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Autoregulation of RANK ligand in oral squamous cell carcinoma tumor cells

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Abstract

Oral squamous cell carcinoma (OSCC) is the most common malignancy among oral cancers and shows potent activity for local bone invasion. Receptor activator of nuclear factor κ B (RANK) ligand (RANKL) is critical for bone-resorbing osteoclast formation. We previously demonstrated that OSCC tumor cells express high levels of RANKL. In this study, confocal microscopy demonstrated RANKL specific receptor, RANK expression in OSCC tumor cell lines (SCC1, SCC12, and SCC14a). We also confirmed the expression of RANK and RANKL in primary human OSCC tumor specimens. However, regulatory mechanisms of RANKL expression and a functional role in OSCC tumor progression are unclear. Interestingly, we identified that RANKL expression is autoregulated in OSCC tumor cells. The RANKL specific inhibitor osteoprotegerin (OPG) treatment to OSCC cells inhibits autoregulation of RANKL expression. Further, we showed conditioned media from RANKL CRISPR-Cas9 knockout OSCC cells significantly decreased osteoclast formation and bone resorption activity. In addition, RANKL increases OSCC tumor cell proliferation. RANKL treatment to OSCC cells demonstrated a dose-dependent increase in RANK intracellular adaptor protein, TRAF6 expression, and activation of IKK and $\text{I}\kappa\text{B}$ signaling molecules. We further identified that transcription factor NFATc2 mediates autoregulation of RANKL expression in OSCC cells. Thus, our results implicate RANKL autoregulation as a novel mechanism that facilitates OSCC tumor cell growth and osteoclast differentiation/bone destruction.

Keywords

autoregulation; NFATc2; oral squamous cell carcinoma; RANK ligand

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

1 | INTRODUCTION

Oral cancer is the sixth most common malignancy, which accounts for over 500,000 cases each year across the world (Bernier, Bentzen, & Vermorken, 2009; Haddad & Shin, 2008). Oral squamous cell carcinoma (OSCC) frequently occurs at the tongue and gingiva and contributes more than 90% of all oral cancers (Attar et al., 2010; Jimi, Shin, Furuta, Tada, & Kusakawa, 2013). Genetic predisposition and environmental factors such as tobacco, alcohol, chronic inflammation and viral infection have been implicated in the pathogenesis of OSCC (Choi & Myers, 2008; Petti, 2009). In spite of extensive research and development for cancer treatments, survival rate of OSCC patients have not improved (Carvalho, Nishimoto, Califano, & Kowalski, 2005; Cunningham, Johnson, Myers, & Thearle, 1986; Guo et al., 2014). It has been demonstrated that OSCC invades into the maxilla or mandibular bone which leads to metastasis and affects the prognosis of patients (Jimi et al., 2011). The bone marrow niche plays a pivotal role in tumor cells growth and progression in the tumor-bone microenvironment (Jatana et al., 2010; Kulasinghe, Perry, Jovanovic, Nelson, & Punyadeera, 2015). Further, constitutive activation of nuclear factor-kappa B (NF- κ B) is involved in the invasive characteristics of OSCC (Furuta et al., 2012). Cytokines including interleukin (IL)-6, tumor necrosis factor (TNF)- α , and parathyroid hormone-related protein (PTHrP) were elevated in OSCC tumor cells. Also, conditioned media from these cells stimulates bone-resorbing osteoclast differentiation in vitro (Nomura, Shibahara, Katakura, Matsubara, & Takano, 2007; Deyama et al., 2008). We established a calvarial murine model of OSCC in athymic mice, which develop tumors in 4–5 weeks and showed tumor cell invasion of bone/osteolysis (Pandruvada et al., 2010).

The stromal/preosteoblast cells along with various cancer cells produce TNF superfamily member, receptor activator of nuclear factor κ B (RANK) ligand (RANKL) (Renema, Navet, Heymann, Lezot, & Heymann, 2016). RANKL-RANK interaction is critical to promote osteoclast formation which resorbs bone (Boyle, Simonet, & Lacey, 2003). It has been demonstrated that RANKL expression in tumor cells contributes to bone metastasis beyond the stromal support (Zhang et al., 2001). RANKL expression in tumor cells further amplifies the osteoclast response in the bone invasion of cancer cells (Dougall, Holen, & Gonzalez Suarez, 2014). We identified OSCC tumor cell expression of RANKL in vivo and in vitro play an important role in tumor osteolysis (Sambandam et al., 2013; Yuvaraj et al., 2009). Also, inhibition of RANKL may reduce bone and lung metastasis in malignant tumor cells (Dougall et al., 2014). These studies implicate the significance of RANKL produced by tumor cells. However, molecular mechanisms, which control the expression of RANKL in OSCC tumor cells, as well as the functional role for RANKL in OSCC tumor growth, are unclear. In this study, we identified for the first time that the expression of RANKL in OSCC cells is self-regulated. Further, RANKL-RANK signaling stimulates the transcription factor NFATc2 that play an important role in autoregulation of RANKL in malignant tumor cells. Our findings suggest that targeting molecules involved in autoregulation of RANKL may be an effective strategy to control both OSCC tumor growth and bone destruction.

2 | MATERIALS AND METHODS

2.1 | OSCC cell culture

Human OSCC tumor-derived SCC1, SCC12, and SCC14a cell lines (Brenner et al., 2010) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin/streptomycin at 37 °C in humidified atmosphere with 5% CO₂.

2.2 | Immunohistochemistry

Human OSCC tumors tissue and control adjacent normal tissue specimens were obtained from the Hollings Cancer Center (HCC) Tissue Biorepository and an Institutional Review Board (IRB) approved the protocol. Formalin-fixed OSCC tumor specimens from human subjects were processed for 5-µm paraffin serial sectioning using a modified Leica RM 2155 rotary microtome (Leica Microsystems, Ontario, Canada). The specimens were incubated with goat primary anti-human RANKL (Santa Cruz, Dallas, TX, SC-377079) and anti-human RANK (Santa Cruz, SC-374360) antibodies for 60 min at room temperature. Immune-staining was performed with horseradish peroxidase (HRP) labeled secondary antibody and 3,3'-diaminobenzidine (DAB) substrate (Vector Laboratories, Burlingame, CA). The slides were counterstained with hematoxylin, dehydrated through graded alcohols to xylene, and coverslipped with a permanent mounting media.

2.3 | Confocal microscopy

SCC1, SCC12, and SCC14a cells (1×10^3 cells/well) were cultured in Lab-Tek four-well chamber slides (Nunc, Rochester, NY). Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, permeabilized with 0.1% Triton X-100 for 10 min at room temperature, and blocked for 1 hr with PBS containing 2% horse serum (HS). Cells were immunostained with primary antibodies against RANK (Santa Cruz, SC-374360 AF488) in PBS containing 2% HS and incubated for 3 hr. After washing (3×) with PBS, cells were incubated with Alexa 488-conjugated anti-rabbit IgG in PBS containing 2% HS for 1 hr. Nuclear staining was performed using DRAQ5 and membrane localization of RANK was visualized with an I×81 confocal microscope (Olympus, I×81; 60×).

2.4 | RANKL suppression by the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas9) knockout plasmids

We used CRISPR double nickase plasmid (Santa Cruz) to suppress RANKL gene expression in OSCC tumor cells. RANKL double nickase plasmid (h) consists of a pair of plasmids, encoding a D10A mutated Cas9 nuclease and a unique, target-specific 20 nt guide RNA (gRNA). One plasmid contains a puromycin resistance gene for selection; the other plasmid contains a GFP marker to visually confirm transfection efficiency. SCC14a cells were transfected using lipofectamine-plus transfection reagent (Invitrogen, Carlsbad, CA) for 24 hr and confirmed the transfection (>80%) by GFP expression under fluorescence microscopy. The conditioned media (CM) collected from control and RANKL knockout (KO) cells were further tested on osteoclast differentiation and bone resorption activity.

2.5 | Osteoclast differentiation and bone resorption assay

Bone marrow from C57BL/6 mice long bones (tibia and femur) was aseptically collected and washed with α -MEM. Cells were then incubated in tissue culture dishes with α -MEM containing 10% FBS for overnight. The non-adherent cells were collected and cultured (2×10^6 cells/ml) in 96-well plate (0.2 ml/well) in the presence of M-CSF (10 ng/ml), RANKL (50 ng/ml) with and without OSCC cell CM (20%). Every alternative day, half of the medium was withdrawn and replaced with fresh medium containing M-CSF, RANKL and OSCC-CM. At the end of culture period, the cells were fixed with 2% glutaraldehyde in PBS and stained for tartrate-resistant acid phosphatase (TRAP) activity using a histochemical staining kit (Sigma, St. Louis, MO) as we described previously (Sambandam, Sundaram, Saigusa, Balasubramanian, & Reddy, 2017). TRAP-positive multinucleated cells (MNC) containing three or more nuclei were scored as osteoclast cells under a microscope. Bone marrow non-adherent cells were cultured as above on dentine slices in 96-well plate (0.2 ml/well) to determine bone resorption activity. After 10 days of culture, TRAP positive MNC osteoclasts formed on the dentine slices were counted under a reflected light microscope (Rao et al., 2006). 1 M NaOH solution was used for the removal of attached cells from the dentine slices and stained with 1% toluidine blue for 5 min. The Olympus microscope was utilized to obtain the digital images of the resorbed area on dentine slices. The percentage of resorption was quantified relative to the total dentine area using a computerized image analysis (Adobe Photoshop). The animal procedures employed were performed as per the approval of Institutional Animal Care and Use Committee (IACUC).

2.6 | Western blot analysis

OSCC cells were seeded (5×10^5 /well) in 6-well plates and supplemented with DMEM containing 10% FBS. Cells were stimulated with or without RANKL or OPG as noted. Total cell lysates were prepared in a buffer containing 20 mM Tris-HCl at pH 7.4, 1% Triton X-100, 1 mM EDTA, 1.5 mM $MgCl_2$, 10% glycerol, 150 mM NaCl, 0.1 mM Na_3VO_4 and 1 $\times$ protease inhibitor cocktail. The protein content of the samples was measured using the Bradford protein assay reagent (Bio-Rad, Hercules, CA). Protein (100 μ g) samples were subjected to SDS-PAGE (4–15% Tris-HCl gels) and blot transferred on to a PVDF membrane. Immunoblotting was performed with anti-RANKL (Santa Cruz, SC377079), anti-proliferating cell nuclear antigen (PCNA) (Santa Cruz, SC-25280), anti-TRAF2 (Santa Cruz, SC-136999), anti-TRAF6 (Santa Cruz, SC-8409), anti-IKK α/β (Santa Cruz, SC-101706), anti-NFATc2 (Santa Cruz, SC-13034), anti-p-IkB- α (Cell Signaling Technology, Danvers, MA, cat #2859), anti-IkB- α (Cell Signaling Technology, cat#4814), and anti- β -actin (Sigma-Aldrich, Cat #A1978) antibodies. The bands were detected using the enhanced chemiluminescence (ECL) detection system and quantified by densitometric analysis using the NIH ImageJ Program.

2.7 | Quantitative real-time RT-PCR

Real-time RT-PCR was performed to analyze the RANKL and NFATc2 mRNA expression in OSCC tumor cells as described (Yuvaraj et al., 2009). Briefly, total RNA was isolated from OSCC cells treated with RANKL or OPG at indicated concentrations for 48 hr using RNazol reagent (Biotech Labs, Houston, TX). Total RNA was treated with DNase I

(Sigma–Aldrich, St. Louis, MO) for 15 min followed by 10 min at 65 °C with the addition of 25 mM EDTA to eliminate the residual genomic DNA contamination. The integrity of RNA samples prepared was evaluated based on the 28S and 18S rRNA bands on agarose gels and the A₂₆₀/A₂₈₀ ratio approximately 2.0. The reverse transcription (RT) reaction was performed with poly-dT primer using total RNA (2 µg), 1× PCR buffer and 2 mM MgCl₂ at 42 °C for 15 min followed by 95 °C for 5 min. The real-time PCR was performed using IQ™ SYBR Green Supermix in an iCycler (Bio-Rad). The primers used to amplify hGAPDH mRNA were 5′-CCT ACC CCC AAT GTA TCC GTT GTG-3′ (sense) and 5′-GGA GGA ATG GGA GTT GCT GTT GAA-3′ (anti-sense); hRANKL mRNA 5′-ACC AGC ATC AAA ATC CCA AG-3′ (sense) and 5′-TAA GGA GTT GGA GAC CT-3′ (anti-sense); NFATc2 (sense) 5′-AAA CTC GGC TCC AGA ATC CA-3′ and (anti-sense) 5′-TGG ACT CTG GGA TGT GAA CT-3′. Thermal cycling parameters employed were 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 1 min, 72 °C for 2 min, and 72 °C for 10 min as the final elongation step. The melt curve analysis of the PCR reaction was performed from 59 to 95 °C with 0.5 °C increments for gene-specific peaks. Relative levels of mRNA expression were normalized with respect to the levels of GAPDH amplification in all the samples analyzed.

2.8 | Cell proliferation assay

SCC14a cells were stimulated with different concentrations of RANKL for 24 hr and incubated with alamarBlue reagent (Life Technologies, Inc, Grand Island, NY) for 4 hr at 37 °C. The fluorescence intensity was read at 560 nm excitation and at 590 nm emission. The alamarBlue reduction was calculated and background absorbance was subtracted using a control media following the manufacturer's protocol.

2.9 | hRANKL gene promoter-luciferase reporter assay

We have previously developed hRANKL promoter-luciferase reporter plasmid construct (hRANKL P#3) as described (Roccisana et al., 2004; Yuvaraj et al., 2009). SCC14a cells were transfected with plasmid DNA construct using lipofectamine-plus transfection reagent (Invitrogen). Transfection efficiency was normalized by co-transfection with pRSV β-gal plasmid (0.2 µg) and β-gal activity was measured in the total cell lysates (Promega, Madison, WI). The SCC14a cells with and without NFATc2 siRNA transfected were stimulated with RANKL for 48 hr. The cells were washed with PBS and incubated for 15 min with 0.3 ml cell lysis reagent at room temperature. A 20-µl aliquot of each sample was mixed with 100 µl of the luciferase assay reagent and light intensity was measured for 10 s using a Sirius Luminometer (Promega, Madison, WI).

2.10 | STATISTICAL ANALYSIS

Results represented as mean ± SD for three independent experiments and compared by Student's *t*-test. Values were considered significant at **p* < 0.05.

3 | RESULTS

3.1 | RANK and RANKL expression in OSCC tumor cells

We previously demonstrated the expression of RANKL in OSCC cells and tumors developed on calvaria in athymic mice in vivo (Sambandam et al., 2013). However, the RANKL specific receptor, RANK expression in OSCC tumor cells needs to be analyzed. As shown in Figure 1a, confocal microscopy analysis revealed RANK expression in OSCC cell lines, SCC1, SCC12, and SCC14a. Further, immunohistochemical staining of OSCC tumor specimens from human subjects ($n = 5$) demonstrated abundant levels of RANKL expression compared to normal adjacent tissues. In addition, OSCC tumor specimens showed a high levels RANK receptor expression; however, very low levels of expression observed in normal adjacent tissue (Figure 1b). These results suggest that RANKL-RANK receptor signaling may play an important role in OSCC tumor cells.

3.2 | Autoregulation of RANKL expression in OSCC cells

Though the OSCC tumor cells showed high levels of RANKL expression, the underlying molecular mechanism remains unclear. Since RANK receptor is expressed in OSCC cells, we next examined self-regulation of RANKL expression in OSCC cells. OSCC cell lines (SCC1, SCC12, and SCC14a) were stimulated with various concentrations of recombinant hRANKL (0–80 ng/ml) for 24 hr. Total cell lysates obtained were subjected to western blot analysis for RANKL expression. Interestingly, RANKL expression is autoregulated in tumor cells. Quantification of these results identified a dose-dependent increase in RANKL expression in SCC12 cells at the concentrations tested, whereas SCC1 and SCC14a cells showed induction of RANKL expression at 0–40 ng/ml concentration (Figures 2a and 2b). We further confirmed autoregulation of RANKL in the presence of OPG, a decoy receptor for RANKL in OSCC cells. RT-PCR analysis of total RNA isolated from tumor cells cultured in the presence of RANKL demonstrated a 6.2-fold increase in RANKL mRNA expression. However, cells cultured in the presence of RANKL with OPG and OPG alone showed a marked inhibition of RANKL expression (Figure 2c). These results indicated an autoregulation of RANKL expression in OSCC tumor cells, which may have implications for tumor growth.

3.3 | Suppression of RANKL in OSCC cells inhibits OSCC-CM enhanced osteoclast formation/bone resorption

To determine the potential of RANKL autoregulation in OSCC cells to stimulate osteoclast formation/bone resorption activity, CM collected from control and RANKL CRISPR-cas9 knockout (KO) SCC14a cells were tested in mouse bone marrow-derived non-adherent cell cultures as described in the methods. As shown in Figure 3a, the GFP expression was analyzed by fluorescence microscopy to determine the transfection efficiency (>80%) of these cells. In addition, we confirmed the suppression of RANKL expression in transfected cells by western blot and RT-PCR analysis (Figure 3b-i and ii). Mouse bone marrow non-adherent cells treated with 20% of OSCC-CM in the presence of RANKL (50 ng/ml) and M-CSF (10 ng/ml) significantly increased the size and number of TRAP-positive multinucleated cells (MNC) with an increased bone resorption activity compared to control cultures treated with RANKL and M-CSF alone. Whereas, the addition of OSCC-CM from

RANKL suppressed cells inhibits osteoclast formation and bone resorption to the control level (Figures 3c and 3d). Osteoclasts cultures stimulated with RANKL (50 ng/ml), M-CSF (10 ng/ml) without OSCC-CM served as a positive control. These data indicate that the autoregulation of RANKL in OSCC cells plays an important role in enhancing osteoclast differentiation/bone destruction at the tumor-bone microenvironment.

3.4 | RANKL stimulation of OSCC tumor cell growth

Our previous studies have been identified that RANKL expression in the OSCC tumor-bone microenvironment plays an important role in bone invasion through osteoclast activation (Sambandam et al., 2013; Yuvaraj et al., 2009). However, a functional role for autoregulation of RANKL in OSCC tumor cell growth is unknown. Therefore, we next examined the capacity of RANKL to stimulate OSCC tumor cells proliferation. As shown in Figure 4a, RANKL (0–100 ng/ml) treatment of OSCC cells for a 24 hr period showed a significant increase in tumor cell proliferation compared to untreated control cells. Western blot analysis further confirmed RANKL stimulation of OSCC tumor cell proliferation with an increased level of PCNA marker protein expression (Figure 4b). In addition, OSCC cells treated with RANKL and OPG decreased the proliferative capacity of the tumor cells (Figures 4c and 4d). Taken together, our results indicate that RANKL-RANK signaling axis plays an important functional role in OSCC tumor progression.

3.5 | Activation of RANKL-RANK signaling molecules in OSCC cells

RANKL ligation to RANK receptors triggers activation of RANK adaptor molecules such as TRAF6 and TRAF2 (Darnay, Besse, Poblentz, Lamothe, & Jacoby, 2007; Ikeda & Takeshita, 2016). Therefore, we next examined the RANKL induced expression of TRAF signaling molecules in OSCC cells. Western blot analysis of total cell lysates obtained from OSCC cells treated with different concentrations (0–100 ng/ml) of RANKL demonstrated a dose-dependent increase in TRAF6 expression (Figure 5a). In contrast, RANKL treatment suppressed the TRAF2 expression in these cells (Figure 5b). We next examined the RANKL activation of IKK α / β and I κ B- α in OSCC cells. As shown in Figures 6a and 6b, OSCC cells (SCC1 and SCC14a) stimulated with RANKL identified the activation of IKK α / β and I κ B- α at 15 min. These results indicate RANKL-RANK signaling is operative in OSCC tumor cells.

3.6 | Participation of NFATc2 in autoregulation of RANKL in OSCC cells

RANKL-RANK signaling has been shown to activate NFAT family of transcription factors (Boyce & Xing, 2007; Takayanagi, 2007). We, therefore, examined the RANKL control of NFATs expression in OSCC cells. Real-time RT-PCR analysis of total RNA isolated from OSCC cells stimulated with RANKL showed a dose-dependent induction of NFATc2 expression (Figure 7a) but no other NFATs screened (data not shown). Western blot analysis of total cell lysates obtained from these cells further confirmed RANKL induction of NFATc2 in a dose-dependent manner (Figure 7b). We then tested the participation of NFATc2 in RANKL autoregulation in OSCC cells. OSCC cells (SCC14a) were transiently transfected with hRANKL-Luc promoter reporter gene plasmid and stimulated with RANKL. Interestingly, siRNA knock-down of NFATc2 in OSCC cells significantly decreased hRANKL gene promoter activity (Figure 7c). These results indicate that NFATc2

transcription factor is a downstream effector of RANKL autoregulation in OSCC tumor cells.

4 | DISCUSSION

Studies have established that bone resorption by osteoclasts is involved with invasion/metastasis of malignant cancer cells (Guisse & Mundy, 1998; Jimi et al., 2011). RANKL is a critical factor for bone-resorbing osteoclasts development/activity, plays an important role in cancer cell invasion and bone destruction. Though the expression of RANKL and its receptor RANK has been identified in cancer types such as breast, prostate, myeloma, and osteosarcoma (Li et al., 2014; Marley, Bracha, & Seguin, 2015; Roux et al., 2002; Yoneda, Tanaka, & Hata, 2013) the significance of RANKL-RANK signaling in oral cancer cells is unclear. In addition, the molecular control of RANKL gene expression in cancer cells is pivotal to our understanding of cancer progression. The poor prognosis for OSCC reflects a limited knowledge of the mechanisms of tumor progression and metastasis in patients (Bernier et al., 2009; Haddad & Shin, 2008). More importantly, the ability of OSCC to invade the maxilla or mandibular bone locally necessitates establishing the appropriate countermeasures against tumor progression and bone destruction (Jimi et al., 2011). We previously reported the high-level expression of RANKL in OSCC tumor cells and osteolysis of calvaria in an athymic mouse model (Sambandam et al., 2013). In this study, we demonstrated that OSCC tumor cell lines, as well as specimens from primary human subjects, express both RANKL and its receptor RANK suggesting that RANKL signaling plays a functional role in tumor cells. We also showed that OSCC production of chemokines such as CXCL13 induces RANKL expression in tumor cells (Yuvaraj et al., 2009). This study highlights that RANKL expression is self-regulated in oral cancer cells indicating a novel mechanism for RANKL gene expression in OSCC cells. Cytokines such as PTHrP, TNF α , and IL-6 produced by OSCC and other cancer cell types (Nomura et al., 2007; Takahashi et al., 2003) which induce RANKL expression could facilitate the RANKL self-regulation process. Therefore, variability in malignancy status of OSCC tumor cells could also be responsible for RANKL gene expression. Our findings that OSCC tumor cells cultured with a decoy receptor of RANKL, OPG inhibit the proliferative capacity of tumor cells indicate the significance of RANKL autoregulation in tumor progression. CRISPR-Cas9 KO of RANKL expression in OSCC cells further reveals the role of RANKL autoregulation in tumor cells to enhance osteoclast differentiation and bone destruction at the tumor-bone microenvironment.

RANK adaptor molecules, TRAFs have been implicated in RANKL-RANK signaling axis activating the downstream effectors of gene expression in osteoclasts (Asagiri & Takayanagi, 2007; Darnay et al., 2007; Wong et al., 1998). TRAF6 and TRAF2 have been linked to multiple myeloma and epithelial cancers respectively (Liu et al., 2012; Shen et al., 2015). Also space TRAF6 implicated in oncogenicity of OSCC tumors (Hung et al., 2013). We observed RANKL induce TRAF6 but not TRAF2 expression in OSCC tumor cells, indicating that more likely TRAF6 is involved in RANKL signaling and may play a significant role in OSCC tumor progression. Nuclear factor-kB (NF-kB) is constitutively activated in several cancer types including OSCC and is involved in the invasive characteristics of OSCC, such as growth, anti-apoptotic activity, and protease production

(Furuta et al., 2012). Earlier, we have shown constitutive activation of c-Jun, which is associated with OSCC tumor progression. Also, chemokine CXCL13 production in OSCC tumor cells increases c-Jun kinase (JNK) activity (Yuvaraj et al., 2009). Therefore, our results that RANKL activation of IKK and IKB- α in OSCC cells suggests the RANKL-RANK signaling axis play a functional role in tumor cell proliferation. In addition, RANKL production by autoregulation in OSCC cells suggests a functional role in constitutive activation of NF- κ B in these cells. MAP kinase signaling and activation of ERK/NF- κ B pathway have been reported to play an important role in resistance to drug therapy (Dhillon, Hagan, Rath, & Kolch, 2007; Shi et al., 2015). OSCC has been shown to be resistant to chemotherapeutic agents such as cisplatin (Yamano et al., 2010; Zhang et al., 2006). Since RANKL has been implicated as a cellular survival factor (Beristain, Narala, Di Grappa, & Khokha, 2012; Cheng & Fong, 2014), RANKL autoregulation in OSCC may have a significant role in resistance to chemotherapy. It is well established that RANKL-RANK signaling activates NFATc1 transcription factor responsible for osteoclast gene expression (Takayanagi et al., 2002). Earlier, we reported that c-Myc participates in the CXCL13 induction of RANKL expression in OSCC tumor-bone microenvironment (Sambandam et al., 2013). However, this study identifies that NFATc2 is the downstream effector of RANKL autoregulation in OSCC tumor cells. We identified the autoregulation of RANKL expression in OSCC tumor cells. Further, RANKL-RANK signaling stimulates NFATc2 transcription factor, which induces RANKL expression in these cells. Thus, our study implicates autoregulation of RANKL as an important process, which may facilitate OSCC tumor cell growth and bone destruction.

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FIGURE 1.

RANKL and RANK expression in human OSCC tumor cells. (a) Confocal microscopy analysis of RANK is shown as detected by Alexa 488-conjugated anti-goat antibody in SCC1, SCC12, and SCC14a cells. Nuclear staining was done with DRAQ5. (b)

Immunohistochemical analysis of RANKL and RANK expression in primary OSCC tumor and adjacent normal tissues from human subjects using anti-RANKL and anti-RANK specific antibodies

FIGURE 2.

Autoregulation of RANKL expression in OSCC cells. (a) SCC14a, SCC1, and SCC12 cells were stimulated with different concentrations of RANKL for 24 hr and total cell lysates were subjected to western blot analysis for RANKL expression. β -actin expression served as control. (b) The band intensities were quantified by ImageJ program. The values are expressed as mean $\pm$ SD of triplicates (* $p < 0.05$). (c) Total RNA isolated from OSCC cells stimulated with RANKL (40 ng/ml) in the presence and absence of OPG or OPG alone for 48 hr were subjected to real-time RT-PCR analysis of RANKL mRNA expression. Relative

mRNA expression level was normalized with respect to GAPDH amplification. The values are expressed as mean $\pm$ SD (* $p < 0.05$)

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FIGURE 3.

Effect of RANKL autoregulation in OSCC cell on osteoclast differentiation/bone resorption. (a) RANKL CRISPR-cas9 knockout (KO) plasmid transfected SCC14a cells as visualized by GFP expression under fluorescence microscopy. (b-i) Western blot analysis of RANKL expression in RANKL-KO OSCC cells. β -actin expression served as control. (b-ii) Total RNA isolated from OSCC cells transfected with CRISPR-Cas9 double nickase plasmid and subjected to real-time RT-PCR analysis of RANKL mRNA expression. Relative mRNA expression level was normalized with respect to GAPDH amplification. The values are expressed as mean $\pm$ SD (* $p < 0.05$). (c and c-i) OSCC-CM effect on osteoclast differentiation. Mouse bone marrow non-adherent cells were incubated with M-CSF (10 ng/ml), RANKL (50 ng/ml) with or without normal and RANKL-KO OSCC-CM (20%) for 7 days. Cells cultured with M-CSF alone served as control. TRAP-positive multinucleated osteoclasts formed at the end of the culture period were scored. The values are expressed as mean $\pm$ SD (* $p < 0.05$). *Compared to control cultures; #Compared to normal OSCC-CM

treated cells. (d) The mouse bone marrow non-adherent cells were cultured as above on dentine slices for 10 days in the presence of M-CSF (10 ng/ml), RANKL (50 ng/ml) with or without normal and RANKL-KO OSCC-CM (20%). (d-i) The resorbed area of dentine slices was calculated relative to the total area. (d-ii) The osteoclasts formed on the surface of dentine slices were counted under light microscope. Each bar represents the mean $\pm$ SD ($p < 0.05$). *Compared to control cultures; #Compared to normal OSCC-CM treated cells

FIGURE 4.

RANKL stimulates OSCC cell proliferation. (a) SCC14a cells were stimulated with different concentrations of RANKL for 24 hr. The proliferation was determined by adding 10 μ l alamarBlue solution and then scanned at 570 nm by a microplate reader. The values are expressed as mean $\pm$ SD (* p < 0.05). (b) RANKL increases proliferative marker PCNA expression in OSCC cells. SCC14a cells stimulated with different concentration (0–80 ng/ml) of RANKL for 24 hr and total cell lysate subjected to Western blot analysis for PCNA. β -actin expression served as control. (c) SCC14a cells were stimulated with RANKL

(40 ng/ml) and OPG (100 ng/ml) for 24 hr. The proliferation was determined by alamarBlue reduction assay as described in methods. (d) SCC14a cells stimulated with RANKL (40 ng/ml) and OPG (100 ng/ml) for 24 hr and total cell lysate subjected to western blot analysis for PCNA. β -actin expression served as control

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FIGURE 5.

Effect of RANKL in TRAF6 and TRAF2 expression in OSCC cells. (a) Western blot analysis of TRAF6 in SCC14a cells treated with different concentration of RANKL (0–100 ng/ml) for 24 hr using anti-TRAF6 antibody. (b) SCC14a cells were stimulated with different concentrations of RANKL (0–60 ng/ml) for 24 hr and total cell lysate was subjected to western blot analysis using anti-TRAF2 antibody. β -actin expression served as control

FIGURE 6.

RANKL activates IKK- α/β and I κ B- α in OSCC cells. SCC1 and SCC14a cells were stimulated with RANKL (40 ng/ml) for the different time period (0–15 min) and total cell lysates were subjected to western blot for (a) p-IKK- α/β and (b) p-I κ B- α expression. Total IKK β and I κ B- α expression were served as control

FIGURE 7.

NFATc2 is a downstream effector of RANKL autoregulation in OSCC cells. (a) SCC14a cells were stimulated with different concentration of RANKL (0–100 ng/ml) for 24 hr. Total RNA isolated was subjected to real-time RT-PCR analysis for NFATc2 mRNA expression using gene-specific primers. GAPDH expression served as control. The band intensities were quantified by ImageJ program and values are expressed as mean $\pm$ SD (* p < 0.05). (b) Cells were stimulated with different concentrations of RANKL (0–100 ng/ml) for 24 hr and total cell lysates were subjected to western blot analysis for NFATc2 expression using

specific anti-NFATc2 antibody. β -actin expression served as control. (c) NFATc2 knock-down inhibits RANKL stimulated RANKL gene promoter activity in OSCC cells. SCC14a cells were transiently transfected with the hRANKL-luc construct and/or NFATc2 siRNA by lipofectamine. Total cell lysates prepared after a 48 hr period were assayed for luciferase activity and normalized for β -gal activity coexpressed. Results represent mean $\pm$ SD of three independent experiments (* $p < 0.05$)