phenotype in Stromal Fibroblasts. The hTERT-hNOFs were treated with rhANGPTL3 (50 ng/mL, 100 ng/mL) to identify tumor promoting cytokines and CAF markers. (A) The mRNA expression of cytokines (CXCL1/Gro-α, IL-6, and IL-8) and CAFs marker (α-SMA) were detected with RT-PCR. (B) Protein expression of CAFs marker (α-SMA and FAP) was detected by western blot. Control is treatment with PBS.

ANGPTL1 to ANGPTL8 (Santulli, 2014). ANGPTLs are widely expressed from many tissues, including the livers, vascular system, and the hematopoietic system. They play multi-biological roles in inflammation, lipid metabolism, and angiogenesis. Among them, ANGPTL3 is a main regulator of lipoprotein metabolism. ANGPTL3 expression is upregulated in hepatocellular carcinoma (Yu et al., 2011; El-Shal et al., 2017) and oral cancer (Koyama et al., 2015). Consistent with previous findings, our results show that ANGPTL3 is overexpressed in OSCC cell lines. Furthermore, ANGPTL3 is significantly overexpressed in monocultured and cocultured OSCC cells (YD10B and YD38) with hTERT-hNOFs, compare to monocultured and cocultured HEK cells with hTERT-hNOFs. It has been reported that ANGPTL3 plays an important role in angiogenesis, and metastasis. According to a previous report (Zhu et al., 2015), cancer cells synthesize and secrete ANGPTL3 protein to promote angiogenesis and to create a suitable microenvironment for cancer cell growth. However, the role of ANGPTL3 in tumor microenvironment has remained elusive as previous reports have only focused on cancer. To address these limitation, we focused on the veiled roles of ANGPTL3 in tumor environment, showing that it contributes to CAF differentiation.

Signals derived from diverse cancer cells or microenvironmental conditions regulate CAF heterogeneity, and various internal cell populations in CAFs can facilitate cancer development and progression (Bu et al., 2019). CAFs can be phenotypically divided two sub-phenotypes, such as myofibroblastic and senescent phenotypes (Prime et al., 2017). Myofibroblasts primarily express the α-SMA protein and usually give rise to CAFs. Several different markers have been widely used to identify this phenotype, including α-SMA, PDGF receptor-β, Fibroblast-specific protein (FSP)/S100A4, and FAP (Prime et al., 2017). To determine whether ANGPTL3 converts fibroblasts into CAF following rhANGPTL3 treatment, we identified α-SMA and FAP CAF markers, as well as tumor-promoting cytokines Gro-α/CXCL1 and IL-6. Treatment with rhANGPTL3 led to increased expressions of Gro-α/CXCL1 and IL-6, α-SMA and FAP in hTERT-hNOFs. These findings indicate that ANGPTL3 can induce phenotypes such as CAFs and thereby may facilitate tumor progression. We further investigated Gro-α/CXCL1 and IL-6 secretion levels following rhANGPTL3 treatment, but there were no ANGPTL3-induced trends seen in hTERT-hNOFs (data not shown). Given a previous study showing that OSCC can induce senescent phenotypes close to fibroblast (Kim et al., 2019), we also identified mRNA expression of senescence marker, p16 and p21, that had not been detected (data not shown). These results suggest that ANGPTL3-stimulated fibroblasts have myofibroblastic phenotypes similar to

traditional phenotypes instead of senescent phenotypes.

Taken together, ANGPTL3 released from oral cancer cells can induce transformation of stromal fibroblasts into CAF-like phenotypes. However, further detailed study is still needed to fully illuminate the mechanism behind ANGPTL3's regulation in fibroblasts. In conclusion, our study reports the first evidence that ANGPTL3 plays a crucial role in the tumor microenvironment by inducing CAF. Therefore, ANGPTL3 holds promise for CAF-targeted therapy, especially in the treating CAF-rich tumors.

Author Contribution Statement

All authors contributed to the study. DK designed and conducted the experiments. SM and JYK contributed to data collection and prepared the manuscripts. JYK, SM, and DK revised the manuscripts. All authors read the approved the final manuscripts.

Acknowledgements

General

The study has been conducted as part of a PhD thesis which was approved by Yonsei University of College of Dentistry, Seoul, Korea.

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Approval

All authors read and approved the final manuscripts.

Ethical Declaration

Not applicable.

Data Availability

All datasets leading to the results of the study are available with the corresponding author on reasonable request.

Study Registration

Not applicable.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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