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High-mobility group box 1 induces bone destruction associated with advanced oral squamous cancer via RAGE and TLR4

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ABSTRACT

Bone destruction of maxillary and mandibular bone by invasive oral squamous cell cancer (OSCC) raises various problems in the management of patients, resulting in poor outcomes and survival. However, the mechanism behind bone destruction by OSCC remains unclear. High-mobility group box 1 (HMGB1), a highly conserved ubiquitous nuclear non-histone DNA-binding protein, has been demonstrated to be secreted by aggressive cancers and regulate osteoclastogenesis, a central player during bone destruction. We therefore reasoned that HMGB1 secreted by OSCCs contributes to bone destruction. Our results showed that HMGB1 is produced by human cell lines of OSCC and promotes osteoclastogenesis via up-regulation of the expression of receptor activator of nuclear factor kappa-B ligand in osteoblasts and osteocytes, and consequently osteoclastic bone destruction in mice. Further, we found that these actions of HMGB1 are mediated via the receptor for advanced glycation end products and toll-like receptors. These findings suggest that HMGB1 of OSCC and its down-stream signal pathways are potential targets for the treatment of bone destruction associated with advanced OSCC.

1. Introduction

Advanced oral squamous cell cancer (OSCC) frequently invades and destroys the maxilla and mandible bones, which is a prognostic factor of poor clinical outcome [1,2]. Broad bone resection, which is currently a first-line surgical treatment for bone destruction associated with OSCC, often disturbs quality of life of a patient [3]. Development of mechanism-based therapeutic interventions has been awaited. However, the pathophysiology of bone destruction associated with OSCCs still remains poorly understood [4]. Recent studies reported that stressed and injured cancer cells release damage-associated molecular patterns (DAMPs), thereby initiating an infection-independent inflammatory response [5,6]. High-mobility group box 1 protein (HMGB1) is one of the most representative DAMPs [7]. HMGB1 was first discovered as a conserved non-histone DNA-binding protein in the nucleus [8], however recent studies revealed that HMGB1 is secreted and mediates inflammatory and immune reactions [9]. Extracellular HMGB1 binds and activates the receptor for advanced glycation end products (RAGE) and toll-like receptor 4 (TLR4) and promotes the activation of immune cells, induction of proinflammatory cytokines, stimulation of cell adhesion and migration, and the promotion of cell proliferation and angiogenesis [10]. Of note, HMGB1 expression was found to be increased in several types of tumors and HMGB1 levels are elevated in the circulation in cancer patients [11]. Further, it is reported that HMGB1 stimulates osteoclastogenesis via regulating the expression of the receptor activator of nuclear factor kappa-B ligand (RANKL) [12].

To determine the molecular mechanism by which invasive OSCCs destroy maxillary and mandibular bone, we investigated the expression of HMGB1 in human OSCC cell lines and the effects of

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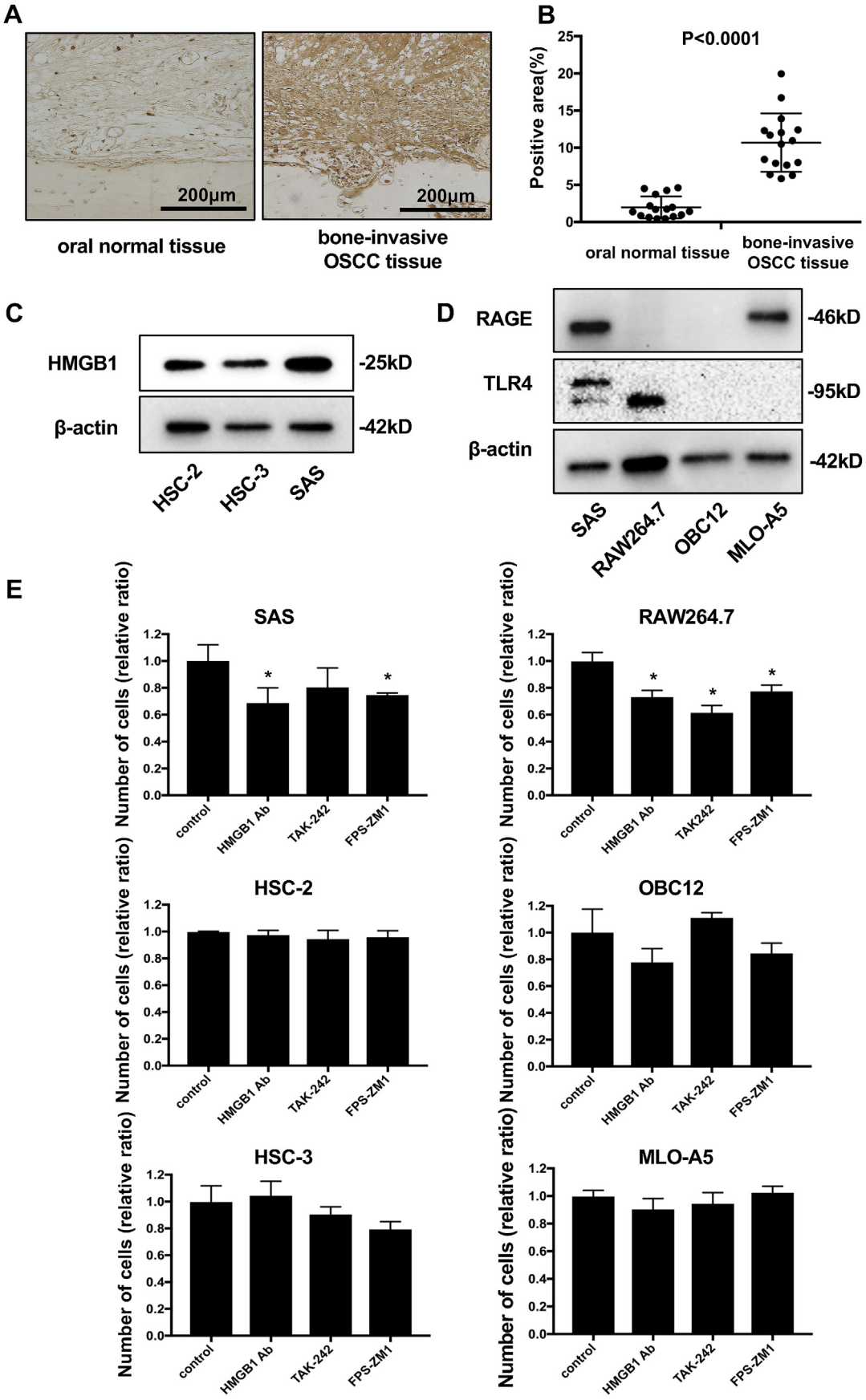

Fig. 1. Expression of HMGB1 in bone-invasive OSCC of patients, OSCC cell lines and the effects of HMGB1 antibody, TLR4 antagonist TAK-242, and RAGE antagonist FPS-ZM1 on human OSCC cell lines. **A:** HMGB1 expression in human oral normal tissue (**left panel**) and in human bone-invasive advanced oral cancer (**right panel**). **B:** Scatterplot analysis of the

HMGB1 on osteoclastogenesis and bone destruction in vitro and in mice. Our results provide the first evidence that OSCCs produce and secrete HMGB1, which in turn increases the expression of RANKL in osteoblasts and osteocytes, thereby promoting osteoclastic bone destruction.

2. Materials and methods

2.1. Reagents

Anti-HMGB1 Chicken IgY neutralizing antibody (polyclonal #326052233) was purchased from SHINO-TEST Corporation (Kanagawa, Japan). RAGE antagonist FPS-ZM1 (#11909) and TLR4 antagonist TAK-242 (#13871) were purchased from Cayman Chemical (Ann Arbor, MI). Anti-HMGB1 antibody (anti-mouse, monoclonal, GTX628834) was purchased from Gene-Tex (Irvine, CA). Anti-NF-κB p65 antibody (anti-rabbit, polyclonal, #ab16502) and Anti-TLR4 antibody (anti-rabbit, polyclonal, #ab13556) were purchased from Abcam (Cambridge, UK). Anti-RAGE antibody (anti-mouse, monoclonal, #sc-80652) was purchased from Sant Cruz Biotechnology (Dallas, TX). Anti-RANKL antibody (anti-rabbit, polyclonal, #bs-0747R) was purchased from BIOSS (Woburn, MA). Horseradish peroxidase (HRP)-conjugated IgG antibody (goat anti-rabbit, monoclonal, #7074), HRP-conjugated IgG antibody (goat anti-mouse, monoclonal, #7076) were purchased from Cell Signaling Technology (Danvers, MA).

2.2. Cell culture conditions

The human OSCC cell lines SAS (#JCRB0260), HSC-2 (#JCRB0622), and HSC-3 (#JCRB0623) were obtained from the Human Science Resources Bank (Osaka, Japan). They were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. Primary mouse osteoblast cells (OBC12) (Cosmo Bio, Tokyo) and the mouse osteocytic cell line MLO-A5 (Dr. T. Bellido, Department of Anatomy and Cell Biology, Indiana University, IN) were cultured in α-minimal essential medium (Thermo Fisher Scientific) containing 5% FBS. The mouse macrophage cell line RAW264.7 was cultured in α-MEM containing 10% FBS. All cell lines were cultured in an atmosphere of 5% CO_2 at 37 °C.

2.3. Immunohistochemical analysis of bone-invasive OSCC samples

We analyzed the expression of HMGB1 in bone-invasive human OSCC tissue and adjacent normal tissue from patients treated at Okayama University Hospital. The specimens were incubated with anti-HMGB1 antibody (1:200) overnight. The immunoreaction was visualized with the use of a DAB peroxidase substrate with VECTASTAIN Elite ABC Kit (Vector Labs, #PK-6102, Burlingame, CA). Quantification was performed using Image J, and the relative integrated density was calculated. The study was approved by the Ethics Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (#206-003).

2.4. Cell proliferation assay

SAS, HSC-2, HSC-3, RAW264.7, OBC12, and MLO-A5 cells were cultured in six-well culture plates at 1×10^5 cells per well with the presence or absence of the HMGB1-neutralizing antibody, TAK-242 or FPS-ZM1. After 48 h, the cells were counted using a TC20 automated cell counter (Bio-Rad, Hercules, CA).

2.5. Western blot analysis

RAW264.7, OBC12, and MLO-A5 were cultured with SAS OSCC-conditioned medium with the HMGB1-neutralizing antibody, TAK-242 or FPS-ZM1. The cell lysate samples were electrophoresed in 4–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories) and blocking with 5% skim milk. Antibodies against HMGB1(1:1000) RANKL (1:500), RAGE (1:1000), TLR4(1:1000) NF-κB p65 (1:1000) were used as a primary antibody. HRP-conjugated anti-rabbit antibody (1:2000) and HRP-conjugated anti-mouse antibody (1:2000) were used as the secondary antibody. A ChemiDoc MP system (Bio-Rad Laboratories) was used for the analysis of western blots.

2.6. Osteoclast differentiation and activity assay

Bone marrow cells were obtained from the femurs and tibiae of 4-week-old male C57BL/6 mice, purchased from Charles River Laboratories (Yokoyama, Japan). Under anesthesia with 0.4 mg/kg of medetomidine, 4.0 mg/kg of midazolam and 5.0 mg/kg of butorphanol, the mice were sacrificed by cervical dislocation. Cut both femurs and tibia ends and flushed out marrow cells with phosphate-buffered saline using 27gauge needle. The cells were then incubated in α-MEM with 30 ng/ml M-CSF (R&D System, Minneapolis, MN) for 24 h. Non-adherent cells were transferred to 48-well plates. The cells were supplemented with 30 ng/mL macrophage colony stimulating factor and 10 ng/mL RANKL (PEPROTECH, Rocky Hill, NJ) and were cultured with SAS OSCC-conditioned medium with the HMGB1-neutralizing antibody or TAK-242 or FPS-ZM1. Following five days of incubation, the cells were fixed and stained for TRAP/ALP Stain Kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The number of TRAP-positive multinucleate cells (nuclear number >3) in each well was counted.

For the osteoclast activity assay, corrected bone marrow cells were transferred to osteo assay surface 24-well multiple well plates (#3987, Corning, Lowell, MA). Following six days of incubation, the cells were fixed and viewed under a fluorescence microscope (IX81, Olympus). The area of pits on the plates was determined using image J.

2.7. Animal experiments

All the animal experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of the Okayama University Graduate School of Medicine and Dentistry (approval no.OKU-2018701 and OKU-2018509).

Mouse models of bone invasion by human OSCC a was established in 7-week-old male BALB/c nude mice (n = 8 per group; n = 32 total; mean body weight, 24.0 g; Charles River Laboratories) by the inoculation of 1×10^5 SAS cells into the bone marrow space of the right tibial metaphysis under general anesthesia with 0.4 mg/kg of medetomidine, 4.0 mg/kg of midazolam and 5.0 mg/kg of butorphanol. At seven days after tumor cell inoculation, the mice were divided into four groups (control, HMGB1 neutralizing

HMGB1-positive areas in oral normal tissues (n = 16) and oral cancers (n = 16). Error bars: Mean ± SD. There was a significantly increased expression of HMGB1 in oral cancers ($p < 0.0001$). **C:** Expression of HMGB1 in human OSCC cell lines including SAS, HSC-2, and HSC-3 by Western analysis **D:** Expression of HMGB1 receptor TLR4 and RAGE in the bone cells by Western analysis. **E:** Effects of HMGB1 neutralizing antibody, RAGE antagonist FPS-ZM1, and TLR4 antagonist TAK-242 on cell proliferation of OSCC cell lines. Live cells excluding trypan blue were counted. Data represent relative ratio over control. *$p < 0.05$.

antibody, TAK-242, and FPS-ZM1). The TAK-242 and FPS-ZM1 groups were administered intraperitoneal of 100 μl of a solution each containing TAK-242 and FPS-ZM1 (1 mg/kg) in PBS five times a week and the control group was administered PBS alone. The HMGB1 neutralizing antibody group was administered 100 μl solution containing HMGB1 neutralizing antibody (1 mg/kg) in PBS three times for two weeks. At the end of the experimental period (day 21), the mice were sacrificed and the right tibias of the mice that had been injected with the cancer cells were excised and then fixed in 4% paraformaldehyde phosphate buffer solution.

2.8. In vivo radiography and measurement of osteolytic lesion areas

Osteolytic bone destruction was assessed on radiographs. The bones were placed against films (22 × 27 cm; Fuji Industrial Film FR: Fuji Photo Film) and exposed to soft X-rays at 35 kV for 15 s with the use of a Sofron apparatus (Sofron). The radiolucent bone lesions were observed microscopically (IX81, Olympus), and the areas were quantified with Lumina Vision/OL (Mitani Corporation). A micro-CT image was obtained with a SKYSCAN scanner (Bruker Japan).

2.9. Immunohistochemical analysis

The bone was decalcified and embedded in paraffin. Serial 3-μm-thick sections were cut cross-sectionally, and the sections were stained with IHFC stain and TRAP stain. The specimens were incubated with RANKL (1:200) or Ki-67 (1:200) antibody, followed by Alexa Fluor 488 anti-rabbit IgG (1:500) as secondary antibodies. Nuclei were counterstained with Fluoroshield mounting medium with DAPI (#ab104139; Abcam).

2.10. Statistical analyses

We analyzed the data using an unpaired Student's t-test for comparisons of two groups and by performing a one-way analysis of variance (ANOVA) and post hoc Tukey's test for the analysis of multiple group comparisons, using Prism ver. 8.0. Results are expressed as the mean ± standard deviation (SD). Probability (p) values < 0.05 were considered significant.

3. Results

3.1. HMGB1 expression in human OSCC tissue

Immunohistochemical examination revealed that HMGB1 expression was increased in the bone-invasive OSCC patient's samples compared to the normal oral tissue (Fig. 1A). The area of HMGB1-positive cells was increased in each OSCC sample compared to that of the normal oral tissue (Fig. 1B).

3.2. Expression of HMGB1 and RAGE and TLR4 in the human OSCC cell lines and bone cells

We then investigated the expressions of HMGB1, TLR4 and RAGE, which are receptors of HMGB1, in the human OSCC cell lines and bone cells. SAS cells strongly expressed HMGB1 compared to the other OSCC cell lines (Fig. 1C). Of interest, pre-osteoclastic cells (RAW264.7) expressed TLR4, whereas osteocyte-like MLO-A5 cells expressed RAGE (Fig. 1D).

3.3. Role of HMGB1 in cell proliferation of OSCC cell lines and bone cells

We next evaluated the role of HMGB1 in cell proliferation of OSCC and bone cells in vitro by testing a neutralizing antibody to HMGB1 and an antagonist to RAGE, FPS-ZM1 and TLR4, TAK242. Cell proliferation of the human OSCC cell line SAS was significantly suppressed by the treatment with FPS-ZM1 and the HMGB1 neutralizing antibody (Fig. 1E). In contrast, neither the HMGB1 antibody nor TAK-242 and FPS-ZM1 showed the effects on cell proliferation of HSC-2 and HSC-3 cells that produce less amounts of HMGB1 than SAS cells. Of interest, cell proliferation of the pre-osteoclastic RAW264.7 cells was suppressed by the treatment with the HMGB1 antibody and TAK-241 and FPS-ZM1. However, there were little effects of the HMGB1 antibody and FPS-ZM1 and TAK-242 on cell proliferation of OBC12 osteoblasts and MLO-A5 osteocyte-like cells. These results suggest that HMGB1 is an autocrine growth factor that stimulates cell proliferation via the binding to RAGE in the human OSCC cell line SAS. Our results also show that HMGB1 promotes cell proliferation of pre-osteoclasts via activation of TLR4 signaling in a paracrine manner.

3.4. OSCC-derived HMGB1 promotes osteoclastogenesis and bone resorption

HMGB1 was reported to promote osteoclastogenesis in rheumatoid arthritis [12]. Therefore, we determined the effects of cancer-derived HMGB1 on osteoclastogenesis in bone marrow cultures and bone resorption in pit assay. We cultured mouse bone marrow cells with suboptimal dose of RANKL (10 ng/ml) and M-CSF (30 ng/ml) in the presence or absence of the conditioned medium (CM, 30% v/v) harvested from SAS cultures, the HMGB1 antibody (100 ng/mL), RAGE antagonist, FPS-ZM1 (25 nM), and TLR4 antagonist, TAK-242 (25 nM) for 5 days. SAS CM significantly increased the formation of TRAP-positive multinucleated osteoclast-like cells (Fig. 2A and B) and resorption pits (Fig. 2C and D). Addition of the HMGB1 antibody, TAK-242, or FPS-ZM1 significantly decreased SAS CM-increased formation of TRAP-positive multinucleated osteoclasts (Fig. 2A and B) and pits (Fig. 2C and D). These results suggest that SAS-secreted HMGB1 promotes osteoclastogenesis and bone resorption through activation of TLR4 and RAGE signal pathways expressed in osteoclasts.

3.5. Cancer-derived HMGB1 regulates RANKL expression in osteoblasts and osteocytes

To determine the mechanism of increased osteoclastogenesis and bone resorption by SAS-secreted HMGB1, the effects of SAS CM on the expression of NF-κB and RANKL in osteoblasts and osteocytes with or without the HMGB1 antibody, FPS-ZM1, or TAK-242 were investigated by Western analysis. SAS CM increased RANKL expression in the mouse primary osteoblasts OBC12 and mouse osteocyte-like cell MLO-A5, while NF-κB expression in pre-osteoclastic RAW264.7 was not changed (Fig. 2E). HMGB1 antibody and FPS-ZM1 significantly reduced those effects of SAS CM. These results suggest that cancer-secreted HMGB1 promotes osteoclast differentiation and bone resorption via increasing RANKL expression in osteoblasts and osteocytes.

3.6. Blocking the HMGB1 signal axis suppressed bone destruction associated with SAS colonization in bone in the mouse model of OSCC bone invasion

We next examined the role of cancer-secreted HMGB1 in bone destruction associated with OSCC colonization in bone by inoculation of SAS cells into the bone marrow cavity of tibiae of mice. Soft X-ray and micro-CT examination demonstrated that intratibial inoculation of SAS cells developed discernible osteolytic lesions three weeks after the inoculation (Fig. 3A and B). Importantly, treatment with the HMGB1 antibody, TAK-242, or FPS-ZM1

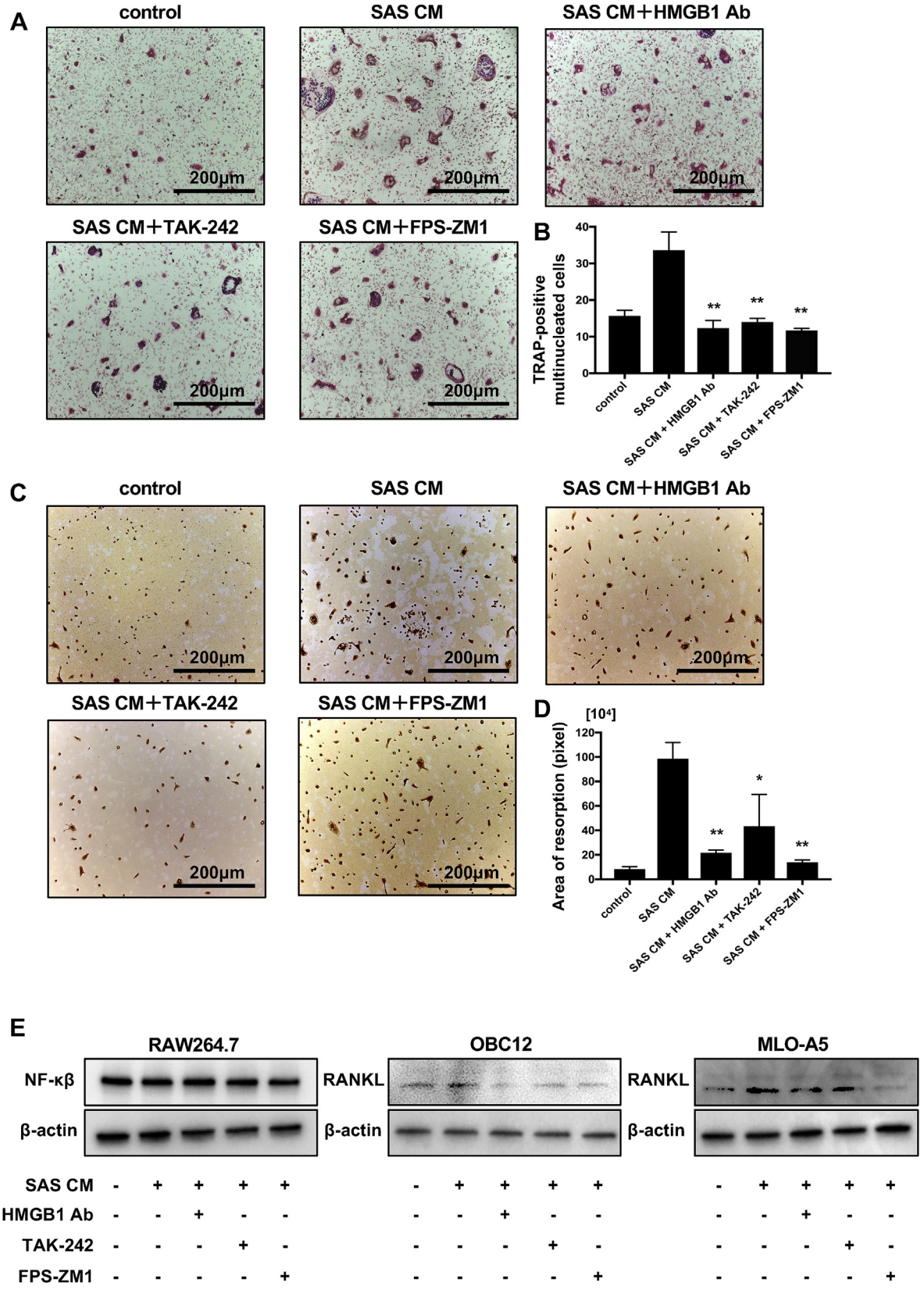

Fig. 2. Effects of OSCC-secreted HMGB1 on osteoclastogenesis and bone resorption. A: Bone marrow cells were cultured with RANKL (10 ng/ml), M-CSF (30 ng/ml) with or without SAS CM (30%, v/v), HMGB1-neutralizing antibody, TAK-242 or FPS-ZM1 HMGB1 in 48-well plates for 6 days. **B:** Number of TRAP-positive multinucleated osteoclast-like cells (nuclear number >3–5) were counted (y-axis). **C:** Bone resorption by osteoclasts differentiated from bone marrow cells was evaluated in pit assay. **D:** Pit area was determined using Image J. *$p < 0.05$; **$p < 0.01$ between the indicated groups. **E:** Effects of HMGB1 antibody, FPS-ZM1 and TAK-242 on the expression of the osteoclast differentiation marker NF-κB in the RAW264.7 pre-osteoclast cell line, and the expression of RANKL in the OBC12 primary osteoblasts and the MLO-A5 osteocytic cells by Western analysis.

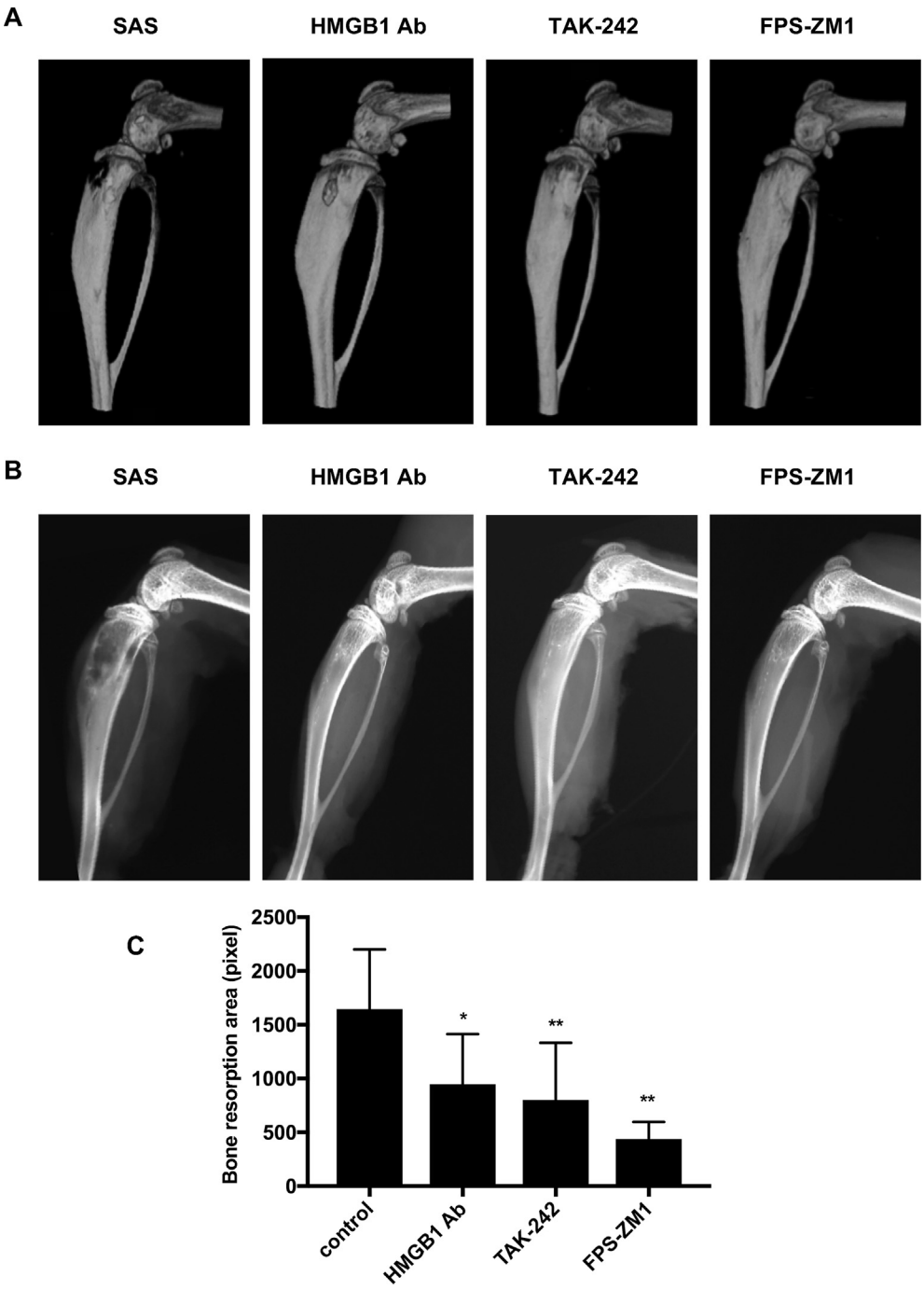

Fig. 3. Effects of the HMGB1 antibody, RAGE antagonist FPS-ZM1, and TLR4 antagonist TAK-242 on SAS-associated osteolysis in tibiae in mice. Representative image of micro-CT **(A)** and radiographs **(B)** of tibia injected with SAS cells at three weeks. **C:** Osteolytic lesion area in tibia from a mouse. Data are mean ± SD, n = 5/group.

significantly decreased the development of osteolytic lesions in tibiae of mice (Fig. 3A–C) compared to those of untreated SAS-inoculated mice. These results suggest that cancer-secreted HMGB1 develops bone destruction associated with OSCC invasion in bone via propagation of HMGB1 through RAGE and TLR4.

Immunohistochemical (IHC) analysis of tibiae injected with SAS cells demonstrated increased RANKL expression in bone colonized by SAS cells (Fig. 4A and B). Consistent with in vitro results, RANKL expression in osteocytes and osteoblasts was decreased by the treatment with HMGB1 antibody, TAK-242, and FPS-ZM1. Further, intratibial inoculation of SAS cells also increased the number of TRAP-positive multinucleated osteoclasts at the tumor-bone interface (Fig. 4C and D), and HMGB1 antibody and FPS-ZM1 significantly decreased the number of TRAP-positive multinucleated osteoclasts. Interestingly, expression of the tumor proliferation marker Ki67 was increased in SAS tumors, which was decreased by the treatment with the HMGB1 antibody, or FPS-ZM1 (Fig. 4E and F). These results suggest that HMGB1 plays a critical role in bone destruction associated with oral cancer and growth of oral cancer in bone.

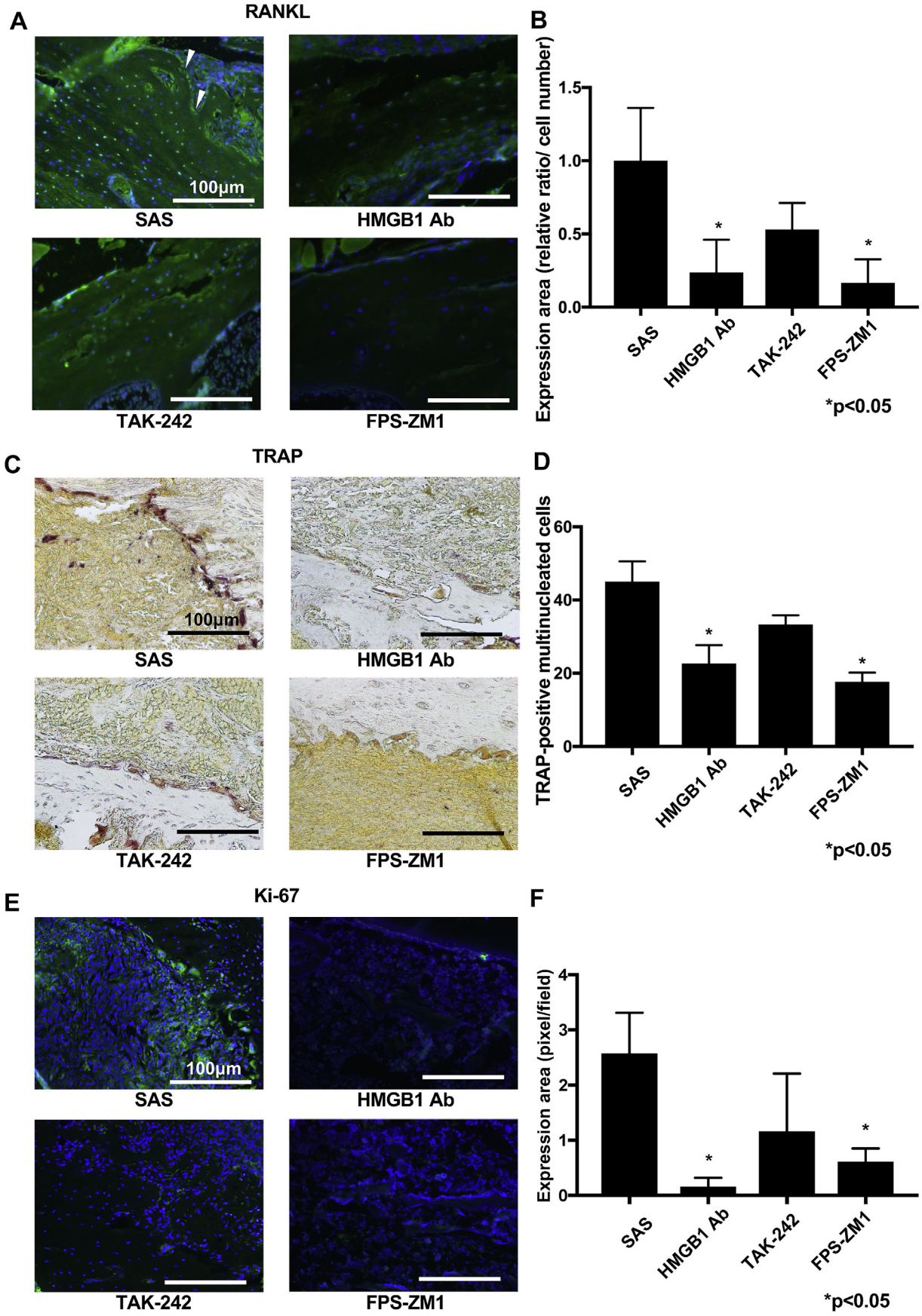

Fig. 4. Expression of RANKL **(A and B)**, TRAP **(C and D)**, and Ki67 **(E and F)** in SAS tumor colonizing bone by histological, histomorphometrical, and immunohistological analysis. n = 8/group.

4. Discussion

A role for HMGB1 in malignant tumors has been described [13], but the involvement of HMGB1 in the bone destruction induced by OSCC is not well understood. Our present experiments revealed the inhibition of HMGB1 signaling inhibited the bone destruction associated with OSCC by suppressing osteoclast differentiation and function.

HMGB1 is a 25-kDa DNA binding protein that is generally distributed in the nucleus [14]. The most extensively studied roles of HMGB1 are those in the immune system. Nuclear HMGB1 acts as a DNA chaperone that regulates DNA repair and transcription [15]. HMGB1 is also known as damage-associated molecular pattern (DAMPs), released from dead cells and dendritic cells [16]. It has been reported that both the level of HMGB1 secreted from various types of cancer cells and the blood serum HMGB1 concentration are correlated with poor prognosis [17,18]. However, the direct effects of HMGB1 on cancer cells have not been established [19]. Our present findings demonstrated that OSCC cells and an OSCC patient's tissue strongly express HMGB1 and subsequently release HMGB1 to the extracellular space. Our data indicate that SAS cells expressed HMGB1 receptor RAGE and TLR4. In addition, the HMGB1 blocking agent decreased the proliferation of the OSCC cell lines in the in vitro experiment. These data suggest that autocrine HMGB1 promotes cancer cell proliferation in OSCC.

HMGB1 is known as an inflammation cytokine. Several reports indicated that lipopolysaccharides (LPS) and other inflammation cytokines enhance osteoclastogenesis [20,21]. HMGB1 and other DAMPs are considered osteoclast differentiation factors [12], but the role of HMGB1 in bone destruction induced by OSCC remains unknown.

As expected, our present results indicated that conditioned medium from HMGB1-rich OSCC SAS cells increased the osteoclast differentiation from total bone marrow cells and increased the bone resorption activity. Further, the HMGB1 neutralizing antibody and the HMGB1 receptor antagonist suppressed the osteoclastogenesis and bone resorption activity. With these results, we evaluated the direct effect of cancer-derived HMGB1 on osteoclast differentiation from macrophages via nuclear factor-kappa B (NF-κB) signaling, which involves canonical and non-canonical Wnt pathways that are essential for osteoclastogenesis [22]. Some reports indicated that HMGB1 directly promotes maturation of osteoclasts independent of RANKL signaling [12]. However, contrary to our expectation the SAS-derived HMGB1 did not affect the expression of NF-kB in the pre-osteoclast cell line RAW264.7.

We also observed that cancer-derived HMGB1 increased the expression of RANKL in osteoblasts, which control osteoclast activation and differentiation. The RANKL expression in osteocytes was proposed to be more important for osteoclastogenesis than that in osteoblasts [23]. Surprisingly, our present findings demonstrated that the expression of RANKL in not only osteoblasts but also in osteocytes was increased by SAS-derived HMGB1. The RANKL expression in both types of cells was decreased by the treatment with neutralizing antibody and the RAGE antagonist but not the TLR4 antagonist. It was reported that advanced glycation end-products that are ligands of RAGE are essential for RANKL in osteoblasts [24]. Our present results indicate that the HMGB1 signaling promote RANKL expression in osteoblasts and osteocytes.

In light of our in vitro data, we expected that blocking HMGB1 signaling would suppress the cancer bone destruction by OSCC SAS cells injected into mouse tibial bone marrow. As expected, the HMGB1 antibody, RAGE antagonist, and TLR4 antagonist significantly decreased the bone destruction and tumor burden in the bone marrow. The number of TRAP-positive multi-nuclear osteoclast cells was significantly decreased in the tibia treated with the HMGB1 antibody, RAGE antagonist, or TLR4 antagonist. Together our data indicate that HMGB1 has a dual effect on the tumor burden in bone: one is a direct effect on tumor progression via HMGB1 autocrine signaling, and the other is an indirect effect of osteoclastogenesis by RANKL regulation in osteoblasts and osteocytes.

Clinically, patients with bone-invasive metastatic malignant tumors, breast cancer, or pancreatic cancer frequently exhibit resistance to cytotoxic chemotherapy such as cisplatin or doxorubicin. Our present findings indicate that anti-HMGB1 agents have not only a direct tumor suppression effect but also a tumor-independent anti-bone destruction effect. Bisphosphonate and other bone-modifying agents have improved the prognosis of patients with bone cancer, but side effects such as medication-related osteonecrosis of the jaw (MRONJ) [25] and atypical femoral fracture (AFF) [26] have become a problem. The results described herein demonstrate that anti-HMGB1 agents could become alternative therapeutic drugs against the development of MRONJ or AFF in patients who have been treated with standard bone-modifying agents for bone cancer.

In conclusion, our results suggest that HMGB1 secreted from OSCC promotes tumor growth in an autocrine manner and osteoclast formation and bone destruction by up-regulation of RANKL expression in osteocytes and osteoblasts in a paracrine manner through activation of RAGE and TLR4 signaling. Thus, blockade of HMGB1 and its downstream signal pathways including RAGE and TLR4 may be a mechanism-based anti-tumor approach for the treatment of advanced invasive oral cancers.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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