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Interaction between oral squamous cell carcinoma cells and fibroblasts through TGF-β1 mediated by podoplanin

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ABSTRACT

Podoplanin is upregulated in the invasive front of oral squamous cell carcinoma (OSCC). Carcinoma-associated fibroblasts (CAFs) may mediate podoplanin expression. However, the role of podoplanin in OSCC cell and fibroblast interaction remains elusive. In the present study, we found that positive podoplanin expression in OSCC cells correlated with smooth muscle actin (α-SMA) expression in CAFs. Using CAFs and normal mucosal fibroblasts (NFs), we established indirect and direct co-culture systems mimicking the structure of OSCC. Podoplanin-overexpressing OSCC cells promoted NF activation; in direct co-culture, but not in indirect co-culture, podoplanin-overexpressing OSCC cells increased fibroblast invasion via matrix metalloproteinase 2 (MMP-2), MMP-14, and αv/β6 integrin receptor (ITGA5/ITGB6) signaling. CAFs also induced podoplanin expression through the transforming growth factor-β1 (TGF-β1)/Smad pathway. TGF-β1 increased the podoplanin-dependent activation of epidermal growth factor receptor (EGFR), AKT, and extracellular signal–regulated kinase (ERK) signaling. Additionally, CAFs promoted OSCC cell invasion by upregulating MMP-2 and MMP-14 expression in both indirect and direct co-culture. Taken together, our findings indicate that podoplanin regulates the interaction between OSCC cells and CAFs via the mutual paracrine effects of TGF-β1.

1. Introduction

Oral squamous cell carcinoma (OSCC) is one of the leading causes of cancer death worldwide, and nearly 50% of patients die from the disease. Despite marked advances in diagnosis and surgery, the 5-year survival rate ranges from 45% to 53% [1]. Although many factors related to OSCC invasion have been identified, the molecular details of how tumor cells acquire metastatic properties are not fully understood. Therefore, improved knowledge of the molecular mechanism underlying the biological progression of OSCC is urgently needed to establish effective therapeutic modalities.

Human podoplanin (PDPN) is a type 1 transmembrane sialomucin-like glycoprotein found in a variety of normal human tissues, including lymphatic endothelial cells, glomerular podocytes, the heart, skeletal muscle, and the placenta [2]. However, its physiological functions are largely unknown. Recent studies have reported podoplanin overexpression in squamous cell carcinoma [2], malignant mesothelioma [3], and solitary fibrous tumor [4]. High podoplanin expression is correlated with nodal metastasis and poor prognosis in human tumors, including squamous cell carcinoma of the esophagus [5], testicular germ cell tumors [6], and mesotheliomas [7]. Previously, we demonstrated that high podoplanin expression correlates with poor prognosis in OSCC, and that the colocalization and interaction of podoplanin and matrix metalloproteinase-14 (MMP-14) at the invasion front of OSCC induce cytoskeletal remodeling, extracellular matrix (ECM) degradation, and tumor invasion during OSCC progression [8]. These findings indicate that cancer cell motility regulation is complex and based on interaction between tumor cells and the tumor stroma, including the ECM components, resident immune system, and vasculature. Previous studies have provided evidence that cancer cells do not exist independently but interact dynamically with local and distant host cells, and tumor progression is a result of evolving cross-talk between different cell types within the tumor and its surrounding supportive tissue [9,10]. As the second most numerous cell type in OSCC, fibroblasts are a dynamic population of cells with functional and phenotypic diversity. Activated fibroblasts, which are referred to as cancer-associated fibroblasts (CAFs), are the most important group, characterized by the expression of α-smooth muscle actin (α-SMA), and are found in association with cancer cell invasion [10]. A variety of cytokines and growth factors, such as transforming growth factor-β (TGF-β), epidermal

Abbreviations: OSCC, oral squamous cell carcinoma; CAF, cancer-associated fibroblast; NF, normal fibroblast; DMEM, Dulbecco's modified Eagle medium; ECM, extracellular matrix; FBS, fetal bovine serum; MMP, matrix metalloproteinase; SMA, smooth muscle actin; TGF, transforming growth factor

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growth factor (EGF), basic fibroblast growth factor (bFGF), and tumor necrosis factor-α (TNF-α), play an important role in tumor–stroma cross-talk [11–13]. In a breast cancer model, Kuperwasser et al. found in humanized stroma that TGF-β overexpression by fibroblasts promoted hyperplastic or tumorigenic outgrowth of normal breast epithelium, whereas normal stromal cells inhibited it [14]. Recent studies have provided evidence that CAFs enhance the invasion and metastasis of ovarian cancer cells through matrix metalloproteinases (MMPs), which destroy the tissue barrier and enhance tumor cell invasion [15,16]. Despite previous studies revealing that activating local residual fibroblasts facilitates the settlement of metastasizing tumor cells in breast cancer [17], little research has focused on evaluating the role of CAFs in OSCC cell metastases.

The present study intends to clarify the role of podoplanin in the interaction between tumor cells and fibroblasts. We co-cultured fibroblasts with podoplanin-transfected OSCC cells to study tumor–stroma interaction in vitro in a 3D co-culture system. Our results demonstrate that podoplanin-positive OSCC cells activate fibroblasts through TGF-β1 secretion and that CAFs enhance OSCC cells invasion through EGF receptor (EGFR), AKT, extracellular signal–regulated kinase (ERK), and MMP signaling, suggesting that podoplanin-positive OSCC cells create a foundation for invasion by activating fibroblasts.

2. Materials and methods

2.1. Tissue samples and immunohistochemistry

Human OSCC tissue samples and the autologous matching normal tissues were obtained from 42 patients treated by surgical resection at the Department of Oral and Maxillofacial Surgery, Peking University, School and Hospital of Stomatology. The study protocol was approved by the Severance Hospital Ethics Committee. Serial sections (4-μm thick) were cut and immunostaining was performed as we have described previously [8]. The primary antibodies were anti-podoplanin (Abcam) and anti–α-SMA antibodies (Abcam). Two independent pathologists blinded to the patient information analyzed the immunostaining results. Reactivity was determined according to the percentage of positive cells: up to 1% positive cells, 0; 2–25% positive cells, 1; 26–50% positive cells, 2; 51–75% positive cells, 3; and > 75% positive cells, 4. Intensity was graded as follows: no signal, 0; weak, 1; moderate, 2; or strong, 3. A total score of 0–12 was calculated and graded as negative (—: score = 0–1), weak (+: 2–4), moderate (++: 5–8), or strong (+++: 9–12). Scores of 0–6 were defined as low positive; scores of 7–12 were considered high positive.

2.2. Isolation and culture of primary human fibroblasts

Primary human cancer-associated fibroblasts (CAFs) and normal fibroblasts (NFs) were isolated from the OSCC tissues of patients. The tumor tissue and paired normal tissues were washed three times with sterile phosphate-buffered saline (PBS) with antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin, 50 mg/ml gentamycin). The tissues were minced with sterile scissors in a fresh sterile culture dish. To isolate fibroblasts, the tissues were digested with 0.1% collagenase type I (Sigma) at 37 °C in two 40-min periods, and carefully pipetted a few times with culture medium. The mixtures were centrifuged and washed with Dulbecco's modified Eagle's medium (DMEM) to remove the fat and tissue debris. Then, the tissues were cultured in DMEM/F12 medium with 20% fetal bovine serum (FBS) for about 2 days. The suspended cells and tissue were removed, and most of the adherent cells were fibroblasts. The primary fibroblasts isolated from tumor tissues were termed CAFs, and those from the paired normal tissues were termed NFs. Cell purity was assessed by vimentin and α-SMA immunofluorescence. The CAFs were further characterized by α-SMA positive expression, whereas NFs were α-SMA–negative. The primary cultures were incubated at 37 °C, and the medium was replaced after 24 h initially and every third day thereafter. All primary fibroblasts used in this study were between passages 2 and 5.

2.3. OSCC cell lines and transfection

Two established OSCC cell lines, WSU-HN6 and CAL27, were kindly donated by the Department of Central Laboratory, Peking University, School and Hospital of Stomatology. The cells were incubated in DMEM containing 10% FBS at 37 °C in a 5% CO_2 atmosphere. The WSU-HN6 cells were transfected with pCMV6-Entry empty vector or pCMN6-AC-GFP vector containing full-length podoplanin with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. On-TARGET plus human podoplanin siRNA SMARTpool and control small interfering RNA (siRNA) were from Dharmacon (Thermo Fisher Scientific). Transfections with podoplanin siRNA or control siRNA were carried out in CAL27 cells according to the manufacturer's instructions. Western blotting was used to identify successful transfection and podoplanin expression in the cells.

2.4. Direct and indirect co-culture system

For direct co-culture, confluent fibroblast and epithelial cell cultures were trypsinized, and 0.5×10^6 cells of each type were plated together in 100-mm tissue culture dishes containing 10 ml co-culture medium (45% DMEM, 45% DMEM/F12, 10% FBS; suitable for the growth of fibroblasts, epithelial cells, and co-cultures). For indirect co-culture, Transwell plates (Corning) with two compartments separated by a polycarbonate membrane with 0.4-μm pores were used. Fibroblasts were seeded in the bottom compartment (0.2×10^5 cells per well) and OSCC cells were seeded in the top compartment (0.2×10^5 cells per well). The two cell types were not in direct contact, but their soluble factors were. The cells were co-cultured for 1–5 days.

2.5. Quantitative real-time PCR

OSCC cell and fibroblast RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions. Then, 2 μg total RNA was reverse-transcribed into complementary DNA (cDNA) using a reverse transcription–PCR (RT-PCR) system (Promega). The quantitative RT-PCR (qRT-PCR) was carried out on a 7500 Real-Time PCR System (Applied Biosystems) with Fast Start Universal SYBR Green Master (Roche) according to the manufacturer's instructions. The following sense and anti-sense primers were used: α-SMA: 5′-CTGTTCCA GCCATCCTTCATC-3′ and 5′-CCGTGATCTCCTTCTGCATT-3′; αv integrin receptor (ITGA5): 5′-GTCGGGGGCTTCAACTTAGAC-3′ and 5′-CCTGGCTGGCTGGTATTAGC-3′; β6 integrin receptor (ITGB6): 5′-AAACGGGAACCAATCCTCTGT-3′ and 5′-GCTTCTCCCTGTGCTTGTA GGT-3′; MMP-2: 5′-CTCCCGGAAAAGATTGATG-3′ and 5′-GGTGCTGG CTGAGTAGAT-3′; MMP-9: 5′-GTGAGTTGAACCAGGTGGACCAAG-3′ and 5′-CACTCCTCCCTTTCCTCCAGAAC-3′; MMP-14: 5′-CCTGCGTCCA TCAACACT-3′ and 5′-GCCCATGAATGACCCTCT-3′; podoplanin: 5′-TGACTCCAGGAACCAGCGAAG-3′ and 5′-GCGAATGCCTGTTACACT GTTGA-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5′-AGAAGGCTGGGGCTCATTTG-3′ and 5′-AGGGGCCATCCACAGTC TTC-3′. Relative gene expression quantification was used to calculate the fold change of mRNA expression according to the comparative threshold cycle (Ct) method. GAPDH was used as the internal control for normalizing different samples. All experiments were confirmed in three independent experiments.

2.6. Western blotting

Each lane on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels was loaded with 30 μg protein. The protein was then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore), which were blocked with 10% skim milk for 1 h and

incubated overnight at 4 °C with the primary antibodies. The membranes were washed three times and incubated with the secondary antibodies (1:10,000) for 1 h, and the protein bands were detected with ECL chemiluminescence. The antibodies used were as follows: GAPDH (ZSGB-BIO); MMP-2, MMP-14, podoplanin (all from Abcam); α-SMA (Epitomics); phosphorylated (phospho)-Smad, Smad, phospho-EGFR, EGFR, phospho-AKT, AKT, phospho-ERK, and ERK (all from Cell Signaling Technology).

2.7. TGF-β1 enzyme-linked immunosorbent assay (ELISA)

Fibroblasts and OSCC cells were cultured monoculture or in co-culture for 48 h, and the supernatant was collected and concentrated with Amicon® Ultra-4 centrifugal filters (Millipore). The amount of TGF-β1 in the medium was measured with a human TGF-β1 immunoassay kit (R&D Systems) according to the manufacturer's instructions.

2.8. Invasion assays

Cell invasion was analyzed using a 24-well Transwell system (Millipore) with a Matrigel–pre-coated filter (BD). Both OSCC cells and fibroblasts were treated with mitomycin C (1 μg/ml) for 1 h. After trypsinization and washing, OSCC cells and fibroblasts were mixed in a 1:1 ratio and placed in the top chamber. Medium containing 20% FBS was placed in the bottom chamber as chemoattractant.

After 48-h incubation at 37 °C, the non-invading cells were removed from the upper surface of the membrane using cotton-tipped swabs, and then the cells on the lower surface of the membrane were fixed in 4% paraformaldehyde, stained with crystal violet, and counted. The invaded cells were also qualified by measuring the fluorescence intensity. All assays were run in triplicate. The invaded cells in six random fields were counted and photographed under a microscope (BX51, Olympus).

2.9. Colony formation assay

OSCC cells in the logarithmic growth phase were seeded in the bottom compartments of 24-well Transwell plates (Corning) at a density of 400 cells/well, and fibroblasts were seeded in the top compartment. Culture medium was changed three times a week. After 2 weeks, colonies were fixed with 10% paraformaldehyde for 30 min, stained with crystal violet for 30 min, and then washed with PBS. The colonies were counted under a microscope; the total number of colonies (> 50 cells/colony) was counted manually.

2.10. Immunofluorescence

OSCC cells and fibroblasts were co-cultured on coverslips, fixed with 4% paraformaldehyde for 30 min, and permeabilized with 0.1% Triton X-100 at room temperature for 10 min. After 30-min incubation with blocking solution at 37 °C (ZSGB-BIO), the cells were incubated with primary antibodies against vimentin (Abcam), podoplanin (Abcam), and α-SMA (Epitomics). Then, the cells were incubated with fluorescent antibodies conjugated with Alexa Fluor 488 or rhodamine (Cell Signaling Technology) at 37 °C for 1 h. Mounting medium containing 4′-6-diamidino-2-phenylindole (DAPI; ZSGB-BIO) was used to counterstain the nuclei, and the immunofluorescence was visualized under a fluorescence microscope (LSM 5, Carl Zeiss).

2.11. Gelatin zymography

Gelatin zymography was used to measure the MMP-2 and MMP-9 activity in the cells. The supernatant was collected and mixed with 5x SDS sample buffer without a reducing agent. Optimized loading volumes were determined by serial dilution of culture medium to ensure that band intensity was proportional to MMP-2 zymographic activity. Then, equal amounts of sample were loaded onto gels for SDS-PAGE (8% polyacrylamide gel containing 0.1% gelatin), and the zymograms were processed as previously described [18]. Lytic bands were analyzed by densitometry.

2.12. Statistical analysis

The experimental data were analyzed using the Statistical Package for the Social Sciences version 13.0 (SPSS, Chicago, IL, USA). The results are expressed as the mean ± standard deviation. The statistical significance of differences was analyzed using the two-tailed Student's *t*-test or one-way analysis of variance. $P < 0.05$ was considered significant.

3. Results

3.1. Podoplanin overexpression correlated with fibroblast activation

We obtained 42 OSCC tumors and their autologous-matched normal oral mucosa tissues from patients who underwent tumor resection. Analysis of podoplanin protein expression in the tissues showed that 90.5% (38/42) of OSCC tissues had high podoplanin expression at the invasion front (Fig. 1A). As fibroblasts in the tumor stroma influence the invasiveness of OSCC cells at the invasion front, we investigated the activation status of fibroblasts adjacent to the tumor cells by staining for α-SMA, an indicator of activated fibroblasts. We found that 95.2% (40/42) of the OSCC tissues were α-SMA–positive and that 94.7% (36/38) of highly podoplanin-positive OSCC tissues were α-SMA–positive (Fig. 1A). Next, primary human fibroblasts were isolated from six OSCC samples (CAFs) and the autologous-matched normal oral mucosa tissues (NFs), followed by morphological and immunocytochemical characterization. The primary fibroblasts initially had a uniform spindle-shape and formed parallel arrays and whorls at confluence (Fig. 1B). CAF purity was identified by the fibroblast biomarker vimentin and the CAF-specific biomarker α-SMA. Immunofluorescence staining showed that both CAFs and NFs were vimentin-positive and that CAFs were identified by their high positive expression of α-SMA, whereas NFs were α-SMA–negative (Fig. 1B). In addition, qRT-PCR revealed that CAF α-SMA expression was higher than that of NFs (Fig. 1C).

Using qRT-PCR, we detected the potential relationship between podoplanin in the epithelium and α-SMA in the tumor stroma in 42 OSCC samples. Podoplanin overexpression in the cancer cells correlated with α-SMA upregulation in the fibroblasts (Fig. 1D). This result indicates that podoplanin overexpression in OSCC cells is involved in NF activation.

3.2. Paracrine TGF-β1 from podoplanin-positive OSCC cells was responsible for NF activation

We used co-cultures to investigate the role of podoplanin in fibroblast activation. NFs were indirectly co-cultured with CAL27 and WSU-HN6 OSCC cells, respectively, and then α-SMA expression was assayed by qRT-PCR. In the indirect co-culture, CAL27 cells demonstrated stronger fibroblast-activating ability than the WSU-HN6 cells (Fig. 2A). Having demonstrated the different fibroblast-activating ability between CAL27 and WSU-HN6 cells, we were interested in the underlying molecular mechanism. Western blotting revealed that CAL27 cells had higher podoplanin expression than WSU-HN6 cells (Fig. 2B). ELISA showed that CAL27 cells had notably higher TGF-β1 secretion than the WSU-HN6 cells (Fig. 2B). To determine whether the TGF-β1 produced by OSCC cells confers NFs with CAF-like properties, NFs from cases 1 and 4 were indirectly co-cultured with OSCC cells in the presence or absence of anti–TGF-β1 neutralizing antibodies in the culture medium. In the absence of anti–TGF-β1 neutralizing antibody, CAL27 cells induced more α-SMA than WSU-HN6 cells did in both cases. Furthermore, treatment with anti–TGF-β1 neutralizing antibodies reduced the effects

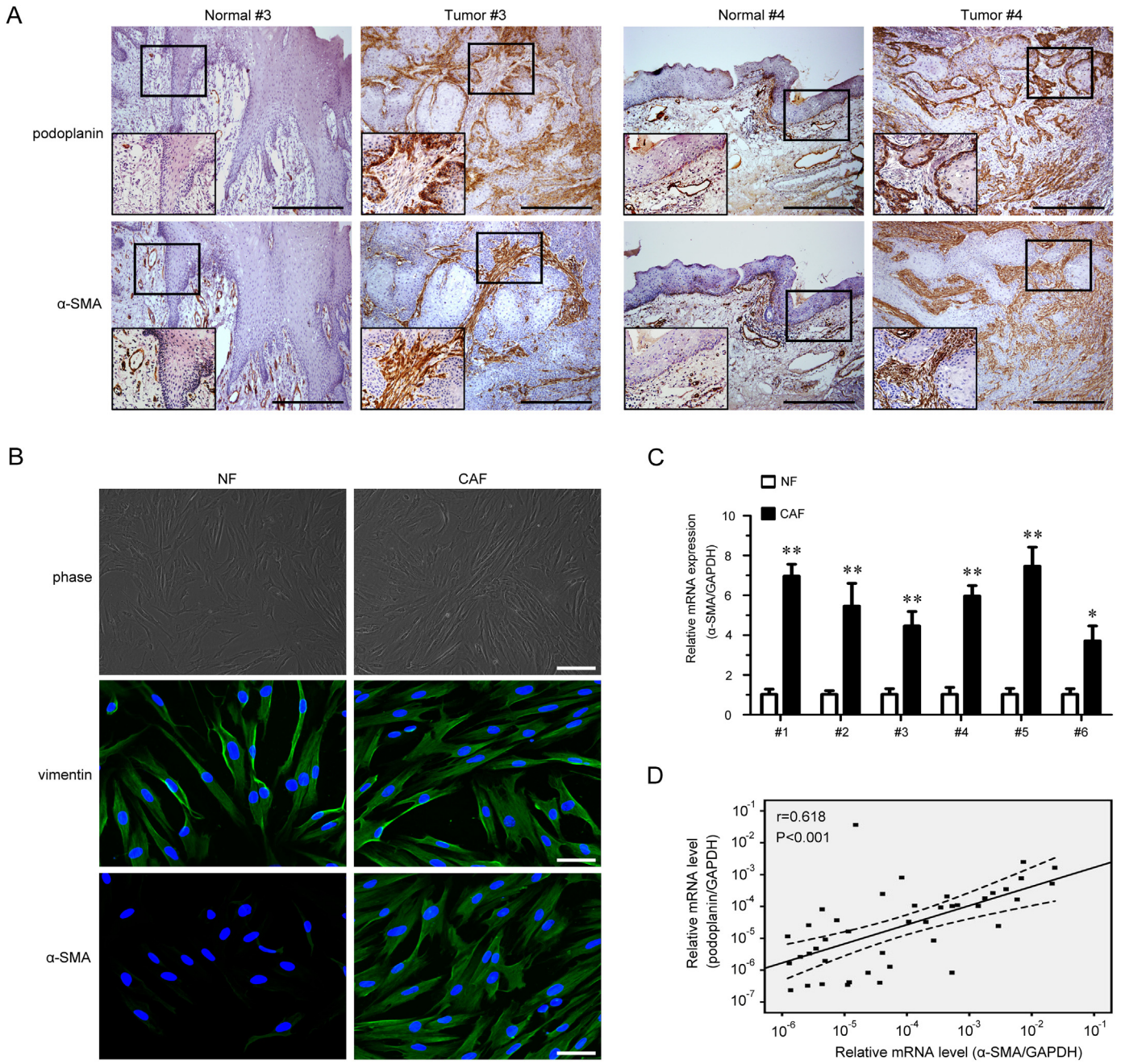

Fig. 1. Fibroblast activation correlated with podoplanin expression in OSCC tissue samples. (A) Representative immunostaining of podoplanin and α-SMA in tumor and donor-matched normal tissue sections. Small and large black frames indicate the original and magnified areas, respectively. Bar, 200 μm. (B) Immunofluorescence staining identification of CAFs using vimentin and α-SMA. Bar for morphological observation, 20 μm; bar for immunofluorescence staining, 100 μm. (C) α-SMA gene expression in CAFs and NFs was re-proved by qRT-PCR. $^{*}P < 0.05$; $^{**}P < 0.01$. (D) The relative expression of podoplanin and α-SMA mRNA normalized to GAPDH mRNA in 42 cancer tissues. The Pearson correlation coefficients (r) are shown.

of OSCC cells on NF activation (Fig. 2C).

To further investigate the effects of podoplanin in NF activation, WSU-HN6 cells were transfected with ectopic podoplanin-over-expressing plasmid (WSU-HN6/PDPN) and CAL27 cells was transfected with podoplanin RNA interference (RNAi) reagents (CAL27/si-PDPN) (Supplemental Fig. 1A and 1B). When NFs from cases 3 and 6 were indirectly co-cultured with podoplanin-transfected OSCC cells, we found that the WSU-HN6/PDPN cells and CAL27 cells transfected with control siRNA (CAL27/si-con) had stronger fibroblast-activating ability than the mock-transfected WSU-HN6 (WSU-HN6/Mock) cells and CAL27/si-PDPN cells, respectively, indicating a significant correlation between podoplanin in OSCC cells and α-SMA in fibroblasts (Fig. 2D). ELISA showed that WSU-HN6/PDPN cells had significantly higher TGF-β1 secretion than WSU-HN6/Mock cells, and TGF-β1 secretion was decreased in CAL27/si-PDPN cells compared with that in CAL27/si-control cells (Fig. 2E). This result implies that TGF-β1 may mediate the OSCC cell-induced α-SMA expression in fibroblasts. To verify this hypothesis, we examined α-SMA expression in NFs treated with 0.5, 1, or 5 ng/ml TGF-β1 for 2 days. The TGF-β1 treatment significantly increased the α-SMA expression in the NFs (Fig. 2F). Furthermore, we directly co-cultured podoplanin-transfected OSCC cells and fibroblasts, co-culturing WSU-HN6/Mock or WSU-HN6/PDPN cells, respectively, with NFs. The cultures organized quickly, typically exhibiting spindle-shaped fibroblasts surrounding tumor cell islets after 2 days and

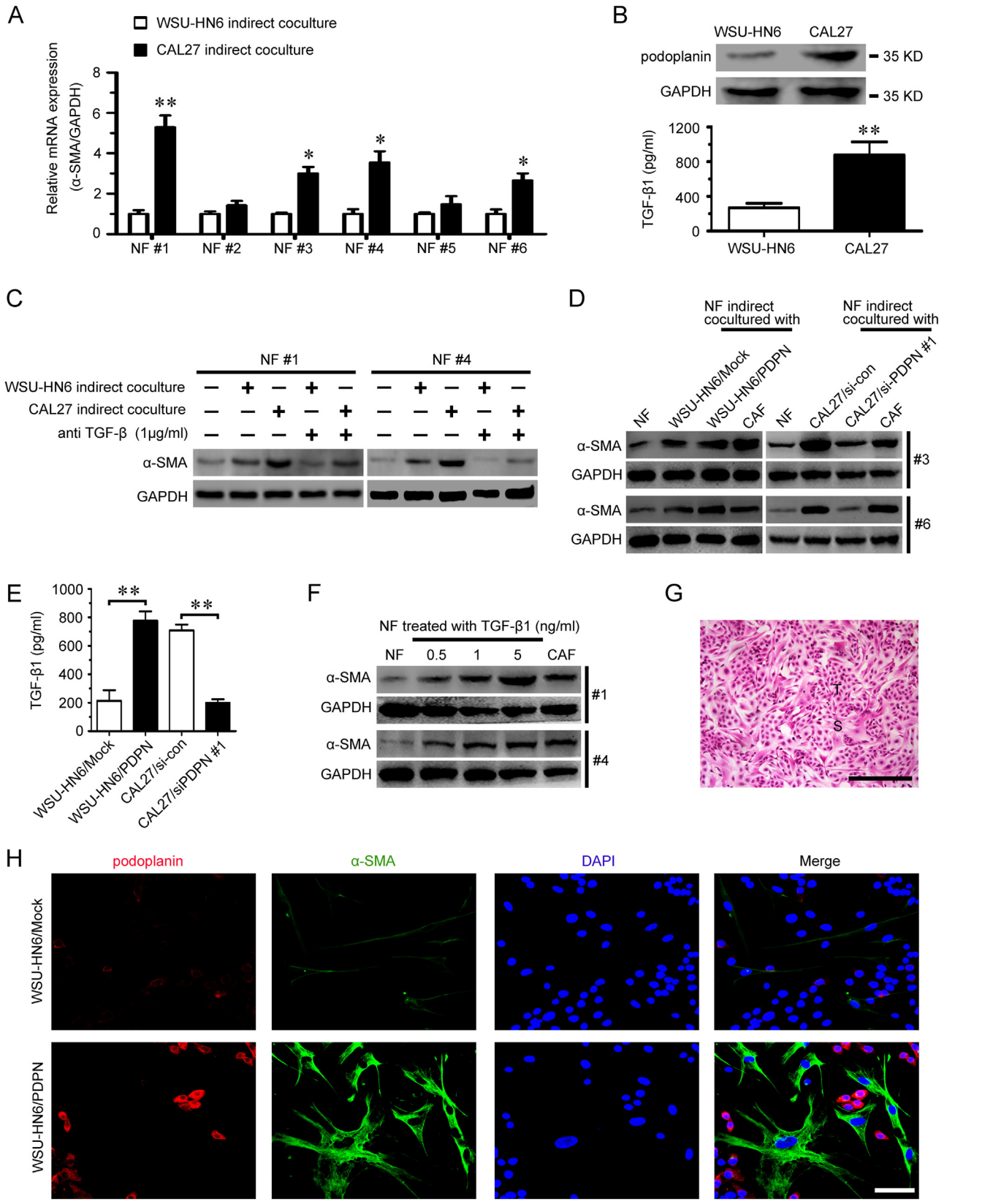

Fig. 2. Podoplanin-positive OSCC cells increase α-SMA expression in fibroblasts through TGF-β1 secretion. (A) α-SMA expression in NFs indirectly co-cultured with OSCC cells analyzed by qRT-PCR. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Podoplanin expression in OSCC cells examined by western blotting (top). ELISA performed to measure TGF-β1 secretion in OSCC cell conditioned medium (bottom). $^{**}P < 0.01$. (C) NFs were indirectly co-cultured with OSCC cells with or without anti–TGF-β1 neutralizing antibodies (1 μg/ml), and α-SMA expression was examined by western blotting. (D) α-SMA expression in NFs indirectly co-cultured with podoplanin-transfected OSCC cells examined by western blotting. (E) ELISA performed to measure TGF-β1 secretion in podoplanin-transfected OSCC cells conditioned medium. $^{**}P < 0.01$. (F) α-SMA expression in NFs treated with TGF-β1 (0.5, 1, 5 ng/ml) for 48 h examined by western blotting. (G) Morphology of direct co-culture of fibroblasts with OSCC cells. Spindle-shaped fibroblasts (S) surround tumor cell islets (T). Bar, 400 μm. (H) α-SMA expression in fibroblasts directly co-cultured with podoplanin-transfected WSU-HN6 cells examined by immunofluorescent staining. Bar, 100 μm.

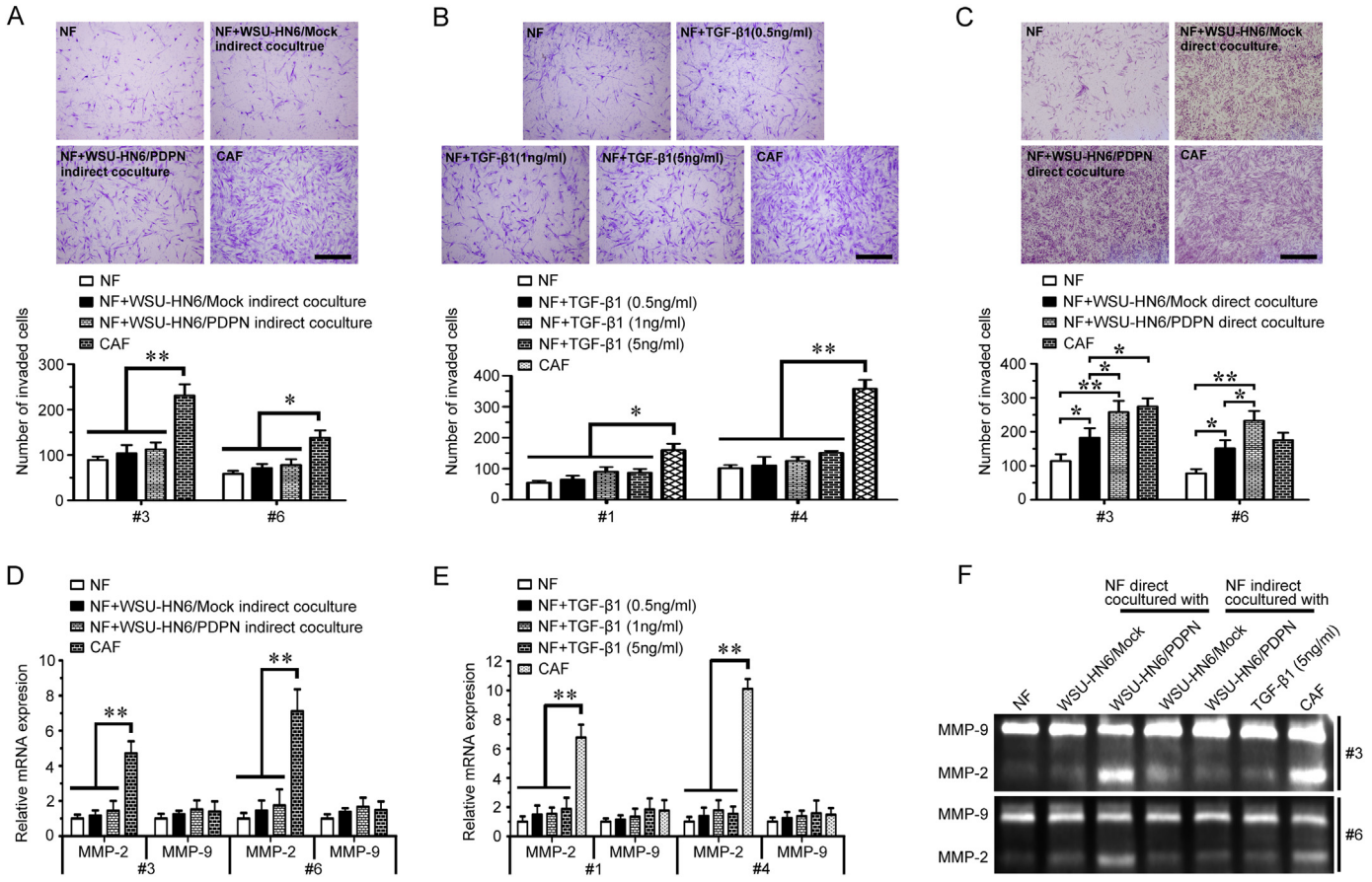

Fig. 3. Podoplanin-overexpressing WSU-HN6 cells promote fibroblast invasion. (A) WSU-HN6/Mock and WSU-HN6/PDPN cells induced NF invasion in indirect co-culture compared with untreated NFs and CAFs. $*P < 0.05$; $**P < 0.01$. Bar, 400 μm. (B) TGF-β1 (0.5, 1, 5 ng/ml) induced NF invasion in indirect co-culture compared with untreated NFs and CAFs. $*P < 0.05$; $**P < 0.01$. Bar, 400 μm. (C) WSU-HN6/Mock and WSU-HN6/PDPN cells induced NF invasion in direct co-culture compared with untreated NFs and CAFs. $*P < 0.05$ and $**P < 0.01$. Bar, 400 μm. (D) MMP-2 and MMP-9 expression in NFs indirectly co-cultured with WSU-HN6/Mock and WSU-HN6/PDPN cells analyzed by qRT-PCR. $**P < 0.01$. (E) MMP-2 and MMP-9 expression in NFs treated with TGF-β1 (0.5, 1, 5 ng/ml) analyzed by qRT-PCR. $**P < 0.01$. (F) MMP-2 and MMP-9 expression in NFs directly or indirectly co-cultured with WSU-HN6/Mock and WSU-HN6/PDPN cells examined by gelatin zymography.

becoming confluent after 4 days (Fig. 2G). NF activation was assessed by immunofluorescence staining (Fig. 2H): WSU-HN6/PDPN cells had stronger ability to induce α-SMA expression in NFs than WSU-HN6/Mock cells (Fig. 2H). These results indicate that paracrine TGF-β1 from podoplanin-positive OSCC cells is responsible for NF activation.

3.3. Podoplanin-positive OSCC cells promoted fibroblast invasion through MMP-2, MMP-14, and ITGA5 signaling in direct co-culture

To examine the role of podoplanin-transfected OSCC cells in fibroblast invasion, we indirectly co-cultured WSU-HN6/Mock and WSU-HN6/PDPN cells with NFs. CAF invasion was significantly higher than that of NFs without OSCC co-culture (Fig. 3A). Surprisingly, both WSU-HN6/PDPN cells and WSU-HN6/Mock cells did not promote NF invasion in indirect co-culture (Fig. 3A). Meanwhile, downregulation of podoplanin in the CAL27 cells did not affect NF invasion in the indirect co-culture (Supplemental Fig. 2A). This result implies that even though α-SMA expression in NFs increased when indirectly co-cultured with podoplanin-transfected OSCC cells, the fibroblasts did not become more invasive than untreated NFs. To determine whether TGF-β1 was involved in fibroblast invasion in the indirect co-culture, we performed Transwell assays of TGF-β1-treated NFs. TGF-β1 (0.5, 1, 5 ng/ml) did not increase the invasiveness of the fibroblasts compared to the control (Fig. 3B).

Next, we directly co-cultured NFs with WSU-HN6/Mock and WSU-HN6/PDPN cells. Interestingly, NF invasion was much higher when directly co-cultured with WSU-HN6/PDPN cells than with WSU-HN6/Mock cells (Fig. 3C). Additionally, the depletion of podoplanin in the CAL27 cells decreased the NF invasion in the direct co-culture (Supplemental Fig. 2B). A previous study reported that stromal fibroblasts produce enzymes to degrade stromal ECM components when moving forward, and that MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are generally involved in degrading denatured collagen and basement membrane components, such as the triple-helical regions of type IV collagen and laminins [19]. Therefore, we investigated MMP-2 and MMP-9 expression in NFs in indirect co-cultures. MMP-2 expression but not MMP-9 expression in CAFs was significantly higher than that in NFs (Fig. 3D and Supplemental Fig. 2C), and MMP-2 expression was not affected in the NFs indirectly co-cultured with the podoplanin-transfected OSCC cells (Fig. 3D and Supplemental Fig. 2C). In addition, treating NFs with TGF-β1 (0.5, 1, 5 ng/ml) did not affect MMP-2 and MMP-9 expression (Fig. 3E). These results imply that indirect co-culture enhances α-SMA expression in fibroblasts, but not MMP expression. However, when NFs were directly co-cultured with podoplanin-transfected WSU-HN6 cells, MMP-2 expression increased markedly (Fig. 3F), and WSU-HN6/PDPN cells exhibited stronger ability to increase MMP-2 expression in NFs than the WSU-HN6/Mock cells (Fig. 3F). We also found that downregulating podoplanin in the CAL27 cells markedly decreased MMP-2 expression in NFs in the direct co-culture compared to that in the CAL27/si-control group (Supplemental Fig. 2D). These results suggest that cell–cell contact between OSCC cells and fibroblasts increases MMP-2 expression and fibroblast invasiveness.

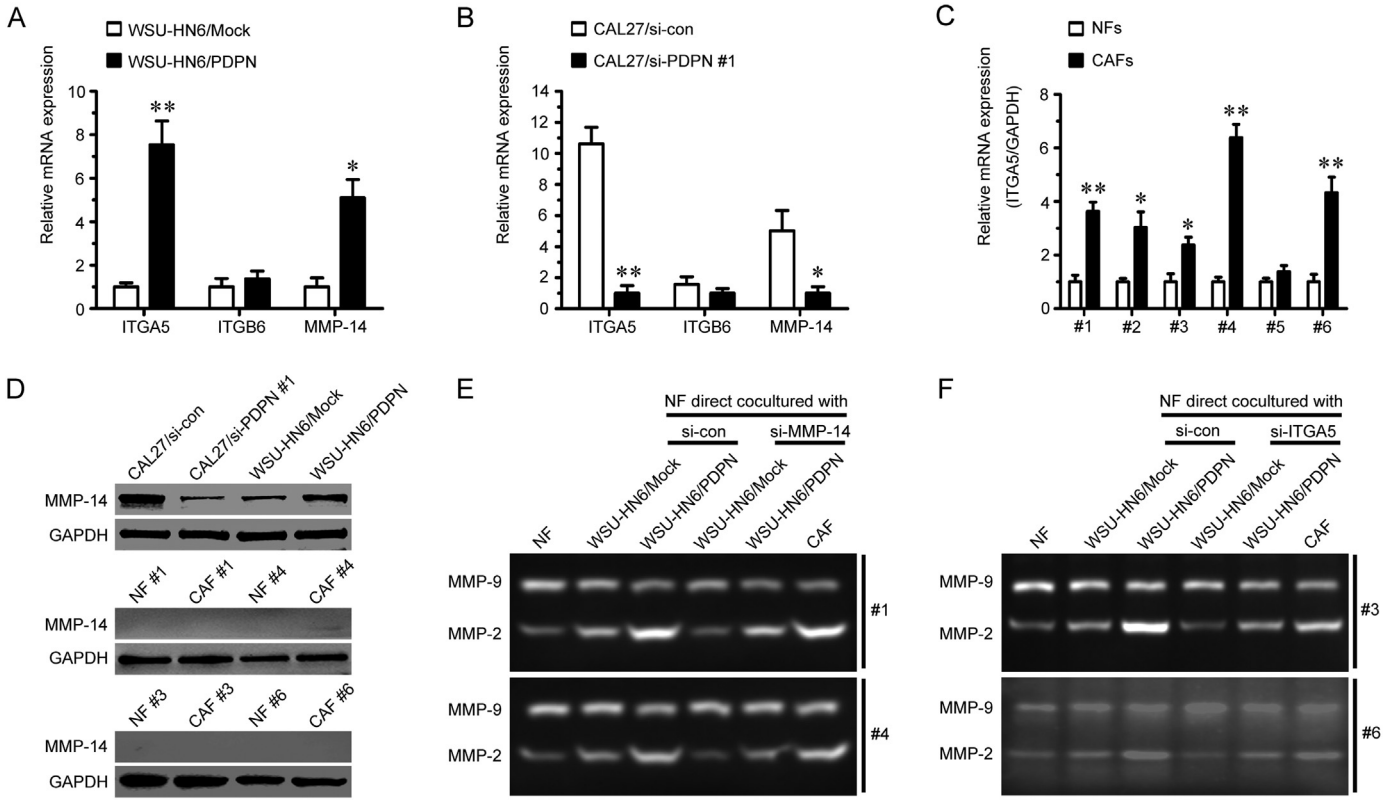

Fig. 4. Podoplanin-transfected OSCC cells promote fibroblast invasion in direct co-culture through MMP-2/MMP-14/ITGA5 signaling. (A) ITGA5, ITGB6, and MMP-14 expression in WSU-HN6/Mock and WSU-HN6/PDPN cells analyzed by qRT-PCR. $*P < 0.05$; $**P < 0.01$. (B) ITGA5, ITGB6, and MMP-14 expression in CAL27/si-con and CAL27/si-PDPN #1 cells analyzed by qRT-PCR. $*P < 0.05$; $**P < 0.01$. (C) ITGA5 expression in fibroblasts analyzed by qRT-PCR. $*P < 0.05$; $**P < 0.01$. (D) MMP-14 expression in fibroblasts and podoplanin-transfected OSCC cells examined by western blotting. (E) MMP-2 and MMP-9 expression in NFs directly co-cultured with WSU-HN6/Mock and WSU-HN6/PDPN cells transfected with MMP-14 siRNA reagents examined by gelatin zymography. (F) MMP-2 and MMP-9 expression in NFs directly co-cultured with WSU-HN6/Mock and WSU-HN6/PDPN cells transfected with ITGA5 siRNA reagents examined by gelatin zymography.

MMP-14 [19] and ITGA5/B6 [20] regulate MMP-2 expression. Therefore, we investigated whether podoplanin-transfected OSCC cells regulate MMP-2 expression in fibroblasts via MMP-14, ITGA5, and ITGB6. MMP-14 and ITGA5 mRNA levels correlated positively with podoplanin expression in the OSCC cells, but ITGB6 expression was unchanged in the podoplanin-transfected OSCC cells (Fig. 4A and B). Furthermore, five of six CAF samples had higher ITGA5 expression than the matched NFs (Fig. 4C). Podoplanin correlated positively with MMP-14 expression in OSCC cells, and MMP-14 expression in both NFs and CAFs was undetectable via western blotting (Fig. 4D). These results imply that MMP-14 and ITGA5 mediate MMP-2 expression in fibroblasts. Subsequently, we used MMP-14 and ITGA5 siRNA to determine whether both genes regulate MMP-2 expression in fibroblasts in direct co-culture. Gelatin zymography showed that silencing MMP-14 and ITGA5 decreased MMP-2 expression in fibroblasts (Fig. 4E and F). Therefore, we conclude that podoplanin-transfected OSCC cells promote fibroblast invasiveness through MMP-2, MMP-14, and ITGA5 signaling.

3.4. Activated fibroblasts increase OSCC cell invasion and proliferation

Immunohistochemistry revealed notably increased podoplanin expression in OSCC cells in contact with the stroma (Fig. 1A). This result implies that CAFs increase podoplanin expression in OSCC cells. To evaluate the effects of fibroblasts on OSCC cells, we co-cultured WSU-HN6 and CAL27 cells with NFs and CAFs, respectively. Western blotting revealed that CAFs had stronger ability to increase podoplanin expression in OSCC cells than NFs did in both direct and indirect co-culture (Fig. 5A). To determine the underlying mechanism, we assessed TGF-β1 secretion in the CAFs and donor-matched NFs. ELISA showed that CAFs had notably increased TGF-β1 secretion in cases 1 (2.3-fold), 3 (2.7-fold), 4 (4.2-fold), and 6 (3.6-fold) compared with the control (Fig. 5B). Having demonstrated the effect of podoplanin-transfected OSCC cells on fibroblast activation through TGF-β1 signaling, we were interested in whether TGF-β1 increases podoplanin expression in OSCC cells. We assessed podoplanin expression in WSU-HN6 and CAL27 cells treated with TGF-β1 (0.5, 5, 25 ng/ml). TGF-β1 upregulated podoplanin and phospho-Smad2/3 expression in a dose-dependent manner (Fig. 5C). A specific TGF-β type I receptor inhibitor (SB431542) was used to verify the role of TGF-β1 in podoplanin expression in OSCC cells. In both WSU-HN6 and CAL27 cells, SB431542 (1, 5, 25 μg/ml) attenuated podoplanin and phospho-Smad2/3 expression, indicating that the TGF-β receptor/Smad signaling pathway is involved in regulating podoplanin expression in OSCC cells (Fig. 5C).

We also assessed WSU-HN6/Mock and WSU-HN6/PDPN cell invasion in both direct and indirect co-culture and found significantly increased invasion by OSCC cells co-cultured with CAFs in comparison with co-culture with NFs. Furthermore, the addition of anti–TGF-β1 neutralizing antibody in the co-culture medium attenuated the invasion of both WSU-HN6/Mock cells and WSU-HN6/PDPN cells (Fig. 5D and E). We investigated the effect of fibroblasts on OSCC cell proliferation: podoplanin overexpression increased the WSU-HN6/PDPN colony counts compared with the WSU-HN6/Mock cells, and CAFs significantly increased the colony counts of both the WSU-HN6/Mock and WSU-HN6/PDPN cells compared with the NF group (Fig. 5F). The inclusion of anti–TGF-β1 neutralizing antibody in the co-culture medium attenuated the colony formation of both the WSU-HN6/Mock cells and WSU-HN6/PDPN cells (Fig. 5F). Our results indicate that CAFs not only

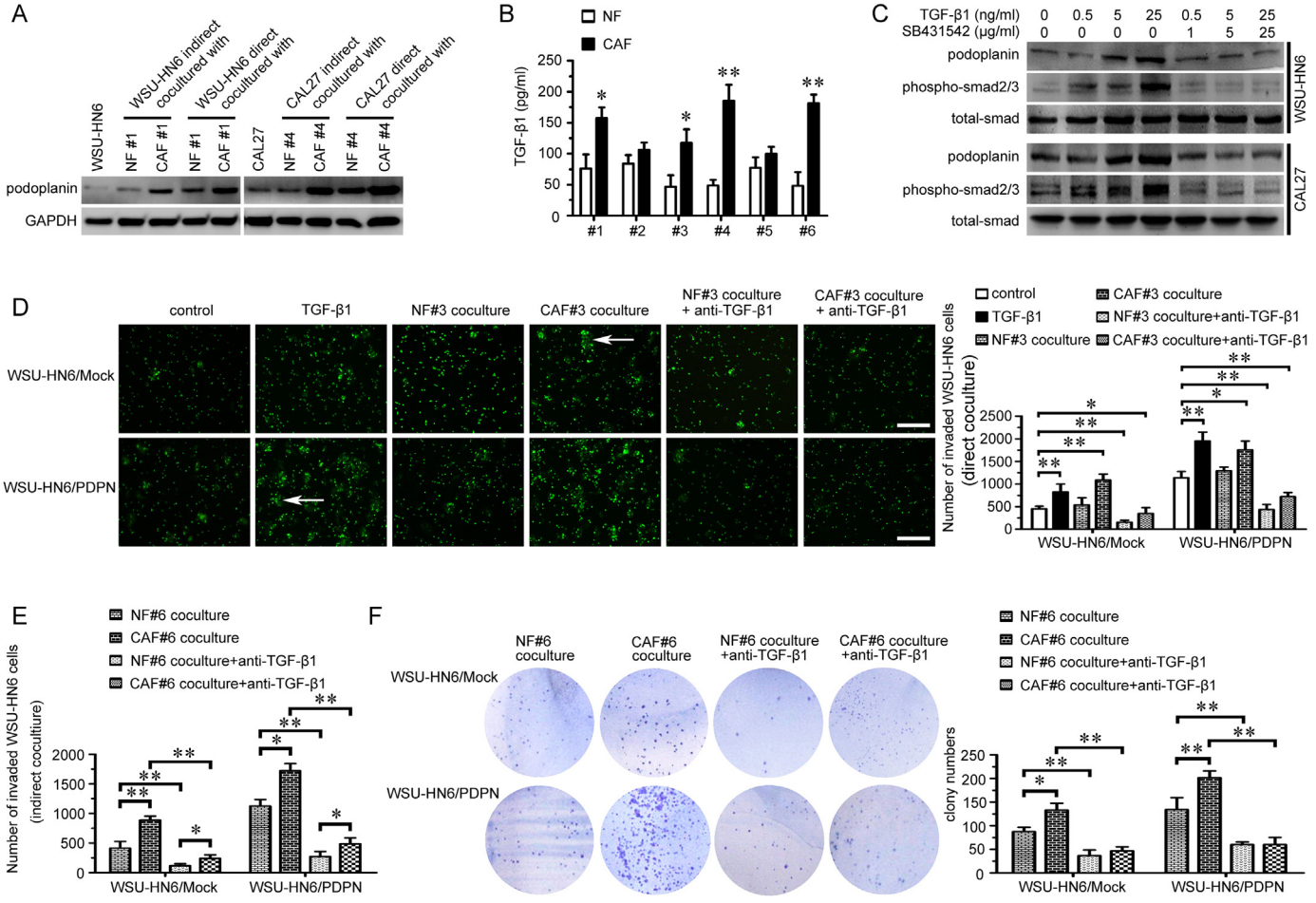

Fig. 5. CAFs promote OSCC cell invasion and proliferation. (A) Podoplanin expression in OSCC cells indirectly and directly co-cultured with NFs and CAFs examined by western blotting. (B) ELISA was performed to measure TGF-β1 secretion in conditioned medium of fibroblasts. $*P < 0.05$; $**P < 0.01$. (C) Podoplanin and phospho-Smad2 expression following treatment with exogenous TGF-β1 (0.5, 5, 25 ng/ml) and SB431542 (1, 5, 25 μg/ml) examined by western blotting. (D) The effect of NF and CAF direct co-culture, TGF-β1, and anti–TGF-β1 neutralizing antibody (1 μg/ml) on invasion by green fluorescent protein (GFP)-labeled WSU-HN6/Mock and WSU-HN6/PDPN cells examined by Transwell assay. Arrows indicate GFP-labeled WSU-HN6 cells. $*P < 0.05$; $**P < 0.01$. Bar, 400 μm. (E) The effect of NF and CAF indirect co-culture, TGF-β1, and anti–TGF-β1 neutralizing antibody (1 μg/ml) on the invasion by GFP-labeled WSU-HN6/Mock and WSU-HN6/PDPN cells examined by Transwell assay. $*P < 0.05$; $**P < 0.01$. (F) The effect of NF and CAF indirect co-culture, TGF-β1, and neutralizing anti–TGF-β1 antibody (1 μg/ml) on colony formation by GFP-labeled WSU-HN6/Mock and WSU-HN6/PDPN cells examined by colony formation assay. $*P < 0.05$; $**P < 0.01$.

increase podoplanin expression, but also increase OSCC cell invasion and proliferation.

3.5. MMP-2 and MMP-14 upregulation and EGFR, AKT, and ERK signaling activation by TGF-β1

The podoplanin-enhanced invasion properties of tumor cells appear to depend on MMP activity [21], so we examined MMP expression in podoplanin-transfected OSCC cells. Podoplanin overexpression correlated with both MMP-2 and MMP-14 significantly (Fig. 6A). We were interested in whether MMP-2 and MMP-14 regulate the invasion of podoplanin-transfected OSCC cells in co-culture. In both direct and indirect co-culture, CAFs demonstrated stronger ability to increase MMP-2 and MMP-14 expression in podoplanin-transfected OSCC cells than NFs did (Fig. 6B). TGF-β1 treatment of podoplanin-transfected OSCC cells increased the expression of both MMP-2 and MMP-14 (Fig. 6B). These results suggest that TGF-β1 secreted by CAFs increases the invasiveness of podoplanin-transfected OSCC cells. TGF-β1 binding to EGFR activates endogenous tyrosine kinase and promotes epithelial invasion [22,23]. We examined whether the EGFR signaling pathway and its downstream effectors are involved in the increased OSCC cell invasiveness caused by CAF co-culture. To address this hypothesis, we examined EGFR, AKT, and ERK expression in podoplanin-transfected OSCC cells treated with TGF-β1 and anti–TGF-β1 neutralizing antibody. Podoplanin overexpression in WSU-HN6/PDPN cells, which increased TGF-β1 secretion (Fig. 2E), increased EGFR phosphorylation as well as its downstream effectors AKT and ERK compared to the WSU-HN6/Mock cells (Fig. 6C). The anti–TGF-β1 neutralizing antibody attenuated the increased activation of these proteins promoted by podoplanin transfection and TGF-β1 treatment (Fig. 6C). These results indicate that in the podoplanin-transfected OSCC cell and fibroblasts co-culture, TGF-β1 secreted both by OSCC cells and CAFs promotes cancer cell invasion and proliferation.

4. Discussion

Podoplanin overexpression has been reported in a variety of cancers [2]. Previously, we indicated that high podoplanin levels are expressed in most microinvasive OSCC cells and the outer edge of OSCCs, indicating that podoplanin expression is associated with tumor stroma. In the present study, we demonstrate bidirectional interactions between fibroblasts and OSCC cells, and explore key factors contributing to fibroblast activation and fibroblast-mediated metastasis of cancer cells.

Activated fibroblasts are known as CAFs, which are characterized by the expression of α-SMA [10]. CAFs are found in almost all solid tumor tissue and play an important role in the malignant progression of

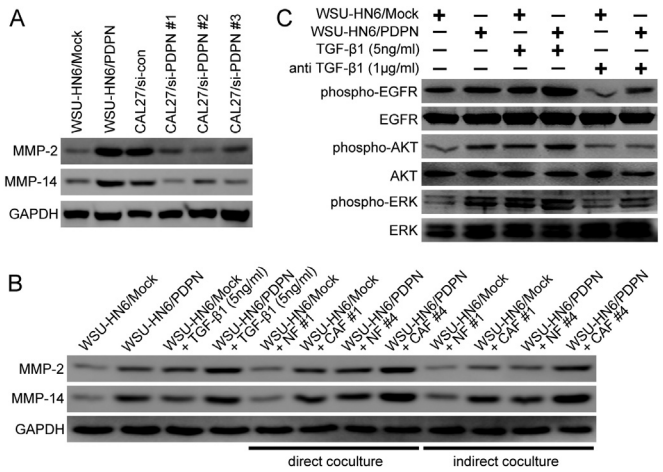

Fig. 6. TGF-β1 activates EGFR, AKT, and ERK and upregulates MMP-2 and MMP-14 expression. (A) MMP-2 and MMP-14 expression in podoplanin-transfected OSCC cells examined by western blotting. (B) The effect of indirect and direct co-culture with fibroblasts and TGF-β1 treatment on MMP-2 and MMP-14 expression in podoplanin-transfected OSCC cells examined by western blotting. (C) The effect of podoplanin transfection, TGF-β1, and neutralizing anti–TGF-β1 antibody on phospho- and total EGFR, AKT, and ERK expression in WSU-HN6 cells examined by western blotting.

cancer. Skobe et al. demonstrated that carcinoma cells induce highly tumorigenic, vasculogenic, and invasive tumor stroma [24]. Chauhan et al. reported that highly polymorphic ductal carcinoma in situ of the breast and non-invasive urothelial carcinoma of the bladder induced NF activation in the stroma surrounding the cancer cells [25,26], demonstrating that cytokines secreted by aggressive tumor cells can activate NFs. One of the best-known cytokines responsible for NF activation is TGF-β1. Many studies have demonstrated that tumor cell autonomous TGF-β1 expression is often increased in human carcinoma, including OSCC and head and neck squamous cell carcinoma (HNSCC) [27,28]. In the present study, we show that fibroblasts adjacent to podoplanin-positive OSCC cells were activated and that TGF-β1 contributes to fibroblast activation in both direct and indirect co-culture. As TGF-β1 secretion was increased in podoplanin-overexpressing OSCC cells, it suggests that podoplanin overexpression in OSCC cells induces NF activation through TGF-β1 signaling.

Many reports have shown that CAFs in the tumor stroma enhance invasion in breast, colon, and prostate cancer and basal cell carcinoma [29–31]. In our study, we demonstrate that CAFs induce podoplanin expression in OSCC cells via TGF-β1/Smad signaling. Using an organotypic culture model, Grugan et al. showed that using fetal esophageal fibroblasts within the esophageal cancer ECM enhanced esophageal cancer cell invasion, whereas fetal skin fibroblasts, which are less activated, produced little evidence of cancer cell invasion [16,32]. Our study combined with these data provide compelling evidence that fibroblast activation is critical in fostering the environment for tumor invasion.

The ECM is composed of a complex network of macromolecules that assemble into 3D structures with distinct biochemical and biomechanical properties that regulate cell survival, motility, and differentiation by ligating specific receptors such as integrin receptors [33]. Macromolecules, including collagen type I, laminins, and fibronectin, compose a major part of the ECM [33], and both collagen type I and laminins are MMP-2 substrates [19]. Cancer invasion through dense matrices is associated with MMP-mediated ECM degradation generating pathways for invasion [34]. In our study, the phenotype activated by

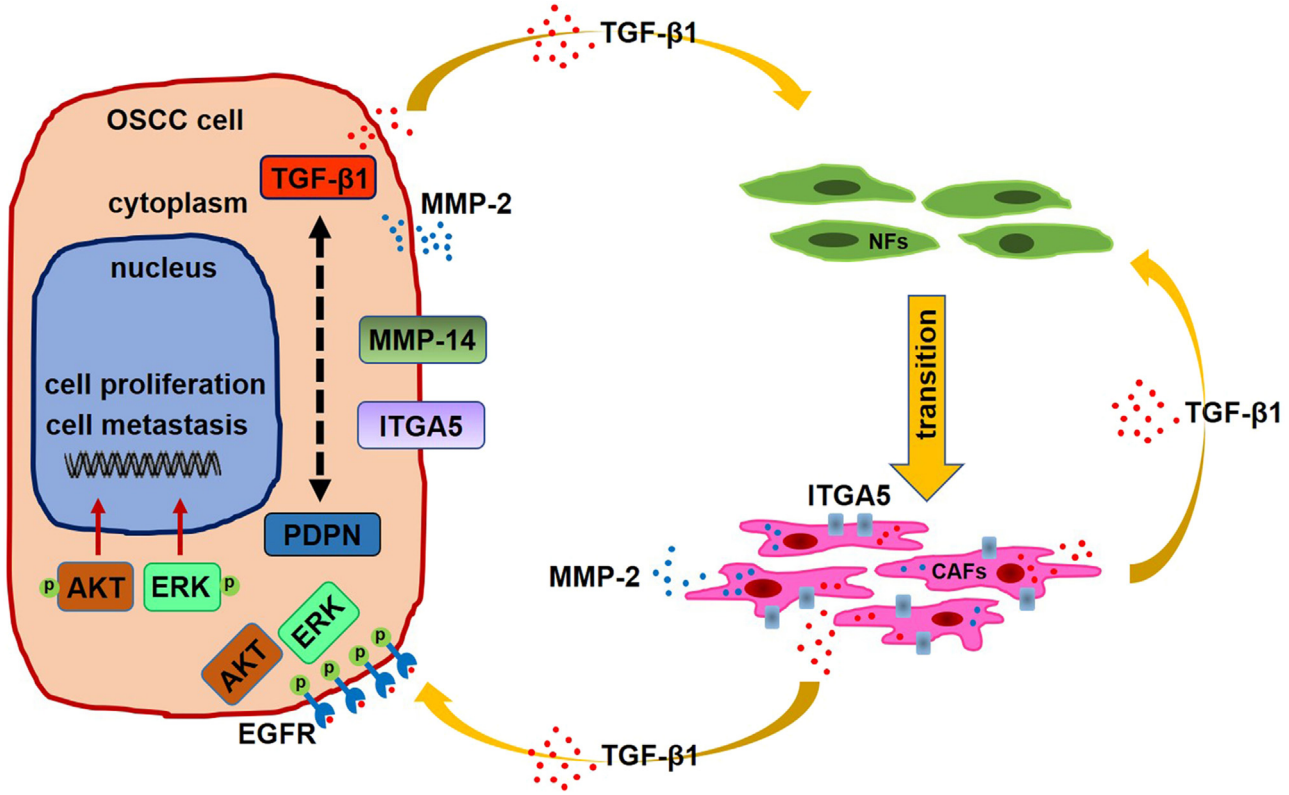

Fig. 7. Schematic representation of the proposed mechanism. In the tumor microenvironment, NFs are stimulated by TGF-β1 and are converted into CAFs. Subsequently, this conversion causes CAF upregulation of TGF-β1 secretion, which elevates podoplanin expression in OSCC cells. Podoplanin overexpression in OSCC cells upregulates TGF-β1 secretion and increases EGFR phosphorylation as well as its downstream effectors AKT and ERK. MMP-14/ITGA5 signaling upregulates MMP-2 expression in both OSCC cells and fibroblasts through direct fibroblast–carcinoma cell contact. OSCC cells gain a more aggressive phenotype in a microenvironment with bidirectional communication with fibroblasts.

indirect co-culture and TGF-β1 treatment did not promote MMP-2 expression. However, MMP-2 was upregulated in fibroblasts surrounding the tumor cells in the direct co-culture. Tang et al. have shown that outside–in signaling mediated by the membrane-anchored MMP-14 is critical for regulating the fate of skeletal stem cells [35]. Tokumaru et al. showed that MMP-14 localized on cancer cells activates MMP-2 expression in stromal fibroblasts [36]. Our study suggests that MMP-14, which was upregulated in podoplanin-transfected OSCC cells, contributes to the increased MMP-2 in fibroblasts when they were co-cultured directly. ITGA5 might upregulate MMP-2 expression [37]. Our study demonstrates that ITGA5 knockdown in podoplanin-transfected OSCC cells reduced MMP-2 expression in fibroblasts in the direct co-culture. Several lines of evidence indicate that the main contribution to the MMP-2 upregulation resides in tumor cells, and might be due to the constitutively expressed, tumor-specific MMP-14. The interplay between CAFs and tumor cells contributes to the upregulation of ITGA5 in tumor cells, indicating that the MMP-2 increase only occurred in the presence of tumor cells.

Cancer-associated ECM is not only an integral feature of a tumor but also actively contributes to its behavior [38]. Similarly, the expression of matrix remodeling genes such as MMPs and collagen cross-linkers predicts poor prognosis for patients with cancer [39]. To identify whether CAFs enhance OSCC cell invasion, we performed Transwell and colony formation assays, and found that CAFs increased OSCC cell invasion and colony formation through TGF-β1 signaling. For the MMP-2 and MMP-14 upregulation in podoplanin-transfected OSCC cells, interaction between fibroblasts and carcinoma cells was required, where the involvement of the TGF-β1 pathway was recognized. TGF-β1 activates EGFR signaling in pancreatic cancer [40], and TGF-β binding to EGFR results in receptor phosphorylation and subsequent activation of the downstream PI3K/AKT and Ras/mitogen-activated protein kinase (MAPK) pathways [41]. We found that EGFR, AKT, and ERK were activated not only by podoplanin overexpression in OSCC cells, but also by co-culture with CAFs. This suggests that AKT and ERK activation through EGFR is an important signaling pathway in podoplanin/TGF-β1–mediated cancer cell invasion. As both fibroblast activation and podoplanin transfection increased TGF-β1 secretion, TGF-β1 plays an important role in the interaction between OSCC cells and CAFs. Fig. 7 depicts the suggested podoplanin/TGF-β1, MMP-2/MMP-14/ITGA5, and EGFR/AKT/ERK signaling regulating OSCC cell proliferation and invasion.

Fibroblast–carcinoma cell communication is bidirectional, and this study sought to address how OSCC cells might affect their surrounding fibroblasts in promoting tumor progression. Our data demonstrate that increased podoplanin in OSCC cells activates NFs by regulating TGF-β1 secretion. Podoplanin-positive OSCC cells promoted fibroblast invasion through MMP-2/MMP-14/ITGA5 signaling in direct co-culture but not in indirect co-culture. When OSCC cells were co-cultured with CAFs or NFs, TGF-β1 secreted from fibroblasts was involved in both podoplanin expression and OSCC cell invasion. Considering the results obtained, we conclude that podoplanin increases OSCC cell and fibroblast invasion by upregulating MMP-2, MMP-14, and ITGA5 in co-culture and that podoplanin overexpression in OSCC cells activates fibroblasts through the TGF-β1 paracrine pathways, leading to the activation of the EGFR, AKT, and ERK pathways in OSCC cells.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2018.04.029.

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