A Novel Function of CXCL13 to Stimulate RANK Ligand Expression in Oral Squamous Cell Carcinoma Cells

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Abstract
Oral squamous cell carcinomas (OSCC) are malignant tumors with a potent activity of local bone invasion/osteolysis. The chemokine ligand, CXCL13, has been identified as a prognostic marker for OSCC development and progression. Here in, we show that recombinant hCXCL13 treatment of OSCC cells stimulates (5-fold) RANK ligand (RANKL), a critical bone resorbing osteoclastogenic factor expression. Anti-CXCR5 chemokine receptor antibody abrogates CXCL13-induced RANKL expression in these cells. Also, CXCL13 stimulated (3.0-fold) hRANKL gene promoter activity in SCC14a cells. SuperArray screening for transcription factors by real-time RT-PCR identified significant increase in the levels of c-Jun and NFATc3 mRNA expression in CXCL13-stimulated OSCC cells. CXCL13 treatment significantly increased (3.5-fold) phospho-c-Jun levels in these cells and a c-Jun-NH2-kinase inhibitor abolished CXCL13-stimulated RANKL expression. Furthermore, we show that CXCL13 stimulation induced nuclear translocation of NFATc3 in OSCC cells. Chromatin-immunoprecipitation assay confirmed NFATc3 binding to the RANKL promoter region. We also show that overexpression of NFATc3 stimulates RANKL expression/promoter activity and that siRNA suppression of NFATc3 abolished CXCL13-stimulated RANKL expression. Thus, our results suggest that NFATc3 is a downstream target of the CXCL13/CXCR5 axis to stimulate RANKL expression in OSCC cells and implicates CXCL13 as a potential therapeutic target to prevent OSCC bone invasion/osteolysis. [Mol Cancer Res 2009;7(8):1399–407]

Introduction
Oral squamous cell carcinoma (OSCC) is the most common oral or pharyngeal malignant tumors estimated ~30,000 cases each year in the United States. Etiologic factors for OSCC includes both a genetic predisposition (1) and exposure to carcinogens such as tobacco, alcohol, chronic inflammation, and the human papilloma virus infection (2). Previously, interleukin (IL)-6 and IL-8 have been implicated as potential biomarkers for OSCC (3). Chemokines are implicated in tumor progression and metastasis (4). More recently, gene expression profiling studies implicated chemokine ligand-13 (CXCL13) in OSCC tumor development/progression (5). CXCL13 (BCA-1) that binds monogamously to the CXCR5 receptor is involved in B-cell chemotaxis (6). Osteoclast is the bone-resorbing cell and RANK ligand (RANKL), a member of the tumor necrosis factor family, which is expressed in bone marrow stromal/pre-osteoblast cells, is critical for osteoclastogenesis (7). Most resorption stimuli in normal and pathologic conditions induce osteoclast formation by modulating RANKL gene expression in marrow stromal/osteoblast cells. Osteoclast activation plays an important role in several malignancies, including oral cancers invasion of bone/osteolysis (8). However, a role for CXCL13/CXCR5 in RANKL expression in OSCC cells is unknown. We previously cloned a 2-kb promoter region of the hRANKL gene and characterized the fibroblast growth factor-2 transcriptional control of RANKL gene expression in human bone marrow stromal/osteoblast cells (9).

The nuclear factor of activated T cells (NFAT) family of proteins exists as phosphorylated proteins in the cytosol in their transcriptionally inactive state. The NFAT transcription factor family includes five members, NFATc1 (NFAT2), NFATc2 (NFAT1), NFATc3 (NFAT4), NFATc4 (NFAT3), and NFAT5. NFAT activation is mediated by calcineurin, a calcium/calmodulin-dependent phosphatase (10). NFAT proteins autoregulate and cooperate with the activator protein (c-Jun/Fos) transcription factors at the cellular level (11). Several genes associated with osteoclast development/bone resorbing activity such as tartrate-resistant acid phosphatase, matrix metalloproteinase 9, OSCAR, calcitonin receptor, and cathepsin K have been shown to be modulated by NFATc1 (10, 12). Recently, NFATc1 and NFATc3 were reported to increase osteixin transcription activity upon coexpression with PIASxα in osteoblast cells (13). In this study, we showed that NFATc3 is a downstream target of CXCL13/ CXCR5 axis to induce RANKL expression in OSCC cells. Thus, our results implicate CXCL13 as a potential therapeutic target to prevent osteolysis associated with OSCC.

Results
CXCL13 Stimulates RANKL Expression in OSCC Cells

Tumor cells activate bone resoring osteoclasts, thereby facilitating the osteolytic process and bone invasion (14). RANKL is a critical osteoelasticogenic factor in the bone microenvironment (7). Microarray analysis for gene expression profiling in OSCC identified gene signatures that include chemokine (CXC motif) ligand-13 (CXCL13) that is implicated
in OSCC development and progression (5). Recent evidence indicated that down-regulation of CXCL5 expression inhibits head and neck squamous cell carcinoma development and invasion (15). Therefore, we examined the potential of CXCL13 to modulate RANKL expression in OSCC cells. Total RNA isolated from SCC14a cells treated with recombinant hCXCL13 (0-25 ng/mL) for a 48-hour period were subjected to real-time RT-PCR analysis to quantify the levels of RANKL.
mRNA expression. SCC14a cells stimulated with CXCL13 showed a dose-dependent increase in the levels of RANKL mRNA expression (Fig. 1A). The mRNA expression was normalized with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification in these cells. The conditioned media obtained from OSCC cells (SCC1, SCC12, and SCC14a) stimulated with and without CXCL13 (25 ng/mL) for 48 hours were measured for soluble RANKL (sRANKL) by ELISA. As shown in Fig. 1B, CXCL13 stimulation significantly enhanced the levels of sRANKL secretion by OSCC cells. SCC14a cells were stimulated with different concentrations of recombinant human CXCL13 (0-25 ng/mL) for 48 hours and total cell lysates obtained were subjected to Western blot analysis of RANKL expression. As shown in Fig. 1C, CXCL13 stimulation resulted in a significant increase in RANKL expression in a dose-dependent manner in these cells. We further examined CXCL13 stimulation of RANKL expression in SCC14a cells at different time points. Total cell lysates obtained from cells stimulated with CXCL13 (25 ng/mL) for different time points (0-48 hours) were analyzed by Western blot for RANKL expression. SCC14a cells stimulated with CXCL13 for 48 hours had a 5-fold increase in the levels of RANKL expression compared with unstimulated cells (Fig. 1D). We further determined the levels of CXCR5 receptor expression in OSCC cells compared with human B lymphocytes (FREV) by real-time reverse transcription-PCR (RT-PCR) analysis. We detected significant levels of CXCR5 mRNA expression in OSCC cells, however, at low level compared with B lymphocytes (Fig. 1E). Furthermore, Western blot analysis of total cell lysates obtained from SCC14a cells stimulated with CXCL13 in the presence of anti-CXCR5 receptor antibody abrogated CXCL13-induced RANKL expression compared with control IgG–treated cells (Fig. 1F). We then examined CXCL13 stimulation of hRANKL gene promoter activity in OSCC cells. SCC14a cells were transiently transfected with hRANKL gene promoter-luciferase reporter plasmid (hRANKL P3) by lipofectamine and treated with CXCL13 for 48 hours. SCC14a cells mock-transfected with the empty vector (EV) served as the control. Total cell lysates prepared after a 48-hour period were assayed for luciferase activity. CXCL13 stimulation of SCC14a cells transfected with hRANKL P3 had a significant increase (3.0-fold) in hRANKL gene promoter activity (Fig. 1G). Transfection efficiency was normalized by measuring β-galactosidase activity coexpressed in these cells. These data indicate that CXCL13 signaling through CXCR5 up-regulated RANKL gene transcription in OSCC cells, which implicates CXCL13 play an important role in OSCC tumor osteolysis.

**SuperArray Screening for CXCL13 Enhanced Transcription Factors**

To identify the transcription factors that may be involved in CXCL13 modulation of RANKL expression, we screened a SuperArray of 84 transcription factors using a set of housekeeping genes as controls by real-time RT-PCR. SCC14a cells were stimulated with CXCL13 (25 ng/mL) for 48 hours and total RNA isolated was used as template for SuperArray screening by real-time RT-PCR and data obtained was analyzed by Web portal as described in Materials and Methods. We thus identified a 3.2- and 2.8-fold increase in c-Jun and NFATc3 mRNA expression in CXCL13-stimulated SCC14a cells, respectively (data not shown). Transfac database search identified the presence of a putative NFAT transcription factor–binding motif at −1434 bp to −1427 bp position in the hRANKL promoter region (9). NFATc1 plays a critical role in RANKL signaling in osteoclast differentiation (11). We further quantified the mRNA expression levels of NFAT transcription factor family members in CXCL13-stimulated SCC14a cells by real-time RT-PCR analysis. As shown in Fig. 2A, SCC14a cells stimulated with CXCL13 showed high levels of NFATc3 mRNA expression and a modest increase in the levels of NFATc1, NFATc2, NFATc4, and NFAT5 mRNA expression compared with unstimulated cells. We then examined CXCL13 stimulation of NFATc3 protein expression in SCC14a cells at different time points. Total cell lysates obtained from cells stimulated with CXCL13 (25 ng/mL) for different time points (0-48 hours) were analyzed by Western blot for NFATc3 expression. Cells stimulated with CXCL13 for 48 hours had a 3.5-fold increase in the levels of NFATc3 expression compared with unstimulated cells (Fig. 2B). These results suggested CXCL13 modulates RANKL expression through specific transcriptional regulatory mechanisms in OSCC cells.

**CXCL13 Enhances Phospho-c-Jun Expression in OSCC Cells**

Previously, activator protein was reported to be constitutively activated in OSCC cells (16, 17). We examined whether...
CXCL13 stimulation elevates phospho-c-Jun (p-c-Jun) expression levels in these cells. Consistent with previous reports, SCC1, SCC12, and SCC14a cells had significant levels of p-c-Jun expression, indicating constitutive activation of c-Jun in these cells (Fig. 3A). Western blot analysis of total cell lysates obtained from SCC14a cells stimulated with CXCL13 (25 ng/mL) for different time points (0-30 minutes) revealed a significant increase (3.5-fold) in the level of p-c-Jun expression at 15-minute period (Fig. 3B). We confirmed CXCL13 stimulation of c-Jun NH2-terminal kinase (JNK) activity in SCC14a cells as described in Materials and Methods. As shown in Fig. 3C, CXCL13 treatment significantly increased (2.5-fold) JNK activity over basal levels of expression in these cells in a time-dependent manner. We further tested if a JNK inhibitor abrogate the CXCL13-stimulated RANKL expression in OSCC cells. Western blot analysis of total cell lysates obtained from SCC14a cells treated with JNK inhibitor (10 μmol/L) for a 6-hour period before the CXCL13 treatment (48 hours) abolished CXCL13-induced RANKL expression in these cells (Fig. 3D). These results suggest that CXCL13 stimulates RANKL gene expression through up-regulation of JNK activity in OSCC cells.

NFATc3 Modulates RANKL Expression

Previously, it has been shown that JNK activation of c-Jun plays a critical role in expression and function of NFAT family (18). Because CXCL13 enhanced p-c-Jun levels and NFATc3 expression in OSCC cells, we next examined the potential of NFATc3 to modulate RANKL expression in these cells. We tested CXCL13-activated NFATc3 translocation to the nucleus in OSCC cells. SCC14a cells were stimulated with CXCL13 (25 ng/mL) for 24 hours and immunostained with anti-NFATc3 antibody. Confocal microscopy revealed that CXCL13 treatment induced nuclear translocation of NFATc3 in these cells. In contrast, unstimulated cells showed cytosolic localization of NFATc3 (Fig. 4A). We further analyzed NFATc3 binding to the hRANKL gene promoter element by chromatin immuno-precipitation (ChIP) assay. SCC14a cells stimulated with CXCL13 were assayed by ChIP with anti-NFATc3 antibodies as described in methods. PCR analysis of chromatin immune complexes using hRANKL gene promoter–specific primers for the NFATc3 binding region identified NFATc3 binding to the RANKL promoter region in these cells (Fig. 4B). These results suggest that NFATc3 is a downstream target for CXCL13 signaling to stimulate RANKL expression in OSCC cells. We then examined the capacity of NFATc3 overexpression to stimulate RANKL expression in OSCC cells. SCC14a cells were transiently transfected with human NFATc3 expression vector, and total cell lysates were analyzed by Western blot for NFATc3 and RANKL expression. As shown in Fig. 5A, overexpression of NFATc3 stimulated RANKL expression (3.5-fold) compared with mock-transfected cells. We further confirmed the specificity of NFATc3 regulation of RANKL expression through siRNA suppression of NFATc3 expression in these cells. Western blot analysis of total cell lysates obtained from cells transiently transfected with double stranded NFATc3 siRNA revealed inhibition of RANKL expression in CXCL13-stimulated cells (Fig. 5B). siRNA suppression of NFATc3 expression in unstimulated cells resulted in inhibition of endogenous levels of RANKL expression in OSCC cells. These results indicate NFATc3 transcription factor plays a critical role in basal and CXCL13-stimulated expression of RANKL in OSCC cells. We next examined whether NFATc3 overexpression stimulates RANKL gene promoter activity. A stable OSCC cell line (SCC11B) with hRANKL-luc construct was transiently transfected with NFATc3 expression plasmid or EV using lipofectamine. Total cell lysates prepared after 48 hours were assayed for luciferase activity. As shown in Fig. 5C, NFATc3 overexpression significantly (4.7-fold) stimulated hRANKL expression.
promoter activity in these cells compared with control EV transfected cells. Collectively, these results suggest that CXCL13 activation of NFATc3 transcription factor modulates RANKL expression in OSCC cells.

Discussion

RANKL is a critical osteoclastogenic factor in the bone microenvironment (7). Activation of bone-resorbing osteoclasts at the tumor-bone interface facilitates the osteolytic process and bone invasion of cancer cells (14). Recent studies indicated CXCR5 chemokine receptor expression by colon carcinoma cells and potential role in tumor growth (19). Therefore, our findings that CXCL13 stimulates RANKL expression in OSCC cells implicates a potential role in osteolysis associated with OSCC and tumor cell invasion of bone. Inflammatory cytokines such as IL-1β have been shown to induce CXCL13 production in differentiated osteoblasts (20) and human osteoblasts have been reported to express functional CXCR5 receptors (21). Although CXCR5 expression by OSCC in situ is yet to be elucidated, we identified the CXCL13-specific CXCR5 chemokine receptor expression in OSCC-derived cells. However, evidence also indicates that CXCL13 has low affinity binding to CCX-CKR expressed in dendritic cells and T cells (22). Thus, CXCL13 may have a paracrine role in regulating RANKL expression in osteoblast and other cell types in the tumor-bone microenvironment. Several osteotropic factors/resorption stimuli including 1,25-(OH)2D3, parathyroid hormone (PTH), IL-1β, IL-11, and prostaglandin E2 have been shown to up-regulate RANKL expression in marrow stromal/osteoblast cells (23-25). OSCC showed mRNA expression of osteotropic cytokines such as IL-6, tumor necrosis factor α, and PTHrP (26). Therefore, RANKL expression in OSCC cells could be regulated through complex autocrine transcriptional regulatory mechanisms operative in tumor cells. Recently, it has been shown that CXCL13 is overexpressed in tumor tissues and in peripheral blood of breast cancer patients (27). Thus, our findings that CXCL13 stimulates RANKL expression implicate a novel function of CXCL13 in the tumor osteolytic process and in metastasis.

Nuclear factor-κB has been reported to be constitutively activated in OSCC, and inactivation of nuclear factor-κB has been shown to suppress a malignant phenotype (28). NFATc2 and nuclear factor-κB cooperatively activate the NFATc1 promoter in response to RANKL stimulation (29). Also, c-Jun activation is associated with transformation and malignancy progression in OSCC cells (30, 31). Our findings that CXCL13 enhances p-c-Jun levels and that JNK activity is up-regulated in SCC14a cells implicate CXCL13 in constitutive activation of activator protein complex transcription factors and in the malignant phenotype in OSCC cells. Studies indicate that STAT-1 transcription factor is up-regulated in oral cancer and implicated in OSCC development and growth (2). Previously it has been shown that signal transducers and activators of transcription 3 activation in stromal/osteoblastic cells is required for induced expression of RANKL by gp130-utilizing cytokines or IL-1 but not 1,25-dihydroxyvitamin D3 or PTH (32). Therefore, CXCL13/CXCR5 axis–independent transcriptional regulatory mechanisms in response to other cytokines such as PTHrP could also play a role in RANKL expression in OSCC cells. We found no significant change in the levels of RANKL mRNA expression in CXCL13-treated cells in the presence of actinomycin D (data not shown), which suggests transcriptional regulation of RANKL expression. Enhanced levels of RANKL expression and nuclear localization of NFATc3 in CXCL13-stimulated OSCC cells indicate CXCL13-specific NFAT activation in these cells. Although the levels of NFATc3 expression is high in CXCL13-stimulated cells compared with unstimulated cells, we cannot exclude the possibility that other NFAT members that are modestly increased may regulate RANKL expression as they have been shown to cooperate with c-Jun signaling to modulate gene expression (18). Our findings that NFATc3 overexpression stimulated RANKL expression and that CXCL13 enhanced NFATc3 binding to the hRANKL promoter region in OSCC cells suggests that NFATc3 is a downstream target to induce RANKL expression in these cells. Thus, our
results implicate CXCL13 as a potential therapeutic target for the prevention of OSCC bone invasion/osteolysis.

Materials and Methods

Reagents and Antibodies

The cell culture and DNA transfection reagents were purchased from Invitrogen. Recombinant Escherichia coli–derived human CXCL13 (purity >95%) with an effective dose at 0.005 to 0.02 μg/mL to chemoattract mouse BaF/3 cells, anti-CXCR5, and anti-human RANKL antibodies were purchased from R&D systems, Inc. Anti-NFATc3, anti–c-Jun, peroxidase-conjugated secondary antibodies, and siRNA against NFATc3 were purchased from Santa Cruz Biotechnology. Super signal-enhanced chemiluminescence reagent was obtained from Amersham Biosciences and nitrocellulose membranes were purchased from Millipore. A luciferase reporter assay system was obtained from Promega. Protease inhibitor cocktail was purchased from Sigma.

Quantitative Real-time RT-PCR

RANKL and CXCR5 mRNA expression levels were measured by real-time RT-PCR as described earlier (12). Briefly, total RNA was isolated from FREV (human B lymphocyte cell line), SCC1, SCC12, and SCC14a cells treated with or without different concentrations of CXCL13 (0-25 ng/mL) for 48 h using RNAzol reagent (Biotex Laboratories). The reverse transcription reaction was done using poly-dT primer and Moloney murine leukemia virus reverse transcriptase (Applied Biosystems) in a 25-μL reaction volume containing total RNA (2 μg), 1× PCR buffer, and 2 mmol/L MgcCl2, at 42°C for 15 min followed by 95°C for 5 min. The quantitative real-time RT-PCR was done using iQ SYBR Green Supermix in an iCycler (iCycler iQ Single-color Real-time PCR detection system; Bio-Rad). The primer sequences used to amplify GAPDH mRNA were 5′-CCTACCCCCCAATGTATCCGTTGTG-3′ (sense) and 5′-GGAGGAATGGGAGTTGCTGTTGAA-3′ (anti-sense); hRANKL mRNA 5′-ACCAGCATCAAAATCCCAAG-3′ (sense) and 5′-TAAGGAGTTGGAGACCT-3′ (anti-sense); CXCR5 mRNA 5′-CTTCGCCAAGTGCTGAGGAG-3′ (sense) and 5′-TGAGATTGGAGGAGG-3′ (anti-sense). Thermal cycling parameters were 94°C for 3 min, followed by 40 cycles of amplifications at 94°C for 30 s, 60°C for 1 min, 72°C for 1 min, and 72°C for 5 min as the final elongation step. Relative levels of mRNA expression were normalized in all the samples analyzed with respect to the levels of GAPDH amplification.

Quantification of sRANKL

sRANKL levels in OSCC cell-conditioned media were measured using an ELISA kit (R&D Systems) following the manufacturer’s protocol.
hRANKL Promoter-Luciferase Reporter Gene Assay

Human OSCC-derived SCC14a cells were cultured in DMEM supplemented with 10% fetal bovine serum and 100 units/mL penicillin/streptomycin in a humidified atmosphere with 5% CO₂ at 37°C. We have previously developed hRANKL promoter-luciferase reporter plasmid construct (hRANKL P#3) as previously described (9). DNA transfections were done using lipofectamine-plus transfection reagent (Invitrogen). The transfection efficiency was normalized by cotransfection with 0.2 μg of pRSV β-gal plasmid and β-galactosidase activity was measured in the cells (Promega). LacZ cytochemical activity staining (Invitrogen, Inc.) indicated the DNA transfection efficiency of 80% in SCC14a cells. The cells were stimulated with E. coli–expressed recombinant hCXCL13 (25 ng/mL) for 48 hours. The cell monolayer was washed twice with PBS and incubated at room temperature for 15 minutes with 0.3 mL cell lysis reagent. The cells were scraped and spun briefly in a microfuge to pellet the debris. A 20-μL aliquot of each sample was mixed with 100 μL of the luciferase assay reagent. The light emission was measured for 10 s of integrated time using a Sirius Luminometer following the manufacturer’s instructions (Promega).

Western Blot Analysis

SCC14a cells were seeded (5 × 10⁵ cells per well) in six-well plates and supplemented with DMEM containing 10% FBS. The cells were stimulated with or without CXCL13 as indicated, and total cell lysates were prepared in a buffer containing 20 mmol/L Tris-HCl at pH 7.4, 1% Triton X-100, 1 mmol/L EDTA, 1.5 mmol/L MgCl₂ 10% glycerol, 150 mmol/L NaCl, 0.1 mmol/L Na₃VO₄, and 1× protease inhibitor cocktail. The protein content of the samples was measured.

**FIGURE 5.** NFATc3 regulates RANKL expression. A, Overexpression of NFATc3 in SCC14a cells. Cells were transiently transfected with NFATc3 expression plasmid. After 48 h, total cell lysates obtained were analyzed with Western blot for NFATc3 and RANKL expression. Data represent three independent experiments. B, siRNA suppression of NFATc3 in SCC14a cells. The cells were transiently transfected with double-stranded NFATc3 siRNA by oligofectamine and stimulated with or without CXCL13 (25 ng/mL) for 48 h. Total cell lysates obtained were analyzed with Western blot for NFATc3 and RANKL expression. β-Actin expression served as loading control. Data represent three independent experiments. The band intensity was quantified by densitometric analysis using the NIH ImageJ Program. C, NFATc3 overexpression stimulates gene promoter activity in hRANKL-luc SCC11B stable cell line. SCC11B cells were transiently transfected with or without NFATc3 expression plasmid by lipofectamine. Total cell lysates prepared after a 48-h period were assayed for luciferase activity per milligram protein. Columns, mean for three independent experiments (*, P < 0.05); bars, SD.
using the BCA protein assay reagent (Pierce). Protein (100 μg) samples were then subjected to SDS-PAGE using 12% Tris-HCl gels and blotted transferred to a polyvinylidene difluoride membrane, immunoblotted with anti-RANKL, anti-NFATc3, and anti–p-c-Jun antibodies. The bands were detected using the enhanced chemiluminescence detection system (Pierce). The band intensity was quantified by densitometric analysis using the NIH ImageJ Program.

SuperArray Screening
SCC14a cells (5 × 10^6 cells per well) were cultured in a 60-mm tissue culture plate with or without CXCL13 (25 ng/mL) for 48 h and total RNA was isolated using RNAzol reagent (Biotecx Laboratories). Reverse transcription reaction was done using poly-dT primer and Moloney murine leukemia virus reverse transcriptase (Applied Biosystems) in 25 μL reaction volume containing total RNA (2 μg), 1× PCR buffer, and 2 mmol/L MgCl₂, at 42°C for 15 min followed by 95°C for 5 min. Real-time PCR was done using 2× SuperArray RT qPCR Master Mix [RT² ProfilerTM PCR Array System, SuperArray (PAHS-075A-02)] in 96-well plate to quantify expression levels of 84 transcription factors. Thermal cycling parameters were 95°C for 10 min, followed by 40 cycles of amplifications at 95°C for 15 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 5 min as the final elongation step. Relative levels of mRNA expression were normalized in all the samples with expression expression levels of housekeeping genes, and data analysis was done using the Web portal.4

JNK Assay
The in vitro nonradioactive stress-activated protein kinase (SAPK)/JNK assay was done using the SAPK/JNK assay kit (#9810; Cell Signaling Technology) following the manufacturer instructions. SCC14a cells were stimulated with CXCL13 (25 ng/mL) for 0- to 30-min period. Total cell lysates (250 μg protein) obtained were mixed with 20 μL of c-Jun 1-89 fusion protein bound to glutathione agarose beads (GST-c-Jun1-89) in 1× lysis buffer, and incubated overnight at 4°C with constant rotation. The immunoprecipitated beads were washed twice with lysis buffer and twice with kinase buffer, and then they were resuspended in 50 μL of kinase buffer containing 2.5 μL of 2 mmol/L ATP. The kinase reaction was carried out at 30°C for 30 min, and terminated by addition of 25 μL of 3× SDS sample loading buffer. The reaction mix was boiled for 5 min and the supernatant was collected and loaded on a precast 4% to 20% SDS-PAGE. The gels were subsequently blotted onto polyvinylidene difluoride membranes, which were processed as described for the Western blots using an enhanced chemiluminescence detection kit. The level of c-Jun phosphorylation by SAPK/JNK was determined using an anti-phospho (Ser 63) c-Jun antibody. The intensity of the phosphorylated c-Jun band represents the relative SAPK/JNK activity precipitated by c-Jun fusion protein agarose beads.

Confocal Microscopy
SCC14a cells were cultured (1 × 10^6/well) in a Lab-Tek four-well chamber slides (Nunc, Inc.). Cells were stimulated with and without CXCL13 (25 ng/mL) for 24 h and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. The cells were permeabilized with 0.1% Triton X-100 for 10 min and blocked for 1 h with PBS containing 2% horse serum at room temperature. Cells were incubated with anti–rabbit-NFATc3 (10 μg/mL) antibody in PBS containing 2% horse serum for 3 h and treated with Alexa 488–conjugated anti-rabbit IgG in PBS containing 2% horse serum for 1 h at room temperature. The nuclear staining was done with DRAQ5 and cellular localization of NFATc3 was visualized by confocal microscopy (LSM 510; Carl Zeiss, Inc.).

ChIP Assay
ChIP was done using the ChIP Assay kit (Upstate) following the manufacturer’s instructions. Briefly, human SCC14a cells were stimulated with and without CXCL13 (25 ng/mL) for 24 h. Cells were cross-linked with 1% formaldehyde for 10 min. Soluble chromatin was prepared following sonication with a Branson-250 digital sonifier (Branson Ultrasonics) to an average DNA length of 200 to 1,000 bp. Approximately 5 × 10⁵ cell equivalent (1/6th) of the sheared soluble chromatin was precleared with blocked Protein G agarose, and 10% of the precleared chromatin was set aside as input control. Immunoprecipitation was carried out with anti-NFATc3 antibody (5 μg) or equivalent concentrations of rabbit IgG as negative control overnight at 4°C. Immune complexes were pulled down using Protein G agarose, washed and eluted twice with 250 μL of elution buffer (0.1 mol/L NaHCO₃, 1% SDS), and cross-linking reversed in 200 mmol/L NaCl at 65°C overnight with 20 μg RNase A (Sigma). DNA was purified following proteinase K treatment (Invitrogen Life Technologies) with the Qiagen PCR purification kit (Qiagen). To analyze the NFATc3 binding to the hRANKL gene promoter region, the immunoprecipitated chromatin DNA samples were subjected to PCR analysis using primer pairs for NFATc3 binding region (−1524 to −1232 bp) in the hRANKL promoter (9): 5′-GAT ACA CAT ATA AAT GCT AA-3′ (sense) and 5′-CGC TAA GTA TTT CTC TA-3′ (anti-sense). Thermal cycling parameters of a 20 μL reaction mixture were 94°C for 30 s, followed by 35 cycles of amplifications at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 5 min as the final elongation step. PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide.

NFATc3 Overexpression and siRNA Interference
SCC14a cells were seeded (5 × 10⁵ cells per well) in six-well plates and supplemented with DMEM containing 10% fetal bovine serum. One day after seeding, cells were transfected with EV or NFATc3 expression construct (2 μg) using lipofectamine or with double-stranded siRNA (10 μmol/L) against NFATc3 (Santa Cruz Biotechnology, Inc.) by oligofectamine (Invitrogen). Nonspecific siRNA-transfected cells served as control. SiRNA-transfected cells were cultured in the presence and absence of CXCL13 (25 ng/mL) for 48 h. Total cell lysates obtained were analyzed by Western blot for NFATc3 and RANKL expression using specific antibodies.

4 http://www.supperarray.com/pcarraydataanalysis.php
Statistical Analysis

Results are presented as mean ± SD for three independent experiments and compared by Student’s t test. Values were considered significant at a P value of <0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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