

Carcinoma associated fibroblasts derived from oral squamous cell carcinoma promote lymphangiogenesis via c-Met/PI3K/AKT *in vitro*

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Abstract. Carcinoma-associated fibroblasts (CAFs) are dominant components of the tumor microenvironment (TME) that promote the development, progression and metastasis of cancer. c-Met is a receptor of the hepatocyte growth factor (HGF), which is involved in lymphangiogenesis. Currently, the roles of CAFs during lymphangiogenesis are unknown. It has been hypothesized that CAFs contribute to lymphangiogenesis of oral squamous cell carcinoma (OSCC) via a HGF/c-Met complex. The expression of HGF in OSCC was determined using CAFs derived from OSCC tissue and it was demonstrated that HGF is overexpressed in OSCC-derived CAFs. It was also revealed that c-Met was highly expressed in human lymphatic endothelial cells (HLECs) when co-cultured with CAFs. Furthermore, it was demonstrated that recombinant human HGF significantly enhanced the proliferation, migration, invasion and tube formation of HLECs. By contrast, the inhibition of c-Met expression suppressed the aforementioned biological activities and also downregulated the expression of c-Met, phosphoinositide 3-kinase and phosphorylated protein kinase B. Taken together, these data demonstrate that c-Met is associated with the regulation of lymphangiogenesis. Thus, the results of the present study indicate that c-Met may be a promising novel therapeutic target to treat patients with OSCC.

Introduction

Metastasis to the regional lymph nodes is a major factor indicating the negative prognosis of patients with cancer. Whether or not metastasis has occurred influences whether a patient receives postoperative therapy, including chemo- and radiotherapy, to reduce the risk of metastatic recurrence (1). Carcinoma-induced lymphangiogenesis within the tumor tissue is one of the causes of lymph node metastasis and it has been revealed that stromal and cancer cells serve a critical role in the induction of lymphangiogenesis by secreting lymphangiogenic factors, including vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) (2). Carcinoma-associated fibroblasts (CAFs) are the most abundant stromal cells in the tumor microenvironment (TME) and promote tumor growth, angiogenesis and the malignant progression of various solid tumors (3). In addition, CAFs use the epithelial-mesenchymal transition, a major biological process that can stimulate cancer progression and metastasis, to regulate the invasiveness of tumor cells (4,5).

The hepatocyte growth factor (HGF) receptor c-Met is encoded by the proto-oncogene MET. MET exhibits tyrosine-protein kinase activity and is overexpressed in numerous types of cancer, such as head and neck squamous cell carcinoma (6,7). Furthermore, the HGF/c-MET complex is widely expressed in cells of epithelial-endothelial origin and regulates multiple cellular processes in cancer, including cell proliferation, invasion and angiogenesis (7). Although a correlation between CAFs and cancer progression and metastasis has been determined (4,5), it remains unknown whether CAFs contribute to lymphangiogenesis in oral squamous cell carcinoma (OSCC) via the HGF/c-Met complex. Therefore, identifying the complex interactions between CAFs and lymphatic endothelial cells may help to identify the underlying mechanisms of CAFs in the lymphangiogenesis of OSCC.

CAFs and normal fibroblasts (NFs) have been successfully isolated and characterized from OSCC as well as from normal oral mucosal tissues (8). A co-culture experiment in a Transwell system revealed that CAFs significantly promote the proliferation, migration, invasion and tube formation of human lymphatic endothelial cells (HLECs) (8). The present study used a chemiluminescent immunoassay (CLIA) to measure

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the secretion of HGF by CAFs and to evaluate the expression of c-Met in HLECs co-cultured with CAFs. Furthermore, the influence of HGF on the proliferation, migration, invasion and tube formation of HLEC was evaluated using recombinant human HGF (rhHGF). An inhibitor of c-Met, JNJ-38877605, suppressed the proliferation, migration and invasion, as well as the tube formation, of HLECs by downregulating phosphatidylinositol 3-kinase (PI3K) and phosphorylated protein kinase B (AKT) levels. Thus, the results of the current study indicate that the c-Met/PI3K/AKT signaling pathway is one mechanism by which CAFs regulate lymphangiogenesis.

Materials and methods

Cell culture. The present study was approved by the Medical Ethics Committee of the West China Hospital of Stomatology of Sichuan University (Chengdu, China). Specimens of OSCC and normal oral mucosal tissue were obtained from 5 patients (2 males, 3 females; mean age, 61.6 years) from November 2015 to December 2015 at the Department of Head and Neck Oncology, West China Hospital of Stomatology, and all patients provided informed consent prior to inclusion in the current study. The isolation and characterization of primary CAFs and NFs was performed according to a previously established protocol (8). CAFs and NFs were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; GE Healthcare, Chicago, IL, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA), penicillin (100 U/ml) and streptomycin (100 µg/ml; GE Healthcare) at 37°C in a humidified incubator with 5% CO₂. HLECs were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured in endothelial cell medium (ECM; ScienCell Research Laboratories) supplemented with endothelial cell growth supplement (ScienCell Research Laboratories), 10% FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in a 5% CO₂ humidified atmosphere. Cell culture duration is described in each specific experiment as follows.

CLIA. CAFs and NFs (third passage, 2x10⁵ cells/well) were plated in 6-well plates in high-glucose DMEM containing 10% FBS. When cells reached 60-70% confluence, they were incubated further at 37°C in FBS-free DMEM-high glucose for 24 h. Culture medium were collected and centrifuged at 1,000 x g, at 4°C for 20 min and the supernatant was evaluated using an HGF CLIA kit (USCN Life Science, Inc., Wuhan, China) following the manufacturer's protocol.

Cell proliferation assay. Cell Counting kit-8 (CCK-8) assays (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) were used to evaluate the proliferation of HLECs. HLECs induced by rhHGF (PeproTech, Inc., Rocky Hill, NJ, USA) or JNJ-38877605 (400 nM; Selleck Chemicals, Houston, TX, USA) were seeded in 96-well plates (2,000 cells/well, 100 µl) and incubated at 37°C for 12 or 24 h. For rhHGF, the blank control was the culture medium without cells. The negative control used the vehicle, deionized water for rhHGF and DMSO for JNJ-38877605. Cells were incubated with 10 µl CCK-8 for 2 h, and optical density (OD) was measured at 450 nm using Varioskan Flash microplate reader (Thermo Fisher Scientific, Inc.).

Cell migration and invasion assays. HLEC migration and invasion assays were performed in a 24-well Transwell system (8 µm pore size; Corning, Corning, NY, USA). In these assays, rhHGF, CAFs or CAFs and JNJ-38877605 (400 nM) were plated in the lower chamber. HLECs (3x10⁵ cells in 300 µl medium) were seeded in the upper chamber coated with Matrigel (BD Biosciences, San Jose, CA, USA) for the invasion assay. The same protocol was used to carry out the migration assay except without coating Matrigel in the upper chamber. Deionized water and CAFs without JNJ-38877605 were taken as the respective negative control in the two assays. Following incubation for 24 h, the non-penetrating cells on the upper surface of the filters were removed using a cotton swab. The migrated and invaded cells were fixed in 4% paraformaldehyde for 5 min at room temperature and stained with 0.1% crystal violet for 15 min. Following each step, filters were washed 3 times in PBS. Three random fields were selected and the cells on each filter were photographed using an inverted phase contrast microscope (Olympus Corporation, Tokyo, Japan) at a magnification of x100.

Matrigel in vitro HLEC tube formation assay. The Matrigel *in vitro* assay was used to evaluate HLEC tube formation following rhHGF treatment or co-culture with CAFs that were supplemented with JNJ-38877605 or not. Matrigel was plated at the bottom of a 24-well plate or the lower chamber of the 24-well Transwell system (0.4 µm; Corning) following thawing overnight at 4°C and then kept at 37°C for 60 min. HLEC cells (1x10⁵) in 500 µl ECM containing 5% FBS and endothelial cell growth supplement were seeded in the Matrigel-coated wells and treated with rhHGF (3, 30 ng/ml) at 37°C for 14 h, or co-cultured with CAFs (upper chamber, 1x10⁵ cells) supplemented with JNJ-38877605 (400 nM) at 37°C for 14 h. Following fixation with 4% paraformaldehyde at room temperature for 30 min, images of the cells were captured using an inverted phase contrast microscope (Olympus Corporation) at a magnification of x100.

Western blot analysis. CAFs (2x10⁵) or NFs in 1 ml FBS-free DMEM supplemented with JNJ-38877605 or not were seeded in the upper chamber of a 6-well Transwell system (0.4 µm; Corning), and 2x10⁵ HLEC cells in 2 ml FBS-free ECM were seeded in the lower chamber. Following 24 or 48 h incubations at 37°C, total proteins were isolated from HLECs using RIPA lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, containing protease and phosphatase cocktails] (Beyotime Institute of Biotechnology, Haimen, China). Protein concentration was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Subsequently, 20 µg proteins were separated using 8% SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane (0.2 µm; EMD Millipore, Billerica, MA, USA). Following blocking with 5% skim milk in Tris-buffered saline and 0.1% Tween-20 (TBST) for 1 h, membranes were incubated with primary antibodies against c-Met (1:2,000), phosphorylated (p)-c-Met (1:500) AKT1 (1:5,000), p-AKT1, (1:5,000), β-tubulin (1:1,000; all from Abcam, Cambridge, UK); PI3K (1:1,000; Tianjin Saierbio Biotechnology, Tianjin, China). Following overnight incubation with primary antibodies

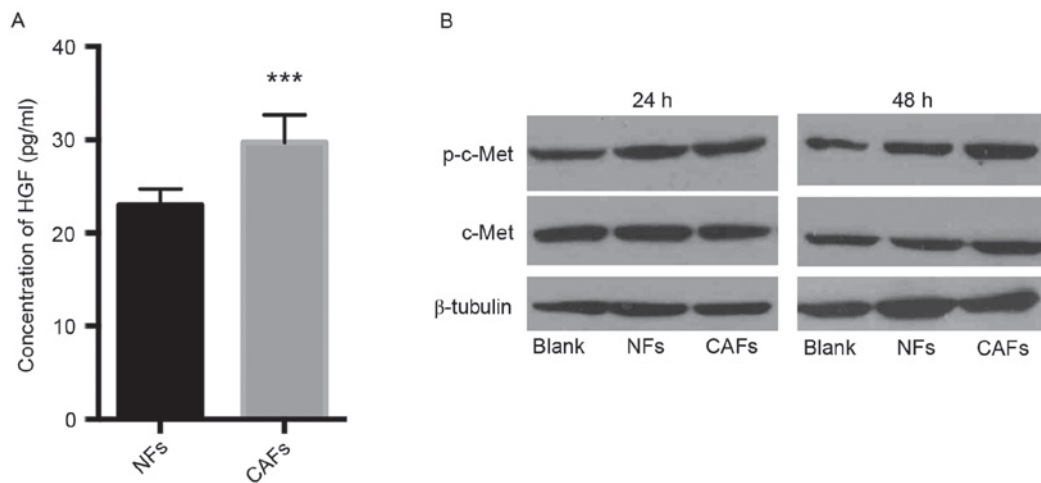


Figure 1. Chemiluminescent immunoassay to determine HGF levels in the supernatant of CAFs and NFs from oral squamous cell carcinoma tissues, and western blots analysis of c-Met and p-c-Met expression in HLECs co-cultured with CAFs and NFs. (A) Representative HGF levels from the supernatant of CAFs and NFs. (B) Representative western blots of c-Met and p-c-Met expression in HLECs co-cultured with CAFs and NFs for 24 and 48 h. *** $P < 0.001$. HGF, hepatocyte growth factor; CAFs, carcinoma-associated fibroblasts; NFs, normal fibroblasts; HLECs, human lymphatic endothelial cells.

diluted in 5% skim milk in TBST at 4°C, membranes were incubated with peroxidase-conjugated goat-anti mouse (cat. no. A0216) or goat-anti rabbit (cat. no. A0208) secondary antibodies (1:2,000; Beyotime Institute of Biotechnology) for 1 h at room temperature. Between each incubation, the membranes were washed 3 times for 5 min each in TBST. Western blots were visualized using Western Lightning Chemiluminescence Reagent (PerkinElmer, Waltham, MA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from HLECs was isolated using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. cDNA was synthesized using a PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan). qPCR was performed using a SYBR® Premix Ex gen, Taq™ II kit (Takara Bio, Inc.) on an ABI 7300 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions used were as follows: 1 cycle of 95°C for 30 sec, 40 cycles of 95°C for 5 sec and 60°C for 31 sec, and tubulin was used as a reference gene. The primers for c-Met and β -tubulin were as follows: c-Met forward, 5'-CATGCCGACAAGTGCAGTA-3' and reverse, 5'-TCTTGCCATCATTTGTCCAAC-3'; β -tubulin forward, 5'-TTG GCCAGATCTTTAGACCAGACAAC-3' and reverse, 5'-CCG TACCACATCCAGGACAGAATC-3'. Data were analyzed using $2^{-\Delta\Delta C_q}$ method (9). Data were analyzed and compared using the following relative quantification method: $R = 2^{-\Delta\Delta C_q}$, $\Delta\Delta C_q = (C_{q1} - C_{q2}) - (C_{q3} - C_{q4})$, where R represents the relative amount of the experimental group relative to the NC group; C_{q1} represents the cycle threshold of c-Met in the experimental group, C_{q2} represents the cycle threshold of tubulin in the experimental group, C_{q3} represents the cycle threshold of c-Met in the NC group and C_{q4} represents the cycle threshold of tubulin in the NC group (9).

Statistical analysis. All experiments were conducted in triplicate and representative data are presented as the mean \pm standard deviation. The comparison of treatment effects

was evaluated using a two-tailed Student's t-test or one-way ANOVA followed by Holm-Sidak's multiple comparisons test. $P < 0.05$ was considered to indicate a statistically significant difference. Image pro plus 6.0 (Media Cybernetics, Rockville, MD, USA) was used to count the cells in the migration and invasion assays as well as the tubes in the tube formation assay. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

Results

CAFs secrete higher level of HGF than NFs. It is currently unknown whether the contribution of CAFs to lymphangiogenesis in OSCC involves HGF/c-Met. Secreted HGF in the supernatant of CAFs and NFs medium were examined using a CLIA kit. Following 24 h, the secretion of HGF by CAFs (29.71 ± 2.963 pg/ml) was significantly higher than the secretion of HGF by NFs (22.99 ± 1.717 pg/ml; $P < 0.001$; Fig. 1A). These data indicate that, compared with NFs, CAFs taken from the TME of OSCC secrete higher levels of HGF.

Overexpression of p-c-Met in HLECs co-cultured with CAFs. To evaluate the expression of c-Met and its phosphorylated derivative p-c-Met in HLECs co-cultured with CAFs or NFs, western blot analysis was performed to identify the expression of c-Met and p-c-Met following 24 and 48 h culture. Co-cultured with CAFs for 48 h, levels of p-c-Met in HLECs were markedly higher than with NFs (Fig. 1B). These data indicate that substances secreted by CAFs promoted the expression of p-c-Met of HLECs. Thus, CAFs may serve an important role in lymphangiogenesis.

HGF promotes the proliferation, migration, invasion and tube formation of HLECs. To determine the regulatory role of HGF on HLECs, HLECs were treated with increasing concentrations of rhHGF and the proliferation, migration, invasion and tube formation of HLECs was subsequently evaluated. A significant dose-dependent difference was observed in the proliferation of HLECs that were treated with rhHGF

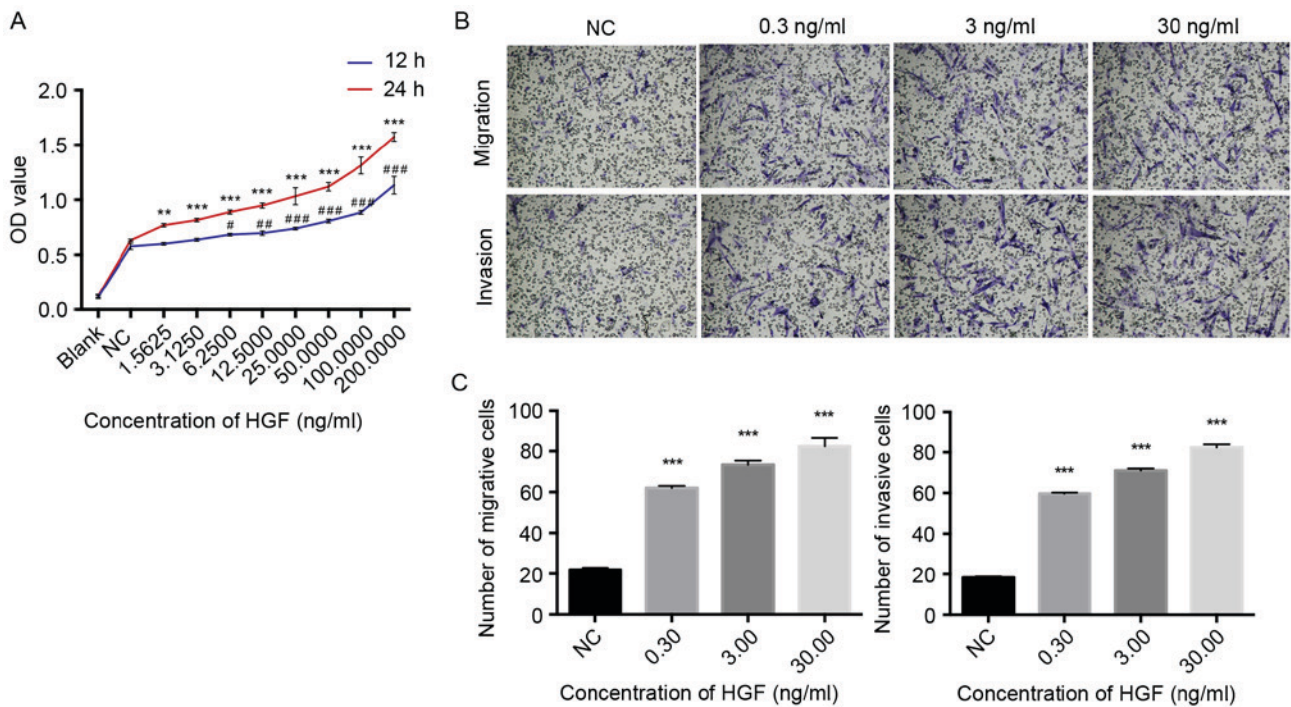


Figure 2. rhHGF promotes the proliferation, migration and invasion of HLECs. (A) Cell Counting kit-8 assay for the growth curve of HLECs treated with 1.5625-200 ng/ml rhHGF for 12 or 24 h; (B) Images of migrating and invading cells and (C) the number of migrating and invading HLECs treated with 0, 0.3, 3.0 or 30 ng/ml rhHGF for 24 h. Cells were identified using an inverted phase contrast microscope (magnification, $\times 100$). Results are the representative of three independent experiments. $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ vs. NC after 12 h; $^{**}P<0.01$ and $^{***}P<0.001$ vs. NC after 24 h. rhHGF, recombinant human hepatocyte growth factor; HLECs, human lymphatic endothelial cells; NC, negative control; OD, optical density; HGF, hepatocyte growth factor.

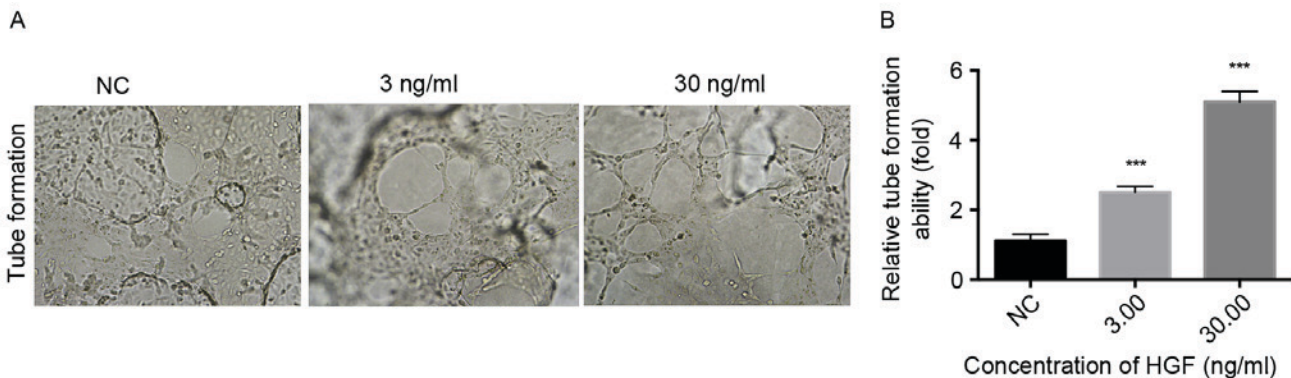


Figure 3. rhHGF promotes the tube formation of HLECs. (A) Matrigel *in vitro* HLEC tube formation assay of cells treated with 3.0 or 30 ng/ml rhHGF for 14 h. Images were visualized using an inverted phase contrast microscope (magnification, $\times 100$). (B) Quantification of tube formation assay. The number of tubes was standardized to relative tube formation ability based on the NC group. Assays were conducted independently in triplicate. $^{***}P<0.001$. rhHGF, recombinant human hepatocyte growth factor; HLECs, human lymphatic endothelial cells; NC, negative control; HGF, hepatocyte growth factor.

(1.5625-200 ng/ml) for 12 and 24 h compared with the NC ($P<0.05$; Fig. 2A). The HLECs were induced by 0, 0.3, 3.0 and 30 ng/ml rhHGF. The number of migrating and invading cells significantly increased in a dose-dependent manner following treatment with rhHGF ($P<0.001$; Fig. 2B and C), indicating that rhHGF promotes the migration and invasion of HLECs. Furthermore, following stimulation with 3.0 and 30 ng/ml rhHGF, HLECs exhibited a significantly higher number of tube formation compared with the NC group ($P<0.001$; Fig. 3), indicating that rhHGF is involved in the tube formation of HLECs. Taken together, HGF serves a critical role in modulating the CAFs-associated proliferation, invasion, migration and tube formation of HLECs.

Inhibition of c-Met attenuates the proliferation, migration, invasion and tube formation of HLECs. To know the role of c-Met in lymphangiogenesis, the proliferation, invasion, migration and tube formation of HLECs co-cultured with CAFs and JNJ-38877605 was investigated. The results of the CCK-8 assay indicated that JNJ-38877605 significantly inhibited the proliferation of HLECs compared with the NC group ($P<0.001$; Fig. 4A). Furthermore, the results of the migration and invasion assays indicated that JNJ-38877605 significantly repressed the migration ($P<0.01$) and invasion ($P<0.001$) of HLECs compared with the NC group (Fig. 4B-D). JNJ-38877605-treated HLECs formed fewer tubes compared with the NC group ($P<0.001$; Fig. 4B and E). These results

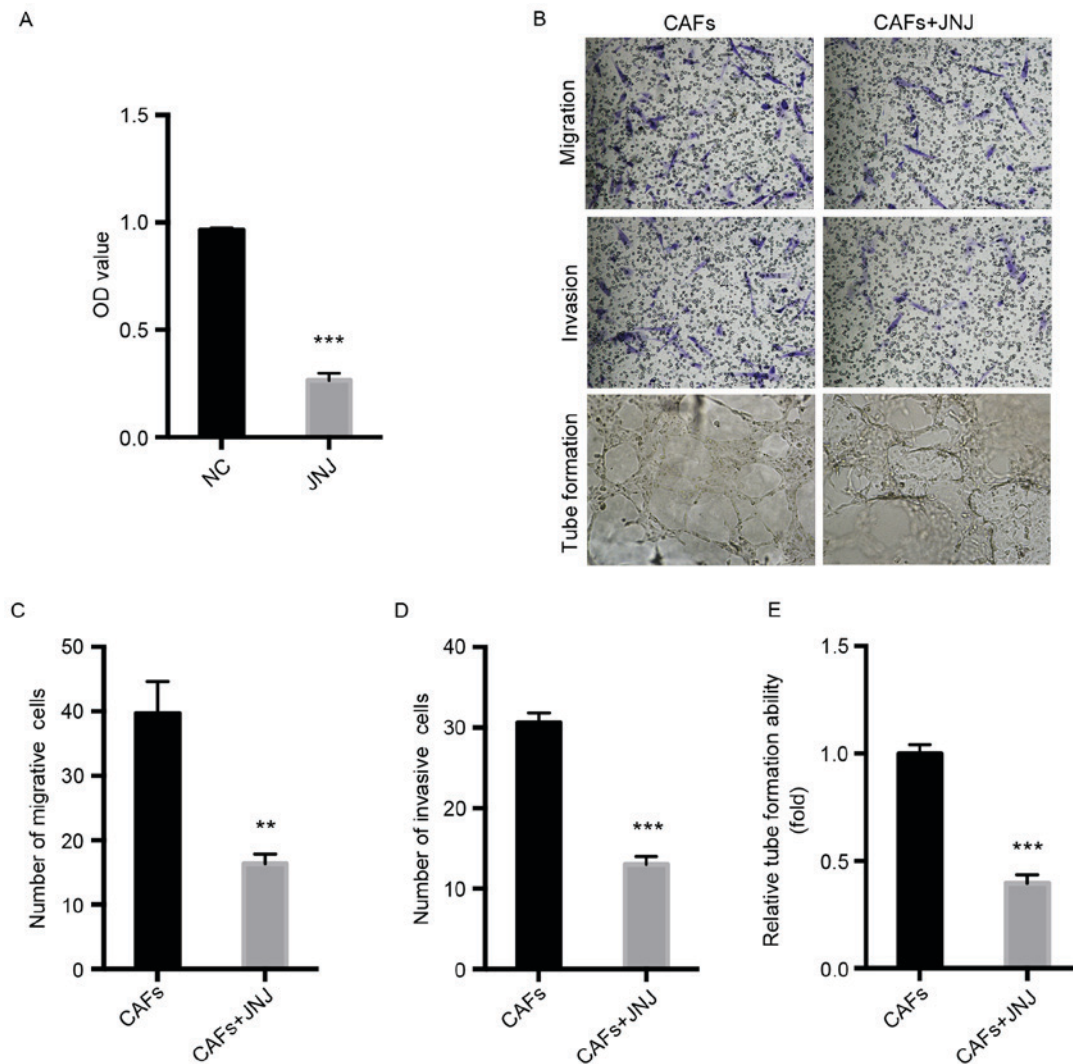


Figure 4. Inhibition of c-Met downregulates the proliferation, migration, invasion and tube formation of HLECs. (A) Cell Counting kit-8 assay measuring the proliferation of HLECs, treated with and without 400 nM JNJ for 24 h. *** $P < 0.001$. (B) Images of migrating and invading cells and the tube formation assays. Number of (C) migrating and (D) invading cells. (E) Relative tube formation ability of HLECs treated with and without 400 nM JNJ. Images were visualized using an inverted phase contrast microscope (magnification, $\times 100$). All quantitative data were averaged from triple independent results. ** $P < 0.01$ and *** $P < 0.001$. HLECs, human lymphatic endothelial cells; NC, negative control; CAFs, carcinoma-associated fibroblasts; OD, optical density; JNJ, JNJ-38877605.

indicate that CAFs-modulated HGF/c-Met is crucial for the proliferation, invasion, migration and tube formation of HLECs.

Inhibitor of c-Met downregulates the expression of c-Met and PI3K/AKT in HLECs. To identify the molecules or proteins involved in the signaling pathway, HLECs were co-cultured with CAFs supplemented with or without the c-Met inhibitor, JNJ-38877605. JNJ-38877605 reduced c-Met mRNA compared with the control group ($P < 0.05$) and markedly downregulated c-Met protein level (Fig. 5A). In addition, western blot analysis indicated that PI3K and p-AKT was also downregulated (Fig. 5B). These data indicate that c-Met/PI3K/AKT may regulate CAF-mediated lymphangiogenesis in the TME of OSCC.

Discussion

Lymphangiogenesis or invasion by pre-existing lymphatic vessels is the prerequisite of lymphatic metastasis (10). The

TME consists of various secreted soluble cytokines or growth factors, non-cellular solid material, ECM and stromal cells, and all of them surround the tumor cells and support tumor hemangiogenesis, lymphangiogenesis and metastasis (3). Stromal cells, including fibroblasts, smooth muscle, endothelial and inflammatory cells, interact with and modify the TME via the release of growth factors and proteases (11-13). Stromal cells also regulate cell proliferation, apoptosis, invasion and metastasis, as well as hemangiogenesis and lymphangiogenesis (11-13). There is increasing evidence that activated fibroblasts, primarily CAFs, are vital to the TME as they promote tumor growth and hemangiogenesis (3,14). Mantovani *et al* (15) reported that CAFs are recruited along with hypoxic inducible factor-1 α and promote tumor growth and angiogenesis in the presence of hypoxia release growth factors, including HGF, VEGF and PDGF. It remains unclear whether CAFs contribute to lymphangiogenesis and the mechanism of CAFs in OSCC remains unknown, although certain lymphangiogenic factors have been identified, including fibroblast growth factor, insulin-like growth factor, angiopoietin,

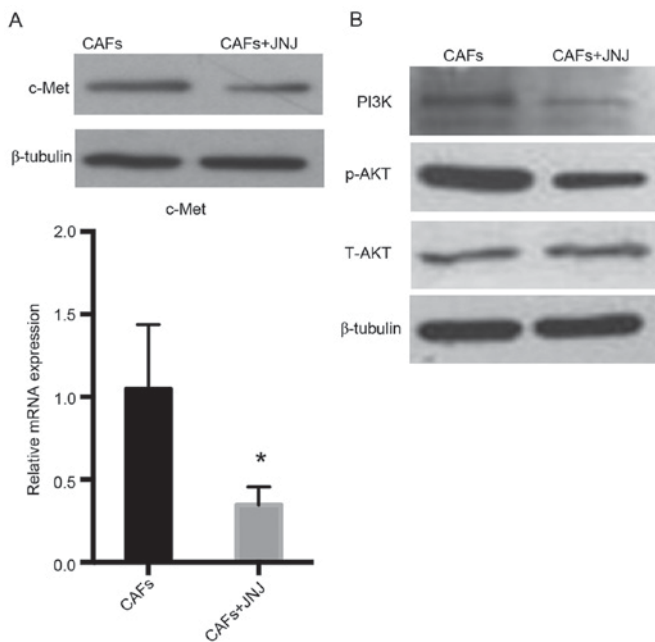


Figure 5. Inhibition of c-Met suppresses the expression of c-Met, PI3K and AKT in HLECs. (A) Reverse transcription-quantitative polymerase chain reaction measuring levels of c-Met mRNA in HLECs co-cultured with CAFs supplemented with or without 400 nM JNJ for 48 h. (B) Western blot assay of PI3K, p-AKT and T-AKT in HLECs co-cultured with CAFs supplemented with or without 400 nM JNJ for 48 h. These data are representative of three independent experiments. * $P < 0.05$. PI3K, phosphoinositide 3-kinase; HLECs, human lymphatic endothelial cells; CAFs, carcinoma-associated fibroblasts; JNJ, JNJ-38877605; T-AKT, total protein kinase B; p-AKT, phosphorylated protein kinase B.

VEGF, PDGF and HGF (16). Cao *et al* (16) successfully induced sprouting and the growth of new lymphatic vessels in a mouse model of corneal lymphangiogenesis via implantation of HGF. In addition, Gibot *et al* (17) used a human 3D lymphatic vascular construct to identify that HLECs, co-cultured with fibroblasts, spontaneously formed a 3D lymphatic capillary network without the use of any other supplemented factors. It was further demonstrated that fibroblast-derived HGF and VEGF-C work together in lymphangiogenesis by activating the extracellular regulated protein kinase (ERK1/2) signaling pathway (17). Additionally, the roles of HGF/c-Met and VEGF-C/VEGF receptor 3 (VEGFR-3) in the formation of lymphatic vessels were identified (17).

The results of the present study revealed that HGF levels were increased in CAFs and that c-Met was overexpressed in HLECs co-cultured with CAFs. Furthermore, it was demonstrated that rhHGF promotes the proliferation, migration, invasion and tube formation of HLECs. A previous study by De Bacco *et al* (18) determined that ionizing radiation induced the overexpression and activation of the MET oncogene that encodes c-Met via ataxia telangiectasia mutated-nuclear factor- κ B (NF- κ B) signaling. This indicated that MET promotes cell invasion and inhibits apoptosis, facilitating the development of radio-resistance. Chen *et al* (19) demonstrated that JNJ-38877605 decreased the migration of retinal pigment epithelial cells, reduced the phosphorylation of ERK and partially suppressed the activation of protein kinase C (PKC) induced by HGF. These data implied that c-Met, the HGF receptor, contributes to the stimulation of the PKC/ERK signaling pathway. The

present study used the co-culture method to simulate cross-talk between CAFs and HLECs in the TME, and the role of HGF/c-Met was subsequently investigated. The results revealed that JNJ-38877605 downregulated the expression of c-Met at the mRNA as well as the protein level.

PI3K, a family of enzymes involved in multiple cell functions, can be phosphorylated and activated by several cell membrane receptor tyrosine kinases, including Met, epidermal growth factor receptors (EGFR), ErbB3 (a type of EGFR), platelet-derived growth factor receptors, VEGFR and insulin-like growth factor 1 receptor (20). PI3K generated by phosphatidylinositol (4,5)-bisphosphate, stimulates AKT via translocation to the plasma membrane and subsequently phosphorylates AKT. As a key effector of PI3K, AKT regulates various cellular responses, including the cell cycle, cell proliferation and cell survival, by phosphorylating the relevant proteins (20–22). Yoo *et al* (23) reported that inhibition of PI3K/AKT blocks sonic hedgehog-induced lymphangiogenesis in gastric cancer. A variety of molecules are involved in the downstream signaling of AKT, including mammalian target of rapamycin, NF- κ B, glycogen synthase kinase 3 β , which regulate a variety of cellular processes including protein synthesis, cell survival and proliferation and make AKT an attractive therapeutic target for cancer (21). Finally, it was demonstrated that the c-Met inhibitor decreased PI3K and p-AKT and attenuated the proliferation, invasion, migration and tube formation of HLECs co-cultured with CAFs.

In conclusion, our present study preliminarily identified that CAFs serves a vital role on lymphangiogenesis via the Met/PI3K/AKT signaling pathway. Future studies should focus on evaluating the downstream mechanisms, which may help to identify novel potential targets for therapies to inhibit metastasis.

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