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Reciprocal activation of cancer-associated fibroblasts and oral squamous carcinoma cells through CXCL1

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ABSTRACT

Objectives: Crosstalk between cancer cells and carcinoma-associated fibroblasts (CAFs) is known to be involved in various aspects of tumor biology, including during invasion. Using oral squamous cell carcinoma (OSCC) cells as a model, we examined whether and how CAFs respond to inflammatory signals to influence cancer cell migration and invasion.

Materials and methods: Chemokine signatures within the human HNSCC datasets from The Cancer Genome Atlas (TCGA) were analyzed together with tissue assessment using immunohistochemical staining (IHC) and real-time PCR. A co-culture system was used to identify reciprocal effects exerted by CAFs and cancer cells upon one another. Recombinant CXCL1, CXCL1 neutralizing antibodies, and CXCR2 antagonist were used to confirm CXCL1/CXCR2 axis-mediated cell behaviors.

Results: Analysis of the TCGA dataset revealed that CXCL1 is associated with poor survival, and IHC demonstrated CXCL1 is highly expressed in OSCC stromal cells. Moreover, real-time PCR showed that in addition to CXCL1, IL-1β and CXCR2 are also highly expressed in OSCC and IL-1β mRNA levels positively correlate with CXCL1 expression. Furthermore, CAFs co-cultured with SAS, a poorly differentiated OSCC cell line, or stimulated with IL-1β exhibit increased CXCL1 secretion in an NF-κB-dependent manner. Treatment of SAS cells with CAF-conditioned medium or CXCL1 increased their invasion and migration capabilities, indicating a reciprocal activation between CAFs and cancer cells. Moreover, CXCL-1 upregulated matrix metalloprotease-1 (MMP-1) expression and activity in CAFs.

Conclusion: The induction of IL-1β following CXCL1 stimulation of CAFs mediates cancer cell invasion, and there is a reciprocal dependency between CAFs and cancer cells in the OSCC microenvironment.

Introduction

Local invasion and lymph node metastasis of oral squamous cell carcinoma (OSCC) are clinical factors considered when determining prognosis [1]. The pathology of metastasis is the invasion of cancer cells into the underlying stroma or extracellular matrix [2]. The invasive tumor front (ITF), a layer of 3–6 tumor cells or detached tumor cell groups at the advancing edge of the OSCC, exhibits distinct morphological and functional characteristics and is directly associated with the aggressiveness and metastasis of OSCC [3–5]. These include decreased expression of E-cadherin [6,7] and increased expression of laminin-5γ_2 and matrix metalloproteinases (MMPs) [8–10]. As our recent histopathological findings showed, the ITF, or the tumor-host interface, is intertwined with a bundle of stromal cells, such as fibroblasts, and infiltrating immune cells, primarily T lymphocytes, with distinct characteristics [11–13]. Crucial interactions between tumor and

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stromal cells at the ITF likely modulate tumor cell invasion [14].

Cancer pathogenesis events take place in imbalanced microenvironments, where pathological states not only affect tumor cells, but also stromal cells. Similar to other mucosal cancers, the OSCC microenvironment is inflammatory and embedded with complex communities of microbiota that trigger chronic immune cell infiltration due to the induction of inflammatory mediators like cytokines or chemokines [15,16]. We previously demonstrated increased expression of interleukin-1β (IL-1β), IL-6, and transforming growth factor (TGF)-β in OSCC, of which are cytokines essential for induction of T helper 17 (Th17) cells and T regulatory (Treg) cells [17]. In addition to T cells, cancer-associated fibroblasts (CAFs) are another stromal component of importance for tumorigenesis. Resident normal fibroblasts (NFs) can be educated and activated by tumor-derived cytokines such as TGF-β and IL-1β to become phenotypically and functionally distinguishable from CAFs [18–20]. In return, upregulated secretion of TGF-β, IL-6, and IL-8 from CAFs further enhances the inflammatory environment to promote OSCC progression [20–22]. Thus, reciprocal activation between CAFs and OSCC cells constitutes the essential basis for tumor growth, invasion, and metastasis. However, how such reciprocal activation is initiated and maintained remains elusive. The putative growth- and invasion-promoting effects of CAFs may be mediated through either direct heterotypic cell-cell contacts [23] or diffusible molecules including chemokines [24]. A previous study surveyed chemokine and chemokine receptor expression in head and neck squamous cell carcinoma (HNSCC) and found that CXCL1 was highly expressed in the vast majority of OSCC cell lines [25], although the specific role of CXCL1 in OSCC remained unclear. In other cancers CXCL1 has been suggested to be involved as a growth and anti-apoptotic factor or as a mediator of tumor invasion [26–28]. Moreover, expression of CXCL1 was also found to be upregulated in CAFs following co-culture with cancer cells or stimulation with inflammatory cytokines [29–31], although the effect of CAF-secreted CXCL1 on the aggressiveness of OSCC has not been fully elucidated.

In the present study, we investigated the activation of CAFs by OSCC cells, and the paracrine effect induced by CAFs in the tumor microenvironment of human OSCC. Our results show that tumor-derived IL-1β stimulates cancer-associated fibroblasts to produce CXCL1, which reciprocally promotes cancer cell migration and invasion.

Materials and methods

Clinical specimens and TCGA analysis

OSCC specimens for CAF isolation were obtained from patients undergoing surgery between 2009 and 2012 (N = 32). Normal gingival tissues for NF isolation were obtained from patients receiving routine third molar extraction (N = 10). The corresponding cancer tissue blocks were then retrieved from the pathology department of National Taiwan University Hospital (NTUH) for immunohistochemistry studies. The study was approved by the Institutional Review Board at NTUH, and a written informed consent had been obtained from patients. Data from 499 HNSCC patients was extracted from the TCGA dataset. This included RNA-Seq data (presented as fragments per kilobase of transcript per million mapped reads [FPKM]) and clinical data (survival status, disease-free status, and tumor source site). Average CXCL1 mRNA expression (52.7FPKM) was used as the cut-off point in Kaplan-Meir survival analysis.

Immunohistochemistry

Paraffin-embedded OSCC tissues were cut into 5-mm-thick slices. Immunostaining was performed using the avidin-biotin-peroxidase method. Colors were developed with diaminobenzidine (Dako) or PolyDetector HRP Blue (Bio SB) and sections were counterstained with Mayer's hematoxylin. Sections were stained with antibodies using anti-human IL-1β (bs-0812R; Bioss), anti-CXCL1 (500-P92; Peprotech), anti-α-smooth muscle actin (GTX100034; Genetex), and anti-CXCR2 (Ab14935; Abcam). Ab titrations and isotype control Ab staining were used to determine optimal staining conditions.

Isolation of RNA and reverse transcription PCR (RT-PCR) analysis

Total RNA was isolated from normal tissues, tumor tissues, or cells using NucleoZOL reagent according to the manufacturer's instructions (Macherey-Nagel). Extracted RNA was converted into cDNA using Superscript IV (Invitrogen) and random hexamers. PCR was performed at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, for a total of 24 cycles in the presence of GAPDH and MMP-1 primers (5′-AAGCGTGTGACAG TAAGCTA-3′ and 5′-AACCGGACTTCATCTCTG-3′).

Quantitative real-time PCR

RNA extraction and reverse transcription were performed as described above. The quantitative real-time PCR was performed in a Bio-Rad CFX Connect™ Real-Time PCR Detection System machine in the presence of GAPDH, IL-1β, CXCL1, CXCR2, and COX-2 primers (Genomics). Target gene transcription levels were measured and normalized to GAPDH expression. The following primer sequences were used: GAPDH, 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGT GATGGGATTTC-3′; IL-1β, 5′-TCAGCCAATCTTCATTGCTCAA-3′ and 5′-TGGCGAGCTCAGGTACTTCTG-3′; CXCL1, 5′-AACCGAAGTCATAGC CACAC-3′ and 5′-GTTGGATTTGTCACTGTTCAGC-3′; CXCR2, 5′-ATCT ATGCCCTGGTATTCCTG-3′ and 5′-GGTAACGGTCCACACTGATG-3′; COX-2, 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′ and 5′-AGATCAT CTCTGCCTGAGTATCTT-3′.

Cancer cell line and fibroblast culture

SAS, established from a patient with poorly differentiated tongue cancer, was obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank. The cell line was authenticated and characterized by the supplier and sent for STR matching analysis by Mission Biotech (Taipei, Taiwan). Cells were cultured in Dulbecco's Modified Eagle's Medium (HyClone) with 10% heat-inactivated fetal bovine serum (Biological Industries) at 37 °C and 5% CO_2. To isolate the stromal cells from tissues, the specimen was minced and semi-purified by Percoll (Sigma) gradient. NFs or CAFs were then cultured from the stromal layer and used in experiments within 10 passages. NFs or CAFs were cultured at 3×10^5 cells/well in 6-well plates and starved in 1% FBS DMEM for 24 h to collect the conditioned medium [32] and defined as NF-CM or CAF-CM.

CAF activation and cytokine analysis

CAFs or NFs were treated with or without 5 ng/ml IL-1β in the presence or absence of 10 or 25 μM anti-oxidant Pyrrolidine dithiocarbamate (PDTC), IL-1β neutralizing antibody (1:100 dilution), IL-1 receptor antagonist (10 μM), COX inhibitors Indomethacin (2 μM), Aspirin (500 μM), or Prostaglandin E2 (500 μM) for 6 h. The culture supernatant was collected to analyze CXCL1 level by ELISA (R&D Systems) and the cells were harvested for gene analysis by real-time PCR.

Transwell co-cultures of CAF and OSCC cells

For the transwell non-contact co-culture system, CAFs or NFs were plated into six-well culture plates, and SAS cells were seeded onto Transwell® permeable inserts (Costar, 24-mm diameter, 0.4-μm pores) placed on top of a six-well plate. The next day, media were replaced with fresh serum-free media and the SAS inserts were moved onto the six-well plate where NFs or CAFs were seeded. After 24 h of co-culture,

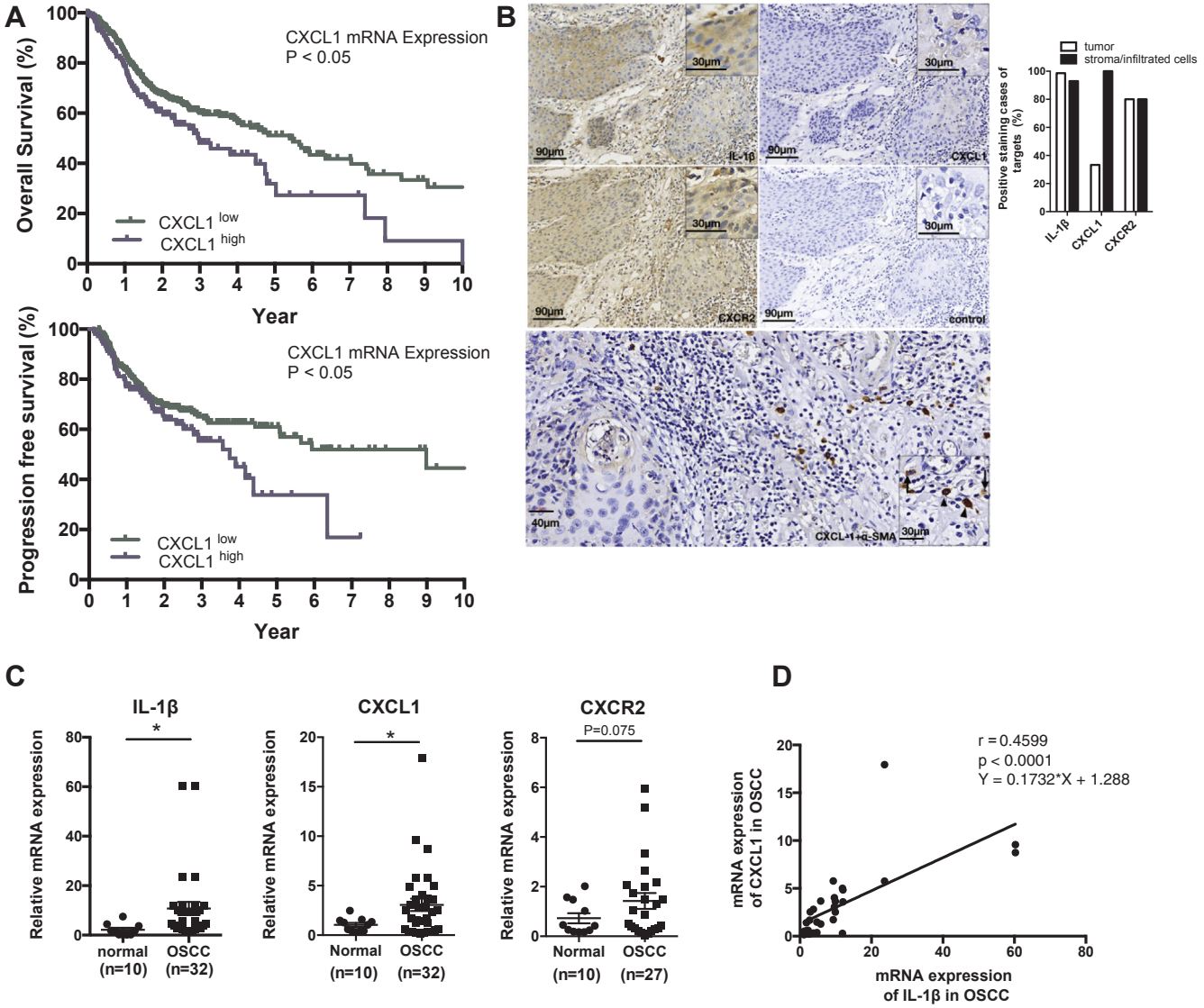

Fig. 1. IL-1β, CXCL1, and CXCR2 are expressed in OSCC tissues. (A) Overall survival (OS) and progression-free survival (PFS) of 350 patients with high CXCL1 expression and 141 with low expression. Results were determined to be statistically significant by log-rank test ($P < 0.05$). Average expression was used as the cut-off point. (B) Representative images of IL-1β, CXCL1, and CXCR2 staining in OSCC tissues. A scale bar and the magnification are shown in each figure. Colocalization of α-smooth muscle actin (grey blue) and CXCL1 (brown). Arrow: cells with CXCL1 staining only, arrow head: colocalized cells. Percentage of positive cases for each antibody is shown. (C) Expression of IL-1β and CXCL1, but not CXCR2 (C, $P = 0.075$), mRNAs were significantly upregulated in OSCC tissues compared to normal gingival tissues ($P < 0.05$). (D) Positive correlation of IL-1β mRNA and CXCL1 mRNA expression in OSCC cancer ($n = 30$) ($p < 0.01$; Spearman rho 0.4599).

the media were collected and defined as NF(co)-CM and CAF(co)-CM for further use in invasion assays and ELISA. The attached cells were also harvested for RT-PCR analysis.

Migration and invasion assays

Transwell® (Costar, 6.5-mm diameter, 8-μm pores) were used to evaluate the migration or invasion capacities of OSCC cells *in vitro*. For cell migration, 1×10^5 SAS cells in 300 μl of serum-free media were seeded into the upper chamber. Conditioned media from NFs or CAFs (chemoattractant) were added to the lower chamber, and the plates were incubated for 16 h at 37 °C in 5% CO_2. For cell invasion, the transwell inserts were coated with growth factor-reduced Matrigel (Corning). The cells that did not penetrate were removed from the top of the filters by wiping with a Q-tip. The cells that penetrated were fixed with 4% paraformaldehyde, stained with 0.5% crystal violet (Sigma), and photographed with an inverted microscope (40× and 100× magnification). The surface areas of penetrated cells were assessed in five randomized high power fields under a microscope and areas were calculated using ImageJ software, and were considered to represent the migration or invasion activity.

Wound healing assay

Cell mobility under CXCL1 stimulation was assessed by an *in vitro* wound healing assay. 80 μl of 4×10^5/ml cells were seeded into a 2-well silicone insert (Ibidi, Germany) with a defined 400 μm cell-free gap, and cultured for overnight. The silicone inserts were removed and washed twice with phosphate-buffered saline (PBS). The cells were then treated with NF-CM or CAF-CM with or without CXCL1 (Peprotech; 5, 10, 50, 100 ng/ml), IL-8 neutralizing antibodies (R&D; 1, 10 μg/ml), CXCL1 neutralizing antibodies (R&D; 2, 10 μg/ml), or CXCR2 antagonist SB 225002 (Merck; 50, 500, 1000 nM). Images of the wounds were taken in the beginning and at 5 h after culture and measured using ImageJ software to calculate the mean and standard deviation. Cell migration was expressed as the wound recovery ratio: (original gap

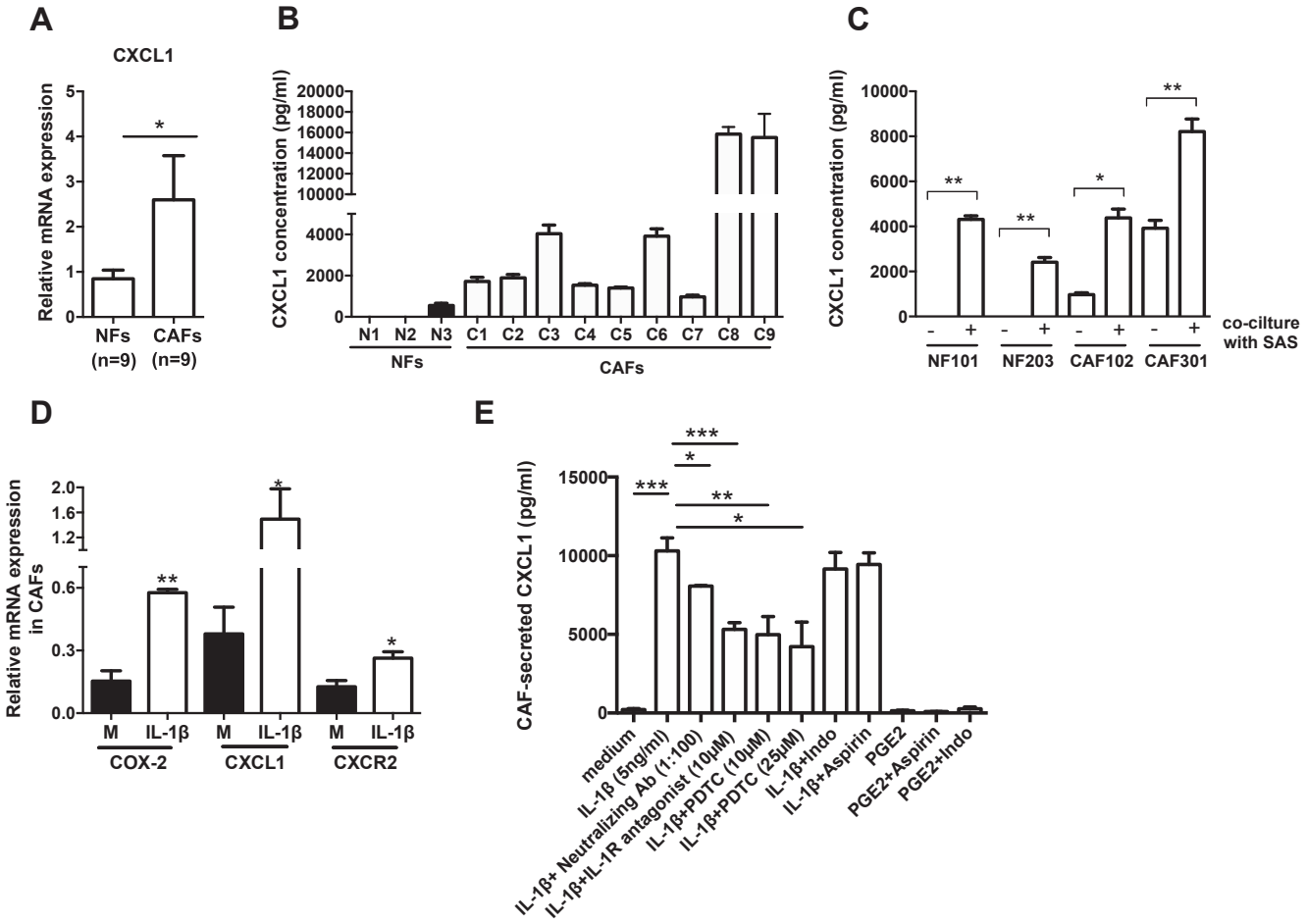

Fig. 2. SAS-conditioned medium and IL-1β enhance CXCL1 production in both normal and cancer-associated fibroblasts. (A) CXCL1 mRNA expression was significantly upregulated in isolated cancer-associated fibroblasts compared to normal fibroblasts ($P < 0.05$). These data are presented as fold related to *gapdh*. (B) CXCL1 secretion (pg/ml) from normal fibroblasts (NFs) of three healthy individuals and cancer-associated fibroblasts (CAFs) of nine patients. Data are presented as mean ± SEM. (C) Changes in CXCL1 secretion of NFs and CAFs after co-culture with SAS cells. (D) Expression of *cxcl1, cxcr2,* and *cox-2* mRNAs in CAFs following IL-1β stimulation (5 ng/ml). (E) Increased secretion of CXCL1 by CAFs following IL-1β stimulation ($P < 0.001$) and inhibition of CXCL1 secretion following treatment with IL-1β neutralizing antibody, IL-1R antagonist, and NF-kB inhibitor (PDTC) ($P < 0.05$).

width - new width)/original gap width.

Western blotting

Cells were washed twice with PBS and dissolved in lysis buffer (Promega) supplemented with protease inhibitor cocktail (Sigma). Approximately 30 μg of protein was denatured and electrophoresed in 10% SDS-PAGE followed by transfer to PVDF membranes, which were then incubated with anti-MMP-1 antibody (Genetex; 1:1000) diluted in 5% BSA/TBST overnight at 4 °C. HRP-conjugated secondary antibodies (Genetex) were diluted 1:10,000 in 5% BSA/TBST and incubated for 2 h at room temperature. Blots were developed using chemiluminescent substrate kit (T-Pro), and analyzed with a Bio-Rad Gel Doc/Chemi Doc Imaging System and Image Lab software. Results of multiple assays were quantified by digitizing the data and normalizing the pixel density of the examined protein against the GAPDH-specific pixel density.

Statistical analysis

All data are presented as the mean ± SEM from at least three independent experiments, unless specifically noted. Statistical analysis was performed using one-way analysis and the Student's *t*-test using Graphpad Prism 6 software (GraphPad Software). Differences with P values < 0.05 were considered statistically significant.

Results

CXCL1 is expressed in OSCC tissues and is associated with patient survival

To test our hypothesis that tumor-secreted IL-1β recruits and activates CAFs to produce CXCL1, we examined the expression of IL-1β, CXCL1, and CXCR2 by immunohistochemical (IHC) staining. We previously demonstrated that the mRNA levels of pro-inflammatory cytokines are upregulated in OSCC tissues [17], including IL-1β, which regulates fibroblast activation [33,34] and stimulates production of CXCL1 in the tumor microenvironment [35]. Analysis of the TCGA dataset revealed that of the chemokines highly expressed in HNSCC, CXCL1 alone was associated with poor survival (Fig. 1A; Supplementary Fig. 1). As shown in Fig. 1B, IL-1β was highly expressed at the tumor site, and CXCL1 was detected at 33% of tumor sites and in 100% of the stromal or infiltrating cells in OSCC tissues. Correspondingly, the CXCL1 receptor, CXCR2, was found to be expressed in 80% of tumor cells and 80% of stromal cells. Hematoxylin and eosin staining of OSCC tissues is shown in Supplementary Fig. 2. To identify the CXCL1 expressing cells in the stroma, we also examined CAFs by costaining for CXCL1 and α-SMA. The colocalization of CXCL1 (brown color) and α-SMA (grey blue color) indicated that a majority of stromal cells expressing CXCL1 are CAFs (Fig. 1B, lower right inset, cells with arrowhead). These data suggested that in OSCC tissues IL-1β is highly

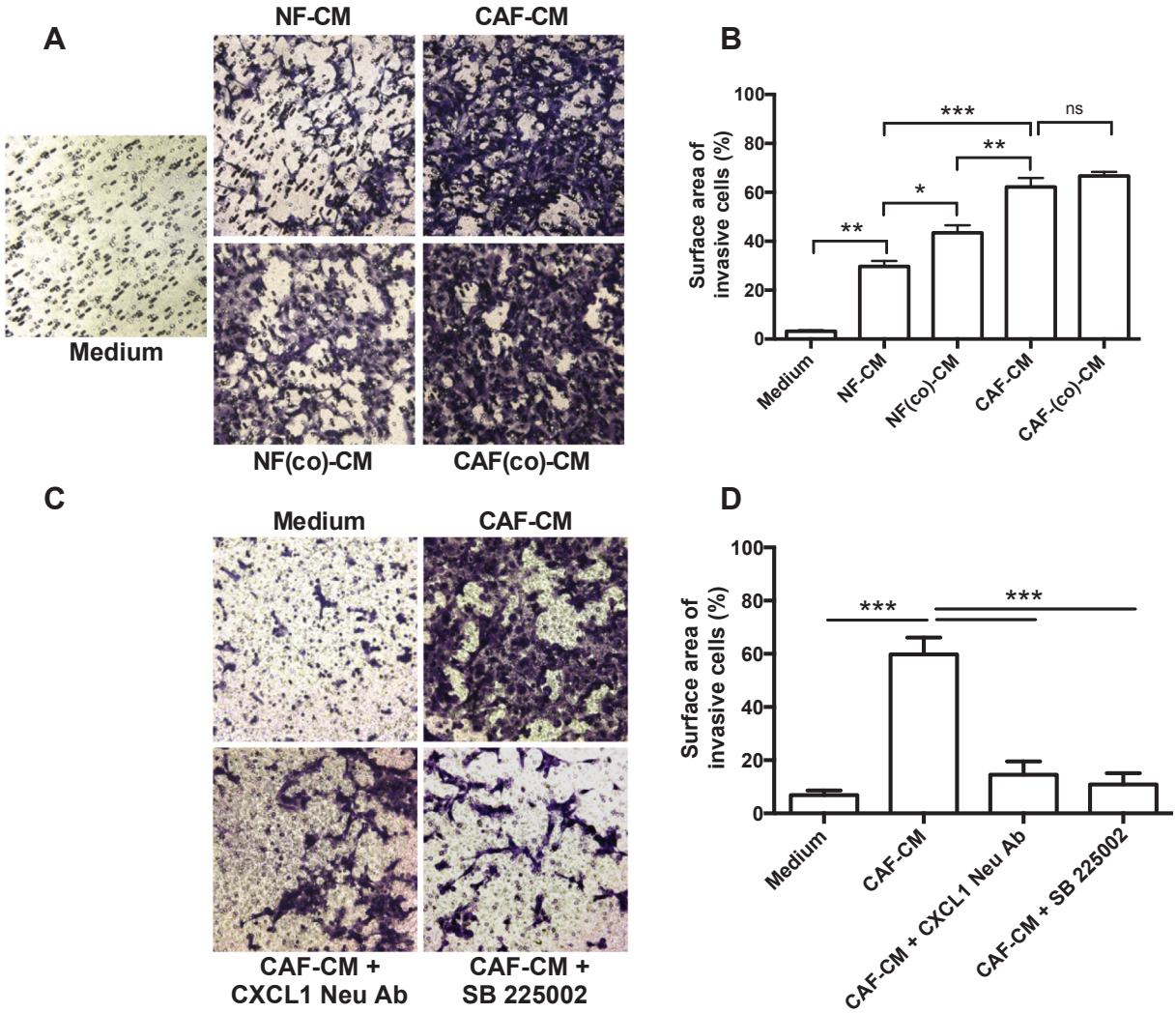

Fig. 3. Alterations in the invasive ability of SAS cells following indirect co-culture with cancer-associated fibroblasts (CAFs) or normal fibroblasts (NFs). (A) For transwell invasion analysis, SAS cells were incubated in the upper transwell chambers for 16 h and conditioned medium from NFs or CAFs was added to the lower chamber. Cells penetrating the Matrigel coating were stained with crystal violet and examined using the high-power field (×100) of a light microscope. (B) Statistical analysis of the mean number of invading cells. (C) SAS cells were pretreated with the CXCR2 antagonist SB 225002 (1000 nM) in the upper chamber or CXCL1 neutralizing antibody was added in conditioned medium to the lower chamber to block CXCL1 signaling. Invading SAS cells were quantitated. (D) Each experiment was carried out in triplicate.

expressed in both tumor and stromal cells, while CXCL1 is mainly expressed in stroma cells including CAFs. Further, both tumor and stromal cells showed positive staining of CXCR2, indicating their potential to respond to CXCL1 stimulation.

Expression of CXCL1 mRNA is upregulated in OSCC tissue and positively correlates with IL-1β expression

To investigate the biological role of IL-1β and CXCL1 in OSCC, their expression in OSCC tissues and normal gingival tissues was examined by quantitative real-time PCR. As represented in Fig. 1C, both IL-1β and CXCL1 were significantly upregulated in OSCC tissues compared with normal tissues ($P < 0.05$). Together with the IHC results (Fig. 1B) this suggests that CAFs are the major producers of CXCL1. Consistent with this assumption, there was a positive correlation between IL-1β and CXCL1 mRNA levels in OSCC (Spearman's rho = 0.4599, $P < 0.01$) (Fig. 1D). Data from The Cancer Genome Atlas (TCGA) was also consistent with our findings (Supplementary Fig. 3, Spearman's rho = 0.52, $P < 0.01$). These data support our hypothesis that tumor-secreted IL-1β mediates the recruitment and activation of CAFs, which produce CXCL1.

Tumor-derived IL-1β enhances CXCL1 production in CAFs

We next isolated and cultured normal fibroblasts (NFs) from gingiva tissues and CAFs from OSCC tissues to characterize their differences. CXCL1 mRNA levels were higher in CAFs compared with the resting NFs (Fig. 2A). While CXCL1 levels in the culture supernatant of NFs and CAFs varied by individual, CAFs were prone to produce high amounts of CXCL1 (Fig. 2B). In addition, immunofluorescence revealed higher expression of CXCL1, α-SMA, vimentin, and prolyl-4-hydroxylase (Supplementary Fig. 4) in CAFs than in NFs, suggesting that CAFs were more active than NFs. To examine whether cancer cells affect the activity of fibroblasts, we co-cultured isolated NFs and CAFs with SAS cells and found that CXCL1 secretion was significantly increased from both NFs and CAFs (Fig. 2C, $P < 0.05$) following stimulation with SAS. Because CAFs constitutively express the IL-1 receptor (Supplementary Fig. 5), IL-1β might be the key mediator modulating CXCL1 production. Thus, we treated isolated CAFs with recombinant IL-1β to mimic the tumor microenvironment. Interestingly, CXCL1, CXCR2, and COX-2 were significantly upregulated in CAFs treated with IL-1β (Fig. 2D, $P < 0.05$), indicating that IL-1β and prostaglandin signaling might mediate CXCL1 production in CAFs. Therefore, we further treated CAFs

with the IL-1β neutralizing antibody, IL-1 receptor antagonist, NFκB inhibitor (PDTC), or COX inhibitors including aspirin and indomethacin and measured their CXCL1 secretion. As presented in Fig. 2E, secretion of CXCL1 in CAFs was significantly elevated upon stimulation with IL-1β ($P < 0.001$), and could be reduced by IL-1β neutralizing antibody ($P < 0.05$) and IL-1 receptor antagonist ($P < 0.001$). Also, PDTC decreased CXCL1 secretion in a dose-dependent manner ($P < 0.05$). COX inhibitors, aspirin, and indomethacin exhibited no effects on IL-1β-induced CXCL1 in CAFs. Consistent with our findings, the proangiogenic regulator PGE2, thought to be an inducer of CXCL1 production in colorectal cancer [36], had no effect on CXCL1 production in CAFs isolated from OSCC tissues. These results demonstrate that tumor-derived IL-1β may activate CAFs to produce CXCL1 in the tumor microenvironment in an NFκB, but not prostaglandin, -dependent manner.

Tumor-enhanced CXCL1 secretion from CAFs reciprocally stimulates tumor cell invasion

As mentioned in Fig. 2C, secretion of CXCL1 was elevated in both NFs and CAFs co-cultured with SAS. Therefore, we co-cultured NFs or CAFs with SAS cells and collected their conditioned medium (defined as NF(co)-CM and CAF(co)-CM, which differed from the pure NF-CM and CAF-CM). Using the stromal–epithelial co-culture strategy, SAS cells were seeded into the upper chamber and conditioned medium from stimulated/unstimulated NFs or CAFs was applied to the lower chamber. This assay demonstrated that the invasive ability of SAS increased significantly following incubation with CAF-CM, compared to medium only and NF-CM (Fig. 3A and B, $P < 0.05$). Moreover, the invasion-promoting effect of NF(co)-CM was greater than that of NF-CM, indicating the reciprocal activation of tumor and stromal cells. Thus, SAS promoted CXCL1 secretion by fibroblasts, and in return, fibroblasts augmented SAS cell invasion. As demonstrated in Fig. 3C and D, the invasion-promoting effect of CAF-CM could be diminished by either CXCL1 neutralizing antibody or the CXCR2 antagonist SB 225002, confirming the importance of CXCL1 in tumor invasion.

Targeting CXCL1 suppresses CAF-augmented cancer cell migration

CAF-CM also promotes SAS cell migration, as demonstrated in a transwell assay without Matrigel coating (Fig. 4A and B). To determine whether CXCL1 could mediate crosstalk between tumor cells and CAFs in the OSCC microenvironment and further regulate the phenotype of tumor cells, recombinant CXCL1 was added to wound healing migration assays. Addition of CXCL1 resulted in a dose-dependent increase in cell migration of SAS cells (Fig. 4C and D). To confirm that CXCL1 acts through binding to CXCR2, SB 225002, the first non-peptide antagonist of CXCR2, was used for blocking. As shown in Fig. 4E, SB 225002 significantly inhibited the induction of SAS cell migration by CXCL1. Similarly, conditioned medium from CAFs also increased cell migration, an effect that decreased in a dose-dependent manner following treatment with SB 225002 (Fig. 4F). To test whether IL-8, another ligand of CXCR2, regulates cell migration, anti-IL-8 (1, 10 μg/ml), anti-CXCL1 (2, 10 μg/ml), and combination antibodies were added to CAF-CM. Anti-CXCL1 antibodies significantly reduced the induction of migration by CAF-CM (Fig. 4G, $P < 0.05$), while anti-IL-8 and combination antibodies did not affect cell migration. These findings indicate that CXCL1 is a key factor in CAF-CM contributing to OSCC cell migration.

CXCL1-induced MMP-1 gene expression in SAS and CAFs

Matrix metalloproteases (MMPs), important factors involved in the degradation of extracellular matrix leading to tumor metastasis, are upregulated in fibroblasts co-cultured with OSCC cells [37] and increase tumor progression in an orthotopic mouse model [38]. Following CXCL1 or SAS-CM stimulation, MMP-1 was significantly induced in CAFs and SAS at both the mRNA and protein levels (Fig. 5A and B). Further, addition of the CXCR2 antagonist SB 225002 to CXCL1 or SAS-CM treated CAFs resulted in a decrease of MMP1 levels (Fig. 5C). These results indicate that MMP-1 might be the downstream effector of CXCL1/CXCR2-mediated invasion in the OSCC microenvironment. A schematic representation of this study is shown in Fig. 5D.

Discussion

CAFs have been reported to contribute to a tumor-permissive inflammatory environment, and display tumor-promoting and prometastatic properties including production of angiogenic factors and MMPs [39]. Although the crosstalk and interactions between stromal and cancer cells have been extensively studied, the complete picture remains obscure, especially in OSCC. OSCC induces an inflammatory microenvironment comprised of cytokines, chemokines, and antitumor immune responses that may not only modulate cancer progression, but also affect invasiveness and metastasis [40,41]. Therefore, the molecular mechanisms underlying the effects of stromal cells, like CAFs, on aggressiveness in OSCC needed to be determined in order to improve treatment strategies.

As illustrated above, cytokines and chemokines exert strong activity in tumor cells and in the crosstalk between tumor cells and their host microenvironment. In previous studies, the recruitment of stromal cells was found to be regulated by tumor cell-derived molecules [42]. We hypothesized that IL-1β signaling from OSCC cells mediated the induction/recruitment of CAFs. To this end, we found that IL-1β is expressed in tumor cells and significantly increases CXCL1 production by CAFs in a paracrine manner. Similarly, Lee et al reported that IL-1β induces CXCL1 production, which in turn activates EGFR through CXCR2 causing an autocrine proliferative response of oral premalignant cells [35]. We also found positive CXCL1 staining on tumor cells from several OSCC patients. This is similar to studies of bladder cancer, where elevated CXCL1 expression with enhanced recruitment of CAFs was observed [43]. It has been well established that the cancer-secreted CXCL1 promotes tumor progression and mediates angiogenesis [36,44]. Recently it was shown that tumor cell–secreted CXCL1 promoted lung cancer growth via recruitment of neutrophils into the tumor *in vivo* [45] and CXCL1 recruited CXCR2-positive myeloid-derived suppressor cells (MDSCs) to form a premetastatic niche that ultimately promoted liver metastases [44]. Also, silencing expression of CXCL1 resulted in decreased proliferation and migration of gastric cancer cells and induce apoptosis [46].

In our study, the chemokine CXCL1 and its receptor, CXCR2, were highly expressed in CAFs compared with NFs. Moreover, the NFs co-cultured with cancer cells could release high concentrations of CXCL1. These findings are supported by a previous study of OSCC that revealed CXCL1 is derived from NFs following exposure to OSCC cells, and induces senescence of CAFs via an autocrine loop [47]. Further, the experiments with conditioned medium and artificially enriched medium, in which purified CXCL-1 was added, demonstrate that CXCL1 is able to affect the migration and invasion status of OSCC cells. Supporting this concept, the biological activity of these cells was blocked by monoclonal antibodies against CXCL1 and its receptor as well as receptor antagonists. In addition, CAFs-secreted CXCL1 contributes to radioresistance in esophageal squamous cell carcinoma [48], indicating that CXCL1 may not only be a target for regulation of cell invasion but also to overcome cancer radioresistance.

In summary, IL-1β secreted by primary tumor cells stimulates cancer-associated fibroblasts to produce CXCL1, which promotes cancer cell migration and invasion by directly targeting cancer cells through the CXCR2 receptor, both on cancer cells and CAFs, resulting in production of MMP1 in the tumor microenvironment. These findings not only shed light on how primary tumor-derived cytokines drive activation of CAFs, but also provide insight into how CAFs contribute to cancer migration and invasion.

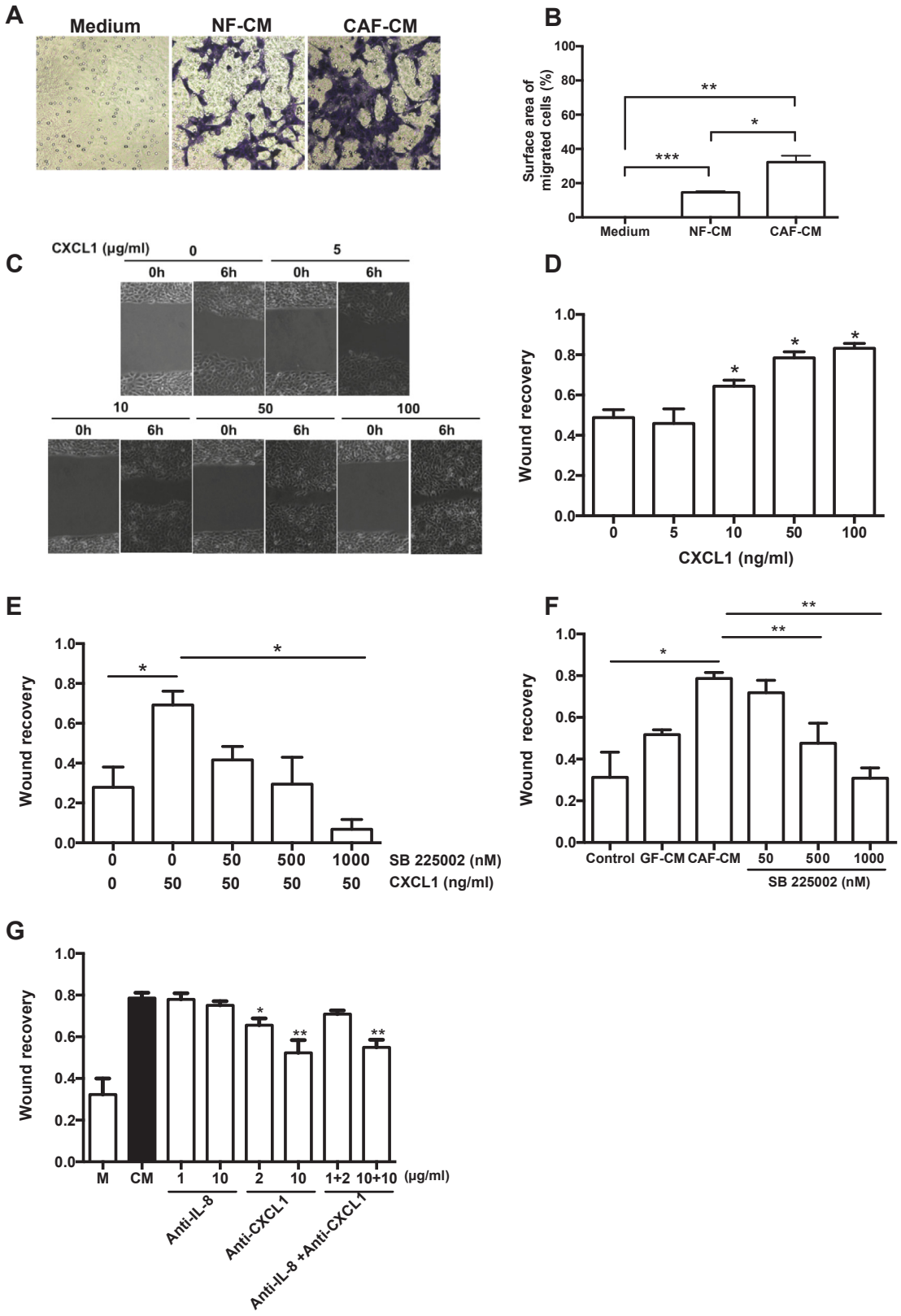

Fig. 4. CXCL1 promotes oral cancer cell migration. (A) The effect of CAF-conditioned medium on SAS migration in a transwell assay without Matrigel coating and (B) the mean number of migrating cells under each condition. (C) Effects of CXCL1 on cell migration as determined by wound-healing assay. SAS cells were seeded in an Ibidi culture insert placed on top of a 6-well plate. Cell migration toward the gap area was photographed 5 h after changing to culture medium containing different doses of CXCL1. (D) Quantitative results of the migration assay. (E) The CXCR2 antagonist, SB 225002, inhibited CXCL1-induced cancer cell migration in a dose-dependent manner. (F) Cancer cell migration was accelerated by incubation with conditioned medium from CAFs and this effect was inhibited by SB 225002. (G) Neutralization of CXCL1 suppresses the CAF-augmented migration ability of SAS cells. The migration results are represented as a wound recovery ratio (0–1). Each experiment was carried out in duplicate.

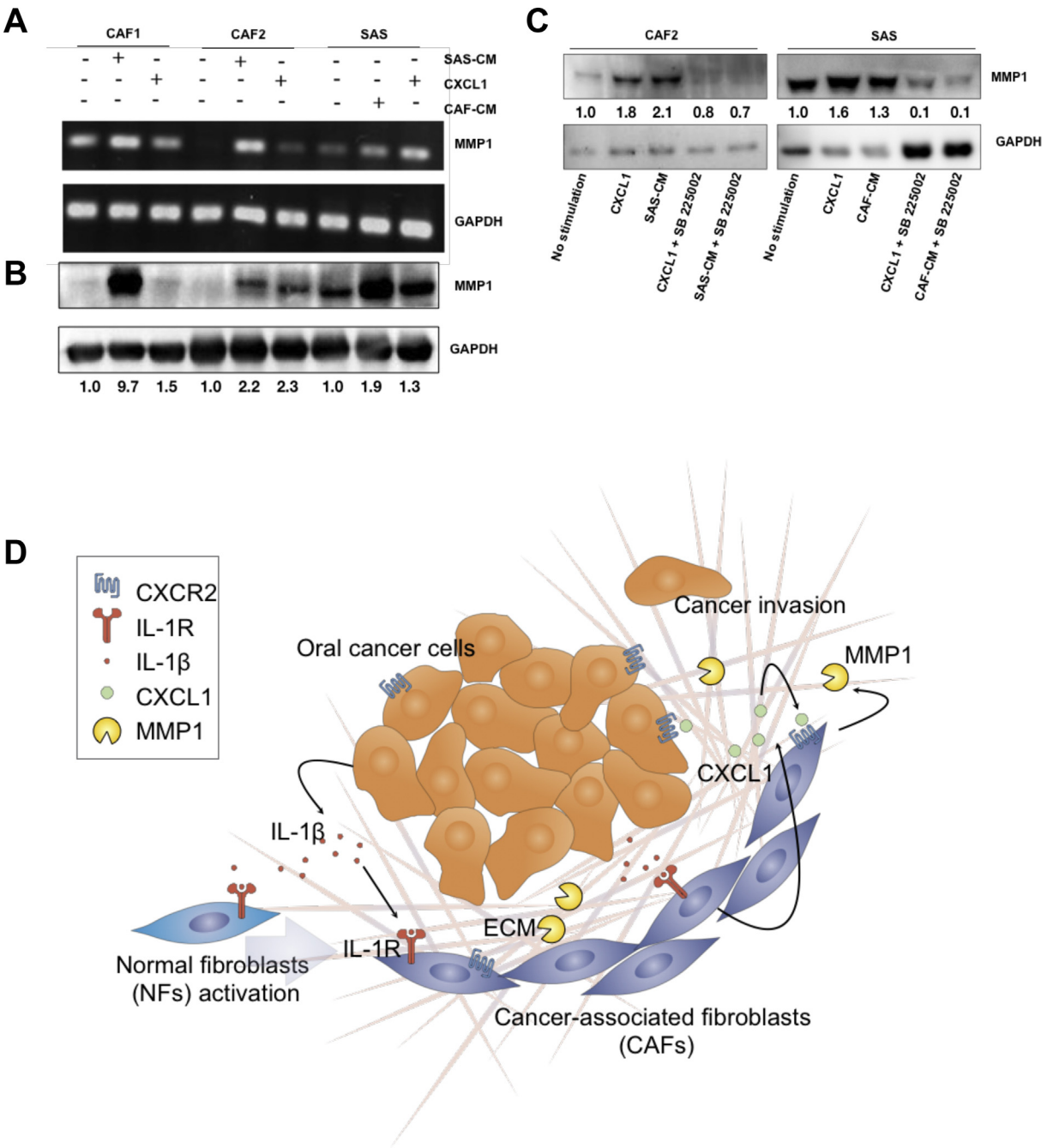

Fig. 5. CXCL1 and SAS-conditioned medium promote MMP1 expression. RT-PCR (A) and western blot analyses (B) of MMP-1 in CAFs following a 24 h treatment with CXCL1 or SAS-conditioned medium or in SAS after a 24 h treatment with CXCL1 or CAF-conditioned medium. GAPDH was used as a loading control and 30 μg of total protein were loaded per lane. (C) Western blot analyses of MMP-1 secretion in CAF-conditioned medium following treatment with SB 225002. (D) Schematic representation of this study.

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Conflicts of interest

None declared.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2018.11.002.

References

[1] Jan JC, Hsu WH, Liu SA, Wong YK, Poon CK, Jiang RS, et al. Prognostic factors in patients with buccal squamous cell carcinoma: 10-year experience. J Oral

Maxillofac Surg 2011;69:396–404.
[2] Clark AG, Vignjevic DM. Modes of cancer cell invasion and the role of the microenvironment. Curr Opin Cell Biol 2015;36:13–22.
[3] Bankfalvi A, Piffko J. Prognostic and predictive factors in oral cancer: the role of the invasive tumour front. J Oral Pathol Med 2000;29:291–8.
[4] Kurokawa H, Zhang M, Matsumoto S, Yamashita Y, Tanaka T, Tomoyose T, et al. The relationship of the histologic grade at the deep invasive front and the expression of Ki-67 antigen and p53 protein in oral squamous cell carcinoma. J Oral Pathol Med 2005;34:602–7.
[5] Piffko J, Bankfalvi A, Ofner D, Rasch D, Joos U, Schmid KW. Standardized demonstration of silver-stained nucleolar organizer regions-associated proteins in archival oral squamous cell carcinomas and adjacent non-neoplastic mucosa. Mod Pathol 1997;10:98–104.
[6] Shinohara M, Hiraki A, Ikebe T, Nakamura S, Kurahara S, Shirasuna K, et al. Immunohistochemical study of desmosomes in oral squamous cell carcinoma: correlation with cytokeratin and E-cadherin staining, and with tumour behaviour. J Pathol 1998;184:369–81.
[7] Wang X, Zhang J, Fan M, Zhou Q, Deng H, Aisharif MJ, et al. The expression of E-cadherin at the invasive tumor front of oral squamous cell carcinoma: immunohistochemical and RT-PCR analysis with clinicopathological correlation. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009;107:547–54.
[8] Ikebe T, Shinohara M, Takeuchi H, Beppu M, Kurahara S, Nakamura S, et al. Gelatinolytic activity of matrix metalloproteinase in tumor tissues correlates with the invasiveness of oral cancer. Clin Exp Metastasis 1999;17:315–23.
[9] Kurahara S, Shinohara M, Ikebe T, Nakamura S, Beppu M, Hiraki A, et al. Expression of MMPS, MT-MMP, and TIMPs in squamous cell carcinoma of the oral cavity: correlations with tumor invasion and metastasis. Head Neck 1999;21:627–38.
[10] Berndt A, Borsi L, Hyckel P, Kosmehl H. Fibrillary co-deposition of laminin-5 and large unspliced tenascin-C in the invasive front of oral squamous cell carcinoma in vivo and in vitro. J Cancer Res Clin Oncol 2001;127:286–92.
[11] Balkwill FR, Capasso M, Hagemann T. The tumor microenvironment at a glance. J Cell Sci 2012;125:5591–6.
[12] Lee JJ, Kao KC, Chiu YL, Jung CJ, Liu CJ, Cheng SJ, et al. Enrichment of human CCR6(+) regulatory T cells with superior suppressive activity in oral cancer. J Immunol 2017;199:467–76.
[13] Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Nat Rev Cancer 2009;9:239–52.
[14] Friedl P, Alexander S. Cancer invasion and the microenvironment: plasticity and reciprocity. Cell 2011;147:992–1009.
[15] Francescone R, Hou V, Grivennikov SI. Cytokines, IBD, and colitis-associated cancer. Inflamm Bowel Dis 2015;21:409–18.
[16] Kramer CD, Genco CA. Microbiota, immune subversion, and chronic inflammation. Front Immunol 2017;8:255.
[17] Lee JJ, Chang YL, Lai WL, Ko JY, Kuo MY, Chiang CP, et al. Increased prevalence of interleukin-17-producing CD4(+) tumor infiltrating lymphocytes in human oral squamous cell carcinoma. Head Neck 2011;33:1301–8.
[18] Erez N, Truitt M, Olson P, Arron ST, Hanahan D. Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-kappaB-dependent manner. Cancer Cell 2010;17:135–47.
[19] Hassona Y, Cirillo N, Lim KP, Herman A, Mellone M, Thomas GJ, et al. Progression of genotype-specific oral cancer leads to senescence of cancer-associated fibroblasts and is mediated by oxidative stress and TGF-beta. Carcinogenesis 2013;34:1286–95.
[20] Kellermann MG, Sobral LM, da Silva SD, Zecchin KG, Graner E, Lopes MA, et al. Mutual paracrine effects of oral squamous cell carcinoma cells and normal oral fibroblasts: induction of fibroblast to myofibroblast transdifferentiation and modulation of tumor cell proliferation. Oral Oncol 2008;44:509–17.
[21] Mirkeshavarz M, Ganjibakhsh M, Aminishakib P, Farzaneh P, Mahdavi N, Vakhshiteh F, et al. Interleukin-6 secreted by oral cancer- associated fibroblast accelerated VEGF expression in tumor and stroma cells. Cell Mol Biol (Noisy-le-grand) 2017;63:131–6.
[22] Bae JY, Kim EK, Yang DH, Zhang X, Park YJ, Lee DY, et al. Reciprocal interaction between carcinoma-associated fibroblasts and squamous carcinoma cells through interleukin-1alpha induces cancer progression. Neoplasia 2014;16:928–38.
[23] Hazan RB, Kang L, Whooley BP, Borgen PI. N-cadherin promotes adhesion between invasive breast cancer cells and the stroma. Cell Adhes Commun 1997;4:399–411.
[24] Orimo A, Tomioka Y, Shimizu Y, Sato M, Oigawa S, Kamata K, et al. Cancer-associated myofibroblasts possess various factors to promote endometrial tumor progression. Clin Cancer Res 2001;7:3097–105.
[25] Wolff HA, Rolke D, Rave-Frank M, Schirmer M, Eicheler W, Doerfler A, et al. Analysis of chemokine and chemokine receptor expression in squamous cell carcinoma of the head and neck (SCCHN) cell lines. Radiat Environ Biophys 2011;50:145–54.
[26] Benelli R, Stigliani S, Minghelli S, Carlone S, Ferrari N. Impact of CXCL1 overexpression on growth and invasion of prostate cancer cell. Prostate 2013;73:941–51.
[27] Ogata H, Sekikawa A, Yamagishi H, Ichikawa K, Tomita S, Imura J, et al. GROalpha promotes invasion of colorectal cancer cells. Oncol Rep 2010;24:1479–86.
[28] Yang G, Rosen DG, Zhang Z, Bast Jr. RC, Mills GB, Colacino JA, et al. The chemokine growth-regulated oncogene 1 (Gro-1) links RAS signaling to the senescence of stromal fibroblasts and ovarian tumorigenesis. Proc Natl Acad Sci USA 2006;103:16472–7.
[29] Kogan-Sakin I, Cohen M, Paland N, Madar S, Solomon H, Molchadsky A, et al. Prostate stromal cells produce CXCL-1, CXCL-2, CXCL-3 and IL-8 in response to epithelia-secreted IL-1. Carcinogenesis 2009;30:698–705.
[30] Tjomsland V, Spangeus A, Valila J, Sandstrom P, Borch K, Druid H, et al. Interleukin 1alpha sustains the expression of inflammatory factors in human pancreatic cancer microenvironment by targeting cancer-associated fibroblasts. Neoplasia 2011;13:664–75.
[31] Jung DW, Che ZM, Kim J, Kim K, Kim KY, Williams D, et al. Tumor-stromal crosstalk in invasion of oral squamous cell carcinoma: a pivotal role of CCL7. Int J Cancer 2010;127:332–44.
[32] St John MA, Li Y, Zhou X, Denny P, Ho CM, Montemagno C, et al. Interleukin 6 and interleukin 8 as potential biomarkers for oral cavity and oropharyngeal squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 2004;130:929–35.
[33] Dudas J, Fullar A, Bitsche M, Schartinger V, Kovalszky I, Sprinzl GM, et al. Tumor-produced, active interleukin-1beta regulates gene expression in carcinoma-associated fibroblasts. Exp Cell Res 2011;317:2222–9.
[34] Elias JA, Freundlich B, Kern JA, Rosenbloom J. Cytokine networks in the regulation of inflammation and fibrosis in the lung. Chest 1990;97:1439–45.
[35] Lee CH, Syu SH, Liu KJ, Chu PY, Yang WC, Lin P, et al. Interleukin-1 beta transactivates epidermal growth factor receptor via the CXCL1-CXCR2 axis in oral cancer. Oncotarget 2015;6:38866–80.
[36] Wang D, Wang H, Brown J, Daikoku T, Ning W, Shi Q, et al. CXCL1 induced by prostaglandin E2 promotes angiogenesis in colorectal cancer. J Exp Med 2006;203:941–51.
[37] Fullar A, Kovalszky I, Bitsche M, Romani A, Schartinger VH, Sprinzl GM, et al. Tumor cell and carcinoma-associated fibroblast interaction regulates matrix metalloproteinases and their inhibitors in oral squamous cell carcinoma. Exp Cell Res 2012;318:1517–27.
[38] Zhang W, Matrisian LM, Holmbeck K, Vick CC, Rosenthal EL. Fibroblast-derived MT1-MMP promotes tumor progression in vitro and in vivo. BMC Cancer 2006;6:52.
[39] Herrera M, Herrera A, Dominguez G, Silva J, Garcia V, Garcia JM, et al. Cancer-associated fibroblast and M2 macrophage markers together predict outcome in colorectal cancer patients. Cancer Sci 2013;104:437–44.
[40] Jewett A, Head C, Cacalano NA. Emerging mechanisms of immunosuppression in oral cancers. J Dent Res 2006;85:1061–73.
[41] Panda S, Padhiary SK, Routray S. Chemokines accentuating protumoral activities in oral cancer microenvironment possess an imperious stratagem for therapeutic resolutions. Oral Oncol 2016;60:8–17.
[42] Osuala KO, Sameni M, Shah S, Aggarwal N, Simonait ML, Franco OE, et al. Il-6 signaling between ductal carcinoma in situ cells and carcinoma-associated fibroblasts mediates tumor cell growth and migration. BMC Cancer 2015;15:584.
[43] Miyake M, Hori S, Morizawa Y, Tatsumi Y, Nakai Y, Anai S, et al. CXCL1-mediated interaction of cancer cells with tumor-associated macrophages and cancer-associated fibroblasts promotes tumor progression in human bladder cancer. Neoplasia 2016;18:636–46.
[44] Wang D, Sun H, Wei J, Cen B, DuBois RN. CXCL1 is critical for premetastatic niche formation and metastasis in colorectal cancer. Cancer Res 2017;77:3655–65.
[45] Yuan M, Zhu H, Xu J, Zheng Y, Cao X, Liu Q. Tumor-derived CXCL1 promotes lung cancer growth via recruitment of tumor-associated neutrophils. J Immunol Res 2016;2016:6530410.
[46] Chen X, Jin R, Chen R, Huang Z. Complementary action of CXCL1 and CXCL8 in pathogenesis of gastric carcinoma. Int J Clin Exp Pathol 2018;11:1036–45.
[47] Kim EK, Moon S, Kim DK, Zhang X, Kim J. CXCL1 induces senescence of cancer-associated fibroblasts via autocrine loops in oral squamous cell carcinoma. PLoS ONE 2018;13:e0188847.
[48] Zhang H, Yue J, Jiang Z, Zhou R, Xie R, Xu Y, et al. CAF-secreted CXCL1 conferred radioresistance by regulating DNA damage response in a ROS-dependent manner in esophageal squamous cell carcinoma. Cell Death Dis 2017;8:e2790.