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CD147 deficiency blocks IL-8 secretion and inhibits lung cancer-induced osteoclastogenesis

Hongkai Wang [1], Yunyun Zhuo [1], Xu Hu, Weiwei Shen, Ying Zhang, Tongwei Chu*

Department of Orthopaedics, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, China

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ABSTRACT

Bone is a frequent target of lung cancer metastasis, which is associated with significant morbidity and poor prognosis; however, the molecular basis of this process is still unknown. This study investigated the role of extracellular matrix metalloproteinase inducer (also known as cluster of differentiation (CD)147) in osteoclastogenesis resulting from bone metastasis, based on the enrichment of this glycoprotein on the surface of many malignant bone tumors. RNA interference was used to silence CD147 expression in A549 human lung cancer cells. Compared with conditioned medium (CM) from control cells (A549-CM), CM from CD147-deficient cells (A549-si-CM) suppressed receptor activator of nuclear factor κB ligand-stimulated osteoclastogenesis in RAW 264.7 cells and bone marrow-derived macrophages. The mRNA levels of osteoclast-specific genes such as *tartrate-resistant acid phosphatase*, *calcitonin receptor*, and *cathepsin K* were also reduced in the presence of A549-si-CM. CD147 knockdown in A549 cells decreased interleukin (IL)-8mRNA and protein expression. IL-8 is present in large amounts in A549-CM and mimicked its inductive effect on osteoclastogenesis; this was reversed by depletion of IL-8 from the medium. Taken together, these results indicate that CD147 promotes lung cancer-induced osteoclastogenesis by modulating IL-8 secretion, and suggest that CD147 is a potential therapeutic target for cancer-associated bone resorption in lung cancer patients.

1. Introduction

Lung cancer is the leading cause of cancer death worldwide [1], and has often spread beyond the initial tumor by the time a diagnosis is made [2,3]. Approximately 30%–40% of advanced lung cancer patients have bone metastases [4]; once these are established, the chance of survival and quality of life of the patient decrease significantly, with a clinical outcome that includes intractable pain, increased risk of fractures, nerve compression syndromes, and hypercalcemia [5,6]. Overt lung–bone metastases are generally osteolytic in nature and rely on the ability of tumor cells to stimulate bone resorption by osteoclasts, which disrupts bone and creates physical space into which tumor cells invade [7]. Cancer cells secrete various factors that stimulate osteoclast-mediated bone resorption; the consequent release of some of these factors from the skeletal matrix promotes cancer cell spreading and bone destruction [8]. Tumor cells secrete interleukin (IL)-1, -6, -8, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β, which contribute to osteoclast-induced bone resorption [9–11]. However, whether there are other factors involved in bone destruction resulting from lung cancer is still unclear.

Extracellular matrix metalloproteinase inducer—also known as cluster of differentiation (CD)147, basigin, M6, or tumor cell-derived collagenase-stimulating factor—is a transmembrane glycoprotein belonging to the immunoglobulin (Ig) superfamily [12] that is normally expressed in the epidermis, breast lobules, retinal pigment epithelium, and ductules. CD147 also plays a key role in tumor progression and metastasis [13]; indeed, high levels of CD147 have been reported in many tumors, including breast

Abbreviations: BMM, bone marrow-derived macrophage; BSA, bovine serum albumin; CalcR, calcitonin receptor; CCK-8, Cell Counting Kit-8; CD147, cluster of differentiation 147; CM, conditioned medium; CtsK, cathepsin K; FBS, fetal bovine serum; Ig, immunoglobulin; IL-8, interleukin 8; M-CSF, macrophage colony-stimulating factor; MEM, Modified Eagle's Medium; PBS, phosphate-buffered saline; RANKL, receptor activator of nuclear factor κB ligand; rhIL-8, recombinant human IL-8; siRNA, short interfering RNA; snc, silencer negative control; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α; TRAP, tartrate-resistant acid phosphatase.

* Corresponding author. Fax: +86 23 68774087.
E-mail address: chtw@sina.com (T. Chu).

[1] These authors contributed equally to this work.

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cancer, oral squamous cell carcinoma, glioma, lymphoma, melanoma, and bladder, lung, and kidney carcinomas [14]. Recent studies have shown that the upregulation of CD147 is an important factor in breast cancer-induced osteolytic lesions [15], which are characterized by excessive bone resorption and are mainly induced by abnormal osteoclast differentiation. There is evidence that bone destruction at sites of bone metastasis result from abnormal osteoclast activity rather than tumor cells per se [16,17]. Based on the well-established role of CD147 in tumor development, the present study investigated the role of CD147 in the ability of lung cancer cells to induce osteoclast differentiation. The findings reveal that CD147 plays a critical role in increased osteoclastogenesis induced by lung cancer-derived IL-8, suggesting that CD147 is a potential therapeutic target for preventing bone resorption in lung cancer patients.

2. Materials and methods

2.1. Cell culture

RAW 264.7 murine macrophage and A549 human lung adenocarcinoma epithelial cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured at 37 °C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (MEM) supplemented with 10% fetal bovine serum (FBS) (both from Gibco, Grand Island, NY, USA) and antibiotics (100 IU penicillin/ml and 100 mg streptomycin/ml).

Bone marrow-derived macrophages (BMMs) were obtained from 4- to 6-week-old male C57BL/6 mice. The tibias and femurs of mice were dissected and flushed with α-MEM containing 10% FBS. Cells were centrifuged for 5 min at 1000 rpm, and the cell pellet was resuspended in α-MEM supplemented with 10% FBS and cultured for 24 h. Non-adherent cells were collected and cultured for 3 days in the presence of macrophage colony-stimulating factor (M-CSF; 20 ng/ml) (PeproTech, Rocky Hill, NJ, USA). Floating cells were discarded, and adherent cells were classified as BMMs [18].

RAW 264.7 cells were seeded in 24-well plates at a density of 2×10^4 cells/well and incubated overnight to allow attachment of the cells. After 24 h, the culture medium was replaced with medium containing receptor activator of nuclear factor κB ligand (RANKL; 50 ng/ml) for 5 days. BMMs were plated at 4×10^5 cells/well in a 24-well plate in the presence of RANKL (50 ng/ml) and M-CSF (20 ng/ml) for 7 days. The culture medium was replaced every 2–3 days.

2.2. Cell proliferation in monolayers

Cells (3×10^3/well) were seeded in 96-well plates and cultured for 24 or 72 h at 37 °C and 5% CO_2. Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. Briefly, 10 μl CCK-8 solution were added to cells, followed by a 2-h incubation, after which the absorbance was measured at 450 nm using a microplate reader. Each assay was conducted in quadruplicate and repeated at least three times.

2.3. RNA interference

Short interfering (si)RNAs against CD147 (CD147-si1, 5′-GTCGTCAGAACACATCAAC-3′ and CD147-si2, 5′-GTTCTTCGTGAGTTCCTC-3′) [19,20] were synthesized by Shanghai GenePharma (Shanghai, China), along with silencer negative control siRNA (CD147-snc). The siRNAs were transfected into A549 cells using Lipofectamine 2000 reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions.

2.4. Preparation of conditioned medium (CM)

CM was prepared by seeding A549, A549-snc,or A549-si cells at 10^6/100-mm dish and culturing until 70% confluence. The medium was replaced with serum-free medium, and after 48 h, the supernatants were collected, centrifuged, and stored at −80 °C until use. To account for differences in cell density due to proliferation, cell number was determined and the volume of CM was normalized to cell number across samples. For IL-8 inhibition experiments, 2 μg/ml of anti-IL-8 neutralizing antibody (R&D Systems, Minneapolis, MN, USA) was used to deplete IL-8 from A549-CM. Recombinant human (rh)IL-8 (PeproTech) was used to stimulate osteoclastogenesis.

2.5. Tartrate-resistant acid phosphatase (TRAP) staining of RAW 264.7 cells and BMMs

Multinucleated (≥three nuclei) osteoclast-like cells were identified after 5 or 7 days of incubation with CM, RANKL, and/or M-CSF by staining for TRAP using a kit (Sigma–Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. TRAP-positive multinucleated osteoclasts were visualized by light microscopy and imaged. The assay was performed at least three times.

2.6. RNA isolation and real-time PCR analysis

Total RNA was isolated from cells with TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Equal amounts of total RNA from each sample were reverse-transcribed to cDNA using the PrimeScript RT Reagent Kit with cDNA Eraser (Takara, Dalian, China). Real-time PCR was performed using FastStart Universal SYBR Green Master Mix (Roche, Shanghai, China) and expression levels were normalized to that of *glyceraldehyde-3-phosphate dehydrogenase*. Sequences of the primers used are listed in Table S1.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The CM was collected as described above and IL-8 levels were quantified with the IL-8 ELISA kit (Boster, Wuhan, China) according to the manufacturer's protocols.

2.8. Immunocytochemistry

A549 and A549-si cells were seeded in 24-well plates and cultured for 24 h. The cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.25% Triton X-100/PBS for 10 min. This was followed by blocking with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 20 min at room temperature. Cells were then stained with anti-CD147 antibody (Abcam, Danvers, MA, USA) in 1% BSA and incubated overnight at 4 °C. After washing with PBS, cells were incubated with Alexa Fluor 568 donkey anti-rabbit secondary antibody (Invitrogen, Grand Island, NY, USA) for 2 h in the dark at room temperature, washed with PBS, then visualized by confocal microscopy (Olympus, Tokyo, Japan).

2.9. Western blotting

Cells were washed with cold PBS and lysed with radio immunoprecipitation buffer with 1 mM phenylmethylsulfoxide. Cell lysates were centrifuged at 12,000 rpm for 30 min, and supernatants were collected. Proteins were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes, which were blocked with 5%

BSA for 2 h in PBS with 0.1% Triton X-100 followed by incubation with primary antibodies overnight at 4 °C. After rinsing, membranes were incubated with secondary antibodies for 2 h at room temperature. Antibodies against CD147 and β-actin were purchased from Abcam and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively.

2.10. Statistical analysis

Experiments were repeated at least three times in triplicate. One-way analysis of variance was carried out to evaluate differences between groups using SPSS v.17.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± standard deviation. $P < 0.05$ was considered statistically significant.

3. Results

3.1. SiRNA-mediated silencing of CD147 expression in A549 cells

Specific siRNAs targeting CD147 mRNA were used to knockdown CD147 expression in A549 cells, which was quantified by real-time PCR. CD147-si2 suppressed CD147 mRNA expression to the greatest extent relative to control-transfected cells (Fig. 1A). CD147 protein expression was also significantly downregulated by CD147-si2-mediated knockdown, as determined by western blotting (Fig. 1B) and immunocytochemistry (Supplementary Fig. S1). A549 cells transfected with CD147-si2 (A549-si) were therefore used in subsequent experiments.

3.2. CM from CD147-deficient A549 cells inhibits RANKL-stimulated osteoclastogenesis

The effects of CD147 silencing on A549 cell proliferation were evaluated by CCK-8 assay. Proliferation was inhibited at 24 and 72 h in CD147-deficient cells as compared to those that were untransfected (A549) or transfected with negative control (A549-snc) constructs ($P < 0.05$; Supplementary Fig. S2), which did not differ in terms of the extent of the knockdown. To determine whether CD147 silencing affected osteoclast formation, CM from A549 cells transfected with A549-si (A549-si-CM) or A549-snc (A549-snc-CM) or from untransfected cells (A549-CM) was diluted by 50% in Dulbecco's MEM and added to RAW 264.7 cell cultures. A549-CM did not promote osteoclast differentiation as compared to the RANKL-treated positive control cells (Supplementary Fig. S3), and therefore cells were treated with CM from tumor cells and RANKL were used in subsequent experiments. An increase in TRAP-positive, multinucleated osteoclasts was observed among RAW 264.7 cells exposed to RANKL (50 ng/ml); this was suppressed by addition of A549-si-CM relative to cells treated with A549-CM or A549-snc-CM ($P < 0.05$; Fig. 2A and C). To further evaluate the inhibitory effect of CD147 silencing on osteoclastogenesis, BMMs were treated with CM in the presence of RANKL (50 ng/ml) and M-CSF (20 ng/ml). TRAP staining showed that CM from CD147-deficient cells suppressed osteoclastogenesis in BMMs induced by RANKL and M-CSF (Fig. 2B and D).

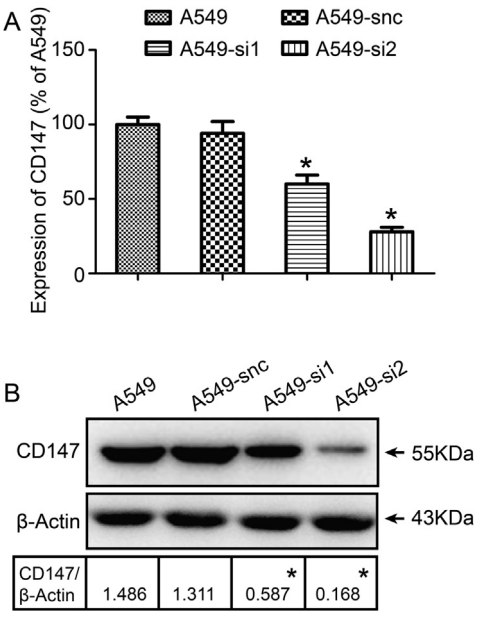

Fig. 1. Effects of siRNA-mediated CD147 silencing in A549 cells. A specific siRNA targeting CD147 mRNA was used to knockdown CD147 expression. (A) CD147 mRNA levels in siRNA-transfected and untransfected cells were quantified by real-time quantitative PCR. (B) CD147 protein levels in the cells were assessed by western blotting, with β-actin used as a loading control; untransfected cells served as a negative control. Values represent mean ± SD of triplicate experiments. *$P < 0.05$.

3.3. Expression of osteoclast-related genes is downregulated by treatment with CM from CD147-deficient A549 cells

The effect of A549-si-CM on osteoclastogenesis was also assessed by evaluating the expression of osteoclast-related genes. During differentiation, osteoclasts express a set of markers including *TRAP*, *calcitonin receptor* (*CalcR*), and *cathepsin* (*Cts*) *K*, which, along with multinucleation and resorption, characterizes the osteoclast phenotype. RAW 264.7 cells were treated with CM and the expression of osteoclast-related genes was examined after 2 and 5 days. *TRAP* mRNA expression was upregulated in cells treated with RANKL as compared to untreated cells. Moreover, *TRAP* mRNA level was downregulated in cells treated with RANKL and A549-si-CM as compared to A549-CM or A549-snc-CM at both time points. Similar trends were observed for *CalcR* and *CtsK* transcript levels (Fig. 3). These results indicate that in osteoclastogenesis is suppressed by CM from cells lacking CD147 expression.

3.4. CD147 depletion reduces IL-8 secretion and inhibits lung cancer-induced osteoclastogenesis

To determine whether osteoclastogenesis-related factorsare secreted by A549 cells to induce osteoclastogenesis, the mRNA expression of various cytokines was evaluated in A549, A549-snc and A549-si cells (Supplementary Fig. S4). Of those that were analyzed, only the expression of IL-8, which reportedly plays an important role in osteoclast formation [21,22], was downregulated in A549-si cells (Fig. 4A). High levels of IL-8 were found in A549-CM and A549-snc-CM but the level was relatively low in A549-si-CM, as determined by the ELISA (Fig. 4B).

The effect of rhIL-8 on osteoclast formation was investigated, since it is known to stimulate osteoclastogenesis independent of RANKL at concentrations ≥50 ng/ml (Supplementary Fig. S5A). However, in the presence of RANKL, 2 ng/ml rhIL-8 was sufficient to promote osteoclast differentiation (Supplementary Fig. S5B). Moreover, IL-8 depletion using a specific antibody (2 μg/ml) suppressed osteoclast formation induced by CM from A549 cells, as compared to treatment with control IgG (Fig. 4C and D). These results demonstrate that CD147 plays an important role in lung cancer-derived osteoclastogenesis by modulating IL-8 secretion.

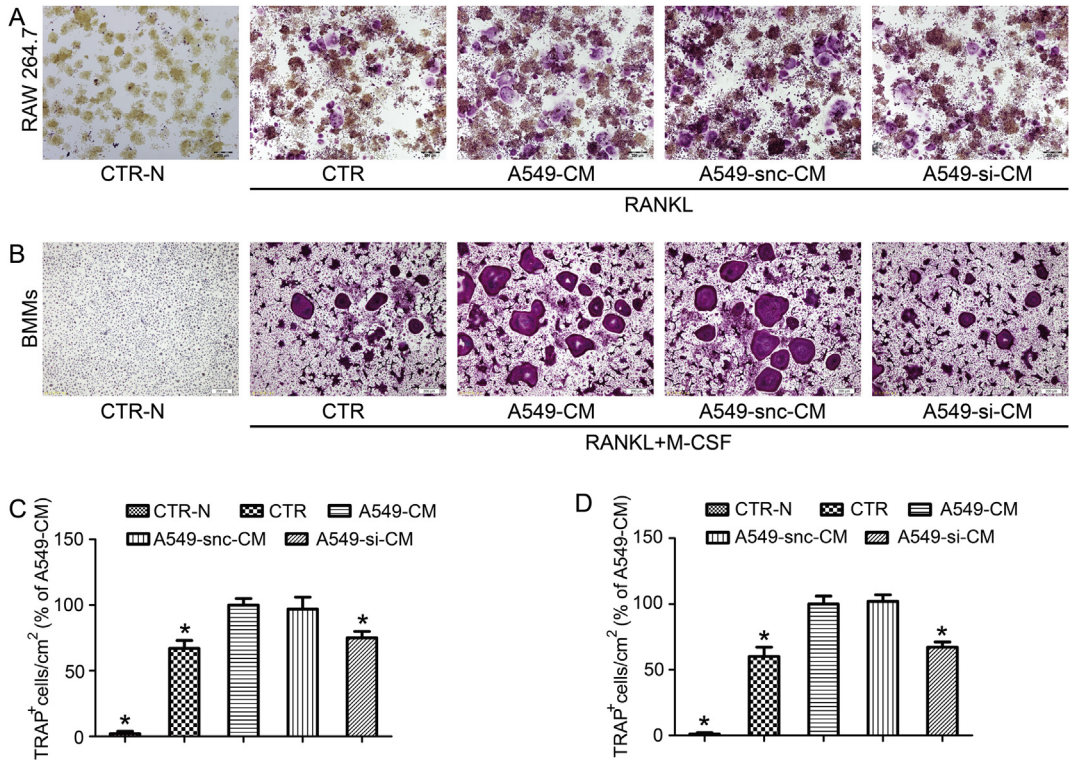

Fig. 2. CM from CD147-depleted A549 cells suppresses RANKL-induced osteoclastogenesis. (A) RAW 264.7 cells were cultured with CM from A549 cells transfected with siRNA against CD147 (A549-si-CM) or a negative control (A549-snc-CM) or from untransfected cells (A549-CM) in the presence of RANKL (50 ng/ml) for 5 days. (B) BMMs were cultured with the various CM in the presence of RANKL (50 ng/ml) and M-CSF (20 ng/ml) for 7 days. (C, D) Quantitative analysis of TRAP-positive multinucleated (≥3 nuclei) RAW 264.7 cells (C) and BMMs (D) cultured with CM. Values represent mean ± SD of experiments performed independently at least three times. *P < 0.05 vs. A549-CM. Scale bar, 200 μm.

4. Discussion

Nearly 90% of cancer-associated deaths are due to tumor metastasis, primarily to the lung, liver, lymph node, and bone. The latter is frequently observed in lung, prostate, and breast cancer. Lung cancer metastasis to bone always leads to osteolytic lesions, and osteoclast differentiation is a key event in lung cancer-induced bone loss [23,24].

Osteoclasts are giant multinuclear cells derived from the monocyte-macrophage lineage that are considered bone-resorbing cells in both normal and pathological bone remodeling [8]. Their differentiation from precursors is induced by RANKL, which also regulates the survival and function of mature osteoclasts. As such, RANKL is used as an inductive factor in studies on osteoclast differentiation and function. In the present study, RAW 264.7 cells and BMMs were induced to differentiate using RANKL. CM from A549 cells lacking CD147 expression decreased osteoclast formation, demonstrating for the first time that loss of CD147-a transmembrane glycoprotein expressed in lung cancer cells-can block lung cancer-induced osteoclastogenesis.

During differentiation, osteoclasts express markers such as TRAP, CalcR, and CtsK; these along with multinucleation and resorption characterize the osteoclast phenotype. TRAP is used as a marker of osteoclast function [25], while CalcR is a cellsurface receptor that modulates osteoclast-mediated bone resorption in vitro and in vivo [26]; its ligand calcitonin is considered as the ideal

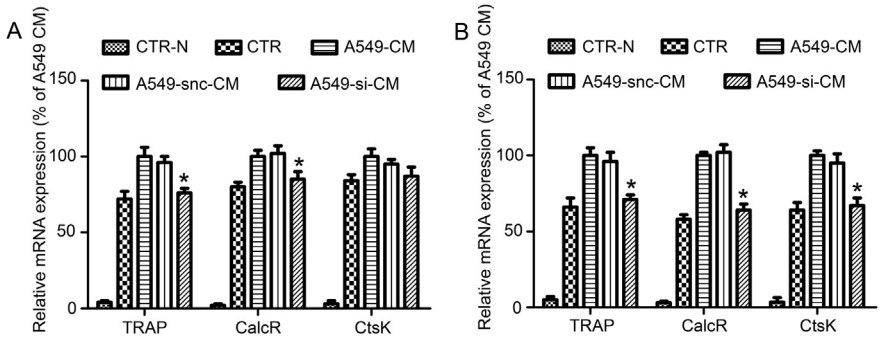

Fig. 3. CM from CD147-depleted A549 cells suppresses the expression of osteoclast-related genes in RANKL-stimulated RAW 264.7 cells. (A, B) Transcript levels of *TRAP*, *CalcR*, and *CtsK* in cells treated with CM from A549 cells transfected with siRNA against CD147 (A549-si-CM) or a negative control (A549-snc-CM) or from untransfected cells (A549-CM) in the presence of RANKL (50 ng/ml) for 2 days (A)or 5 days (B). Data represent fold changes in target gene expression normalized to that of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and are expressed as a percentage of the level in cells treated with A549-CM, which was set to 100%. Values represent mean ± SD (n = 4). *P < 0.05.

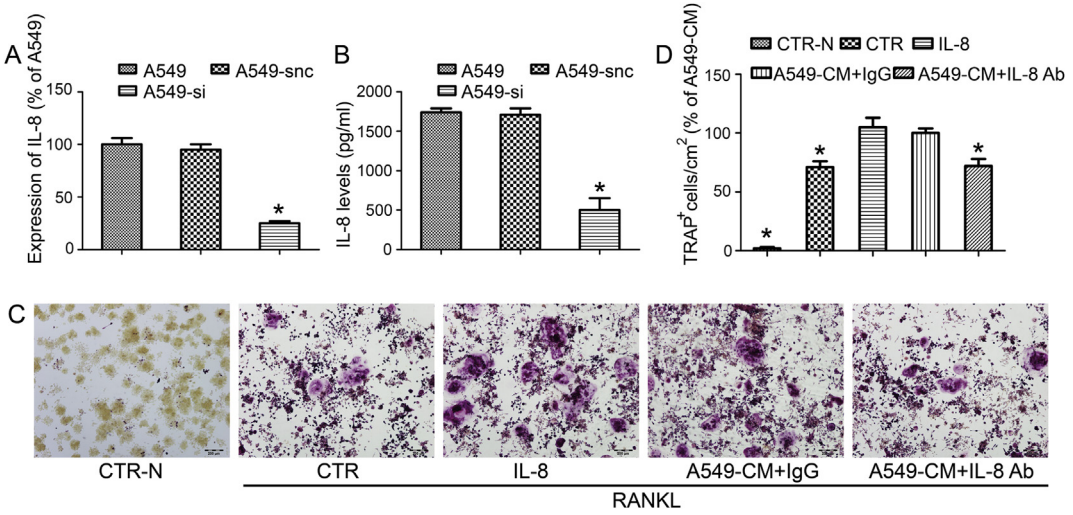

Fig. 4. Role of IL-8 in lung cancer-mediated osteoclastogenesis. (A) IL-8 mRNA expression in A549 cells transfected with siRNA against CD147 (A549-si) or a negative control siRNA (A549-snc) or in untransfected cells was quantified by real-time PCR. (B) IL-8 levels in the CM of transfected (A549-si-CM or A549-snc-CM) or untransfected (A549-CM, positive control) cells were assessed by ELISA. (C, D) Decrease in osteoclastogenesis caused by depletion of IL-8 from A549-CM. RAW 264.7 cells were treated with rhIL-8 (10 ng/ml) or A549-CM followed by treatment with IL-8 antibody (2 μg/ml) in the presence of RANKL (50 ng/ml) (C); TRAP-positive multinucleated cells were quantified following treatment (D). Values represent mean ± SD of at least three independent experiments. *$P < 0.05$ vs. A549-CM. Scale bar, 200 μm.

differentiation marker for osteoclasts [27]. The high level of CtsK expression and its secretion into the resorption lacuna suggest that it plays a key role in the resorption process [28]. In this study, CM from CD147-deficient cells suppressed RANKL-induced expression of these osteoclast markers, suggesting that loss of CD147 decreases the release of a soluble factor that is required for RANKL-induced osteoclastogenesis.

In addition to RANKL, other tumor-derived secreted factors such as IL-1, -6, -8, and parathyroid hormone-related peptidecan induce osteoclast differentiation [9,29]. IL-8 secreted from lung cancer cells increases osteoclast formation in vitro [21,30]; moreover, sera from lung cancer patients with bone metastasis contain high levels of IL-8 [31]. We demonstrated that high levels of IL-8 were present in CM from untransfected or control-transfected A549 cells, but IL-8 was relatively low in CM from cells in which CD147 was depleted. These results suggest that IL-8 is the secreted factor that is required for RANKL-induced osteoclastogenesis. This was confirmed by treating RAW 264.7 cells with rhIL-8 or CM from untransfected cells in the presence of RANKL. Compared to cells exposed to RANKL alone, treated cells showed a greater tendency to differentiate into osteoclasts, while depletion of IL-8 using a specific antibody reduced osteoclast formation. These data indicate that lung tumor cells lacking CD147 do not secrete IL-8, which is required at a high level for RANKL-induced osteoclastogenesis.

In summary, the present study showed that CD147 deficiency in lung cancer cells results in a reduction in secreted IL-8, which in turn inhibits the osteoclastogenic effects of RANKL. Additional studies are needed to determine whether other soluble factors block RANKL-induced osteoclast differentiation; however, the findings suggest that CD147 is a potential therapeutic target for preventing secondary bone loss due to lung cancer metastasis.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.097.

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