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Induction of osteoclast-like cell formation by leptin-induced soluble intercellular adhesion molecule secreted from cancer cells

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Abstract

Background: Leptin is considered a tumorigenic adipokine, suggested to promote tumorigenesis and progression in many cancers. On the other hand, intercellular adhesion molecule-1 (ICAM-1) shows altered expression in a variety of benign and malignant diseases. Histologically, ICAM-1 expression is reported as proportional to cancer stage and considered as a potential diagnosis biomarker. The altered expressions of ICAM-1 and its soluble form in malignant diseases have gained interests in recent years.

Material and methods: The expression of ICAM-1 and its regulatory signaling were examined by Western blot or flow cytometry. The effect of soluble ICAM-1 on osteoclast formation was investigated by tartrate-resistance acid phosphatase staining of RAW cells and tumor-induced osteolysis *in vivo*.

Results: In our study, we found that leptin enhanced soluble ICAM-1 production but not surface ICAM-1 expression in lung and breast cancer cells, and this effect was regulated through leptin receptor (ObR), while silencing ObR abrogated leptin-induced soluble ICAM-1 expression. In addition, we revealed that leptin administration provoked the JAK1/2, STAT3, FAK, ERK, and GSK3αβ signaling cascade, leading to the elevation of ICAM-1 expression. Moreover, soluble ICAM-1 secreted by leptin-stimulated cancer cells synergize with the receptor activator of nuclear factor kappa-B ligand (RANKL) in inducing osteoclast formation. Soluble ICAM also enhanced tumor-induced osteolysis *in vivo*.

Conclusion: These findings suggest that soluble ICAM-1 produced under leptin treatment enhances osteoclast formation and is involved in tumor-induced osteolysis.

Leptin plays an important role in physiology in health and diseases. Leptin affects immune responses that may induce inflammation and carcinogenesis. Leptin is also considered as a tumorigenic adipokine suggested to promote tumorigenesis and progression in many cancers. On the other hand, intercellular adhesion molecule-1 (ICAM-1) shows altered expression in a variety of benign and malignant diseases. Histologically, ICAM-1 expression is reported to be proportional to cancer stage and considered as a potential diagnosis biomarker. It has been reported that soluble ICAM-1 allows tumor cells to escape from immune recognition and stimulates angiogenesis and tumor growth. The altered expressions of ICAM-1 and its soluble form in malignant diseases have gained interests in recent years. In our study, we found that leptin enhanced soluble ICAM-1 production but not surface ICAM-1 expression in lung and breast cancer cells, and this effect was regulated through leptin receptor (ObR), while silencing ObR abrogated leptin-induced soluble ICAM-1 expression. In addition, we revealed that leptin administration provoked the JAK1/2, STAT3, FAK, ERK, and GSK3αβ signaling cascade, leading to the elevation of ICAM-1 expression.

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Moreover, soluble ICAM-1 secreted by leptin-stimulated cancer cells synergize with receptor activator of nuclear factor-kappa B ligand in inducing osteoclast formation. Soluble ICAM also enhanced tumor-induced osteolysis *in vivo*. These findings suggest that soluble ICAM-1 produced under leptin treatment is possibly involved in lung and breast cancer bone metastasis.

Keywords: cancer cell, leptin, osteoclast, soluble intercellular adhesion molecule

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Introduction

Adipose-tissue-derived molecules, including adipokines, are emerging as pivotal regulators that link obesity with cancer. Accumulating evidence indicates that obesity adversely impacts on cancer treatment and the development of drug resistance, particularly through leptin and the leptin receptor.[1–3] Leptin is a tumorigenic adipokine suggested to promote tumorigenesis and therapeutic resistance in many cancers.[4] Leptin also shapes the tumor microenvironment to potentiate angiogenesis and metastasis.[5] In addition, leptin is associated with the expansion of the cancer stem-cell subpopulations,[4,6] and the expression of the leptin receptor is necessary for maintaining its cancer stem-cell-like property.[7] It is also reported that leptin upregulates proinflammatory cytokines and promotes the immune escape of cancer cells.[8] In the past decade, leptin has been largely investigated in hormone-related cancers; however, the role of leptin in other types of cancer is gathering interest.

Invasion and distant metastasis of cancer cells are the sequential events responsible for the majority of cancer deaths. Leptin has a novel impact on the spread of cancers. Several clinical studies have revealed that lymphatic invasion is more frequently observed in cancers with higher leptin expression in different tumor types.[9–11] There is growing evidence that leptin enhances invasion and metastasis in breast, ovarian, lung, gallbladder, and pancreatic cancers, etc.[12–16] In our previous study, we demonstrated that leptin is upregulated in migration-prone cell lines, and exogenous leptin also enhances the migration ability of glioma cells.[17] Furthermore, leptin is reported to be involved in activities of cancer-associated fibroblasts and expression of matrix metalloproteinases, which greatly influence the metastatic behaviors of cancer cells.[18–20] Moreover, accumulating evidence also shows that leptin promotes the epithelial–mesenchymal transition through several signaling mechanisms, leading to tumor invasion and metastasis.[14,21,22]

In malignant diseases, the altered expression of adhesion molecules leads to impaired adhesive properties and cell–cell communication.[23] Deviant expression of adhesion molecules has been reported in various cancer types and associated with cancer progression.[23,24] Cancer cells interacting with intercellular adhesion molecules (ICAMs) or vascular adhesion molecules (VCAMs) of stroma or endothelium are believed to be essential for metastatic processes.[25,26] ICAM-1 (CD54) is a type I membrane-bound glycoprotein which belongs to the immunoglobulin (Ig) superfamily. ICAM-1 also plays an essential role in leukocyte trafficking, lymphocyte activation and other immune functions.[27] Alternative splicing generates different isoforms of ICAM, including the soluble form of ICAM-1 (sICAM-1), while matrix metalloproteinases (MMPs), cathepsins and neutrophil elastase may be responsible for the shedding of sICAM-1.[28] ICAM-1 shows altered expression in a variety of benign and malignant diseases. In malignant cell types, the expression of ICAM-1 has been associated with facilitated invasion.[29] Histologically, ICAM-1 expression is reported as proportional to cancer stage and considered as a potential diagnosis biomarker.[30,31] Moreover, elevated levels of sICAM-1 are associated with adverse clinical features and poor prognosis.[32] It has been reported that sICAM-1 allows tumor cells to escape from immune recognition and stimulates angiogenesis and tumor growth.[33,34] One study also suggest that increased serum levels of soluble ICAM-1 or VCAM-1 may serve as biomarkers for detecting preclinical or early cancer.[35] The altered expressions of ICAM-1 and its soluble form in malignant diseases have gained interests in recent years, although there are still many questions unanswered. In our study, we found that leptin enhanced sICAM-1 production in cancer cells, and detailed signaling pathways are also investigated.

Materials and methods

Materials

Anti-p-JAK1 (Tyr1022/1023), p-JAK2 (Tyr1007/1008), p-FAK (Tyr397), and p-GSK3α/β (ser21/9) antibodies were purchased from Cell Signaling Technology (Danvers, MA, US). JAK1 and JAK2 were obtained from Elabscience (Houston, TX, US). U0126 was purchased from MCE (Monmouth Junction, NJ, US). JAK1 was purchased from Merck Millipore (Temecula, CA, US). Leptin was obtained from PeproTech (Rocky Hill, NJ, US). Anti-ICAM-1, ObR, FAK, p-ERK1/2, ERK, GSK3α/β antibodies, and ObR and control small interfering (si)RNAs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Stattic was purchased from Selleckchem (Houston, TX, US). AG490, PF573228, SB216763, anti-GAPDH, α-tubulin, and β-actin antibodies were obtained from Sigma-Aldrich (St. Louise, MO, US). Anti-p-GSK3β (Tyr216/279) and anti-ICAM-1 antibodies were obtained from Thermo Scientific (Waltham, MA, US). Recombinant soluble ICAM-1 was purchased from KingFisher Biotech (Saint Paul, MN, US).

Cell culture

H292, MCF7, and MDA-MB-231 were purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan). RAW264.7 cell line was a generous gift from Professor Chih-Hsin Tang (Graduate Institute of Basic Medical Science, China Medical University). H292 cells were cultivated in RPMI-1640 medium with 1 mmol/l sodium pyruvate and 4.5 g/l glucose. MCF7 and RAW264.7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM). MDA-MB-231 cells were cultivated in Leibovitz's L-15 medium. All the media were supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin–amphotericin B solution (Thermo Fisher Scientific, Waltham, MA, US). Cells were maintained in a 37°C humidified incubator containing 95% air and 5% CO_2, except for MDA-MB-231 cells, which were in 95% air without CO_2.

Collection of conditioned medium (CM)

Conditioned medium (CM) was collected from H292 and MCF7 cells. About 10^7 cancer cells seeded in a 10 cm dish were treated with leptin (100 ng/ml) or equal volume of distilled water (vehicle for leptin) for 6 h, then cells were refreshed with 10 ml DMEM. After 18 h, CM was collected and designated as control CM (conCM, without leptin treatment) and activated CM (acCM, with previous leptin treatment), respectively. Collected CM were stored at −80°C before treating on RAW264.7 cells.

Assays for cell viability

MTT (3-(4,5-cimethylthiazol-2-yl)-2,5-dephenyl tetrazolium bromide) assay was performed similarly to our previous study.[36] After treatment, cells were washed with warm phosphate buffered saline (PBS) and incubated with MTT (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (0.5 mg/ml in PBS) for 1 h in a 37°C incubator. Cells were lysed by dimethyl sulfoxide (DMSO) after removing MTT solution, and absorbance was evaluated at 550 nm by a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, UK).

Monocyte adhesion assay

The medium of H292 cells was removed, and THP-1 monocytes which had been labeled with 0.1 μg/ml BCECF/AM (Invitrogen, Carlsbad, US) were added to a monolayer of H292 cells. After incubating for 30 min at 37°C, wells were washed with culture medium for removal of nonadherent THP-1 cells. The adherent THP-1 was photographed under Leica fluorescence microscope.

Transfection

Lipofectamine (LF)3000 (Thermo Fisher Scientific) and siRNA were premixed in serum-free medium for 5 min before applying to the cells. Medium was then refreshed with serum-free medium without LF3000 after 24 hours' transfection before undergoing the experimentation.

Immunoprecipitation and Western blot analysis

Cells were lysed on ice by radioimmunoprecipitation assay buffer (RIPA) for 30 min. For immunoprecipitation, 500 μg of protein samples, 1 μg antibody and 20 μl magnetic Protein G slurry were mixed at 4°C overnight. The sepharose-bound complexes were then washed by buffer solution (10 mM Tris, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100) and boiled with sample buffer (62.5 mM Tris-HCl

(pH6.8), 10% glycerol, 2% (w/v) sodium dodecyl sulfate, 5% 2-beta-mercaptoethanol, 0.05% (w/v) bromophenol blue) at 95°C. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, US) followed by blocking in 7.5% skimmed milk for 1 h. The membrane was incubated in primary antibodies overnight at 4°C and secondary antibodies for 1 h. Protein signals were visualized by enhanced chemiluminescence (EMD Millipore, Billerica, MA, US) using Fujifilm Super RX-N films (Valhalla, NY, US). Signal intensities were analyzed and quantitated by ImageJ .[37]

Enzyme-linked immunosorbent assay (ELISA)

Culture medium was collected after indicated treatment. Secretion of ICAM-1 was measured by ELISA kit (R&D systems, Minneapolis, MN) according to the manufacturers' protocol. The absorbance was measured at 450 nm and corrected at 540 nm by the SpectraMax M5 plate reader.

Quantitative PCR

By using a cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA), total ribonucleic acid (RNA) was extracted by TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA) and was reverse transcribed into deoxyribonucleic acid (DNA). Polymerase chain reaction (PCR) reaction using SYBR® Green Master Mix was performed using StepOne Plus Real-Time PCR Systems (Applied Biosystem, Singapore) under the following conditions: 95°C for 10 min, 45 cycles at 95°C for 10 s and then 60°C for 1 min. The sequence of primers was as follows:

(1) ICAM-1: 5'-CCCCCCGGTATGAGATTGT-3' and 5'-GCCTGCAGTGCCCATTATG-3';
(2) (colony-stimulating factor receptor): 5'-TGGCATCTGGCTTAAGGTGAA-3' and 5'-GAATCCGCACCAGCTTGCTA-3';
(3) NFATc1: 5'-AGGCTGGTCTTCCGAGTTCA-3' and 5'-ACCGCTGGGAACACTCGAT-3';
(4) TRAP: 5'-GATCCCTCTGTGCGACATCA-3' and 5'-CCAGGGAGTCCTCAGATCCA-3';
(5) 36B4: 5'-AGATGCAGCAGATCCGCAT-3' and 5'-GTTCTTGCCCATCAGCACC-3'.

Flow cytometry

After fixation by 4% formaldehyde, cells were incubated with anti-ICAM-1 antibody or isotype immunoglobulin G (IgG) control for 1 h. After a brief wash, cells were incubated with fluorescein isothiocyanate (FITC) conjugated secondary antibody for 30 min. Detection of cell-surface ICAM-1 expression by recording 10 thousand events was performed using a NovoCyte flow cytometer (ACEA Biosciences, San Diego, CA, US).

Tartrate-resistance acid phosphatase (TRAP) staining

RAW264.7 cells were treated with indicated treatment for 6 days. Tartrate-resistance acid phosphatase (TRAP) staining was conducted using the Acid Phosphatase, Leukocyte (TRAP) kit (Sigma, St. Louis, MO, US) for staining osteoclasts, then cells were subjected to hematoxylin counterstaining. The number of TRAP-positive multinucleated osteoclasts (with three or more nuclei) was counted under microscope.

Animals and treatments

The studies were conducted under a protocol approved by the Institutional Animal Care Committee of China Medical University (No. 2017-092-1, Taichung, Taiwan). Female BALB/c mice were purchased (BioLASCO Taiwan Co., Ltd., Taipei, Taiwan). Animals were housed in standard laboratory cages under 12 h light/dark cycle and provided with free access to food and water throughout the experiments. Mice were injected with 4T1-Luc breast cancer cells (5×10^5 in 20 μl PBS) into the bone marrow cavity of tibia under pentobarbital anesthesia. After 7 days, mice were intratibially injected with saline (10 μl), mouse sICAM-1 (0.25 mg/kg), control IgG (1 mg/kg), or ICAM-1-neutralizing antibody (1 mg/kg) twice per week. At day 21, luciferase activity was used to measure tumor growth. After sacrificing the mouse, blood sera were collected for evaluating TRAP activity, and the tibia were analyzed using micro-CT by Skyscan 2211 at 8.8 μm. The voltage was 50 kVp and current was 630 μA at 8 W output. Image reconstruction was performed using reconstruction software, Instarecon. Reconstructed cross-sections were realigned and

region of interest (ROI) was further selected. We standardized the ROI at 12–13 mm (114 slices) above the medial malleolus, and the fibula was excluded. Analysis was performed with ROI of isolated tibia. Thresholding and trabecular bone morphometric analysis were performed using CTAn software (Bruker).

Statistics

Statistical analysis was carried out by GraphPad Prism (San Diego, CA) and SigmaPlot (Karnataka, India) software. Values are expressed as mean ± standard deviation (SD) of at least three independent experiments, with significance defined as $p < 0.05$ analyzed by student's paired t test.

Results

Leptin induces soluble ICAM-1 expression rather than surface ICAM-1

To examine the effect of leptin on the expression of adhesion molecules, H292 cells were treated by different dosages of human recombinant leptin for 6 h or 24 h, and the messenger RNA (mRNA) and protein expressions of ICAM-1and VCAM-1 were evaluated. We found that leptin dose dependently enhanced mRNA and protein expressions of ICAM-1 to 2.21 ± 0.28-fold and 2.09 ± 0.22-fold more than control at 100 ng/ml leptin, respectively [Figure 1(a), (c)] in H292 lung cancer cells. However, protein expression of VCAM-1 was not altered [Figure 1(b)]. In addition, the expression of ICAM-1 on cell surface was evaluated by flow cytometry. From Figure 1(d), we demonstrated that under 100 ng/ml leptin treatment, cell surface ICAM-1 was not affected after 24 h or 48 h. Moreover, examined by monocyte adhesion assay, the number of monocytes adhered to H292 cells was not significantly altered between control group and leptin-treated group [Figure 1(e), (f)]. Since ICAM-1 may be shed as sICAM-1, the supernatant of culture medium was then investigated. We found that the amount of sICAM-1 was significantly increased to 1.87 ± 0.20-fold of control at 100 ng/ml leptin [Figure 1(g)]. On the other hand, leptin also increased ICAM-1 protein expression and soluble ICAM-1 production in MCF7 and MDA-MB-231 breast cancer cells [Figure 3(a–d)]. Leptin dosages we used had been tested for not affecting cell viability by MTT assay (data not shown). These findings suggest that leptin induces sICAM-1 production but not cell-surface ICAM-1 expression.

Leptin-induced increase of soluble ICAM-1 expression is mediated through ObR

The physiological effects of leptin are mediated through its receptor, also known as ObR. We hypothesized that the observed ICAM-1 enhancement induced by leptin is also mediated through ObR. Here, we used siRNA against ObR (siObR) to investigate whether leptin-induced ICAM-1 expression is altered. From Figure 2(a) and (c), protein and mRNA expression of ICAM-1 induced by leptin were markedly decreased by transfection of siObR, but not influenced by control siRNA (siCon) in H292 lung cancer cells. The increased production of sICAM-1 in culture medium induced by leptin was also abolished by siObR [Figure 2(d)]. The silencing effects of siObR and siCon were confirmed by examining protein expression of ObR [Figure 2(b)]. As shown in Figure 3(e–h), we demonstrated that leptin-induced ICAM-1 protein expression and sICAM-1 production were also diminished by silencing ObR in MCF7 and MDA-MB-231 breast cancer cells. These findings indicate that leptin-induced production of sICAM-1 is mediated through the leptin receptor, ObR.

Leptin-induced increase of ICAM-1 expression is mediated through JAK1/2/STAT3/FAK/ERK/GSK3αβ

Leptin plays a vital role in appetite regulation through the Janus kinase (JAK)/signal transducer of activators of transcription (STAT) pathway.[38] In addition, in obesity-linked cancer, leptin-induced JAK/STAT signaling is also involved.[39] In order to explore the leptin signaling pathway leading to increased ICAM-1 expression, we next examined whether JAK/STAT signaling is involved. First, leptin treatment induced significant increase of JAK1 and JAK2 phosphorylation, which peaked within 5 min and attenuated after that [Figure 4(a, b)]. In order to examine the direct interaction between ObR and JAK1/2 upon leptin administration, whole-cell lysates were immunoprecipitated by anti-ObR antibody followed by immunoblotting with phospho-JAK1 and phospho-JAK2. As shown in Figure 4(c), leptin time dependently increased the direct association between ObR and phospho-JAK1 and

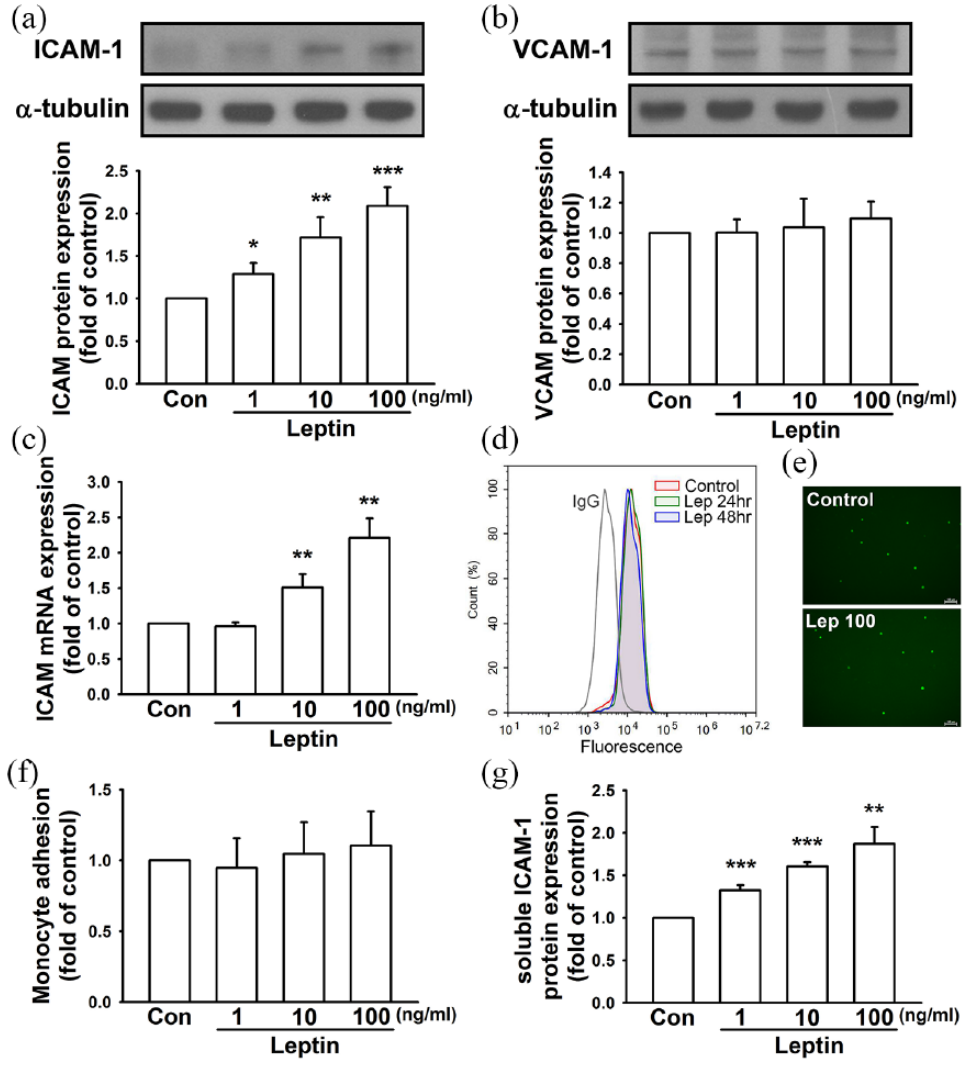

Figure 1. Leptin induces ICAM-1 expression in lung cancer cells.
Leptin dose dependently induced ICAM-1 (a) but not VCAM-1 (b) protein expression examined by Western blot; (c) the expression of ICAM-1 mRNA was significantly enhanced by leptin examined by quantitative PCR; (d) by using flow cytometry, cell-surface expression of ICAM-1 was not affected by leptin (100 ng/ml) treatment for 24 h or 48 h; (e, f) examined by monocyte adhesion assay, the number of THP-1 monocytes adhered to H292 cells was not significantly different between the control and leptin-treated groups; (g) measured by ELISA, sICAM-1 secreted by H292 cells was elevated in a dose-dependent manner. Graphs show mean ± SD of at least three independent experiments. Control values were used as baseline to normalize the treatment group values.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with the control group.
Con, control; ELISA, enzyme-linked immunosorbent assay; ICAM-1, intercellular adhesion molecule-1; mRNA, messenger ribonucleic acid; PCR, polymerase chain reaction; sICAM-1, soluble ICAM-1; SD, standard deviation; VCAM-1, vascular adhesion molecule 1.

phospho-JAK2. Moreover, by inhibiting JAK1/2 by JAK inhibitor I (JAKi), JAK2 by AG490, and STAT3 by Stattic (Selleckchem, Houston, TX, US)., leptin-increased ICAM-1 expression was reversed dose dependently [Figure 4(d)].

ICAM-1 is reported to be upregulated by focal-adhesion kinase (FAK)-driven extracellular signal-regulated kinase (ERK) activation in Toll-like receptor signaling in eosinophils during the inflammatory response,[40] and glycogen synthase kinase 3 beta (GSK3β) activation is also essential for inflammation stimulator-induced ICAM-1 expression.[41] Hence, we next investigated whether FAK, ERK, and GSK3 are involved in leptin-induced ICAM-1 expression. As shown in Figure 5(a–c), leptin treatment markedly increased FAK, ERK, and GSK3αβ phosphorylation, and we

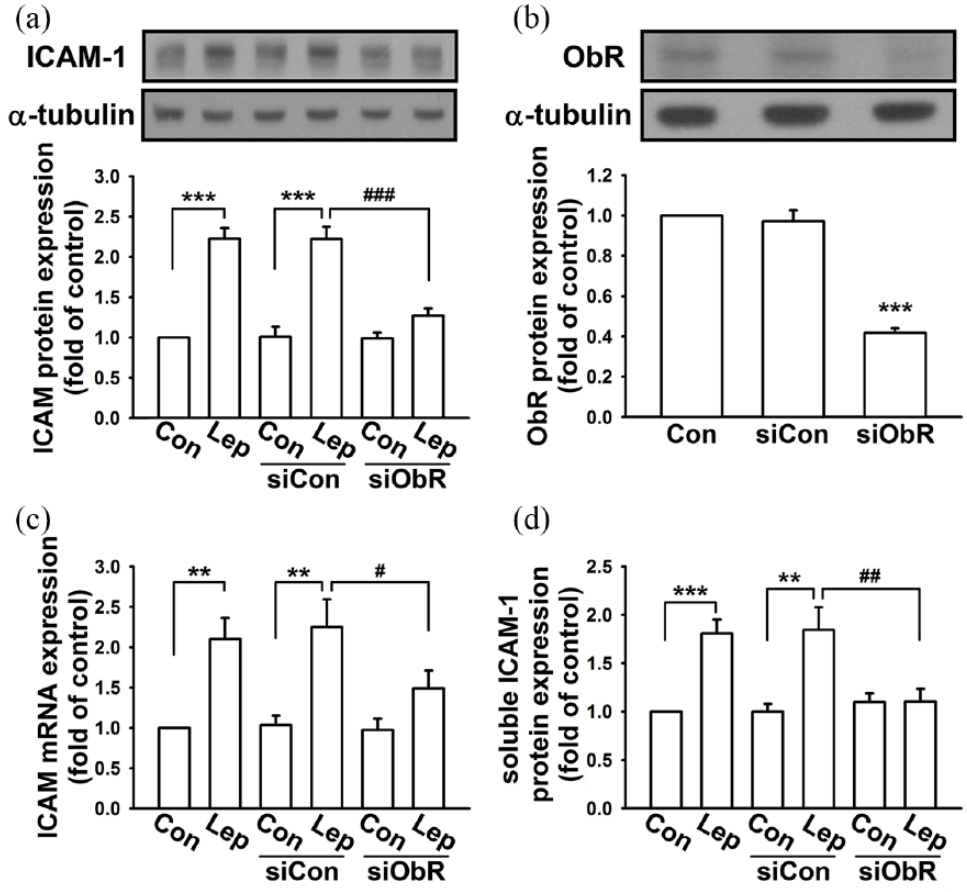

Figure 2. Leptin-induced ICAM-1 expression is mediated through ObR in lung cancer cells.
Leptin-induced increase of ICAM-1 protein (a) and mRNA (c) expression was abolished when ObR was silenced; (b) the silencing effect of siRNA on ObR (siObR) protein expression was confirmed; (d) secreted ICAM-1 enhanced by leptin (100 ng/ml) was reduced by silencing ObR. Graphs show mean ± SD of at least three independent experiments. Control values were used as baseline to normalize the treatment group values.
$^{**}p < 0.01$; $^{***}p < 0.001$ compared with the control group.
$^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ compared with the siCon-transfected leptin-treated group.
Con, control; ICAM-1, intercellular adhesion molecule-1; Lep, leptin; mRNA, messenger ribonucleic acid; SD, ObR, leptin receptor; standard deviation; sICAM-1, soluble ICAM-1; siRNA, small interfering ribonucleic acid; siCon, scrambled control siRNA; siObR, siRNA against ObR.

noticed that phospho (p-)ERK and p-GSK3αβ were continuously increased up to 30 min. Moreover, by inhibiting FAK by PF573228, ERK by U0126, and GSK3αβ by SB216763, leptin-increased ICAM-1 expression were decreased [Figure 5(d)]. These findings suggest that JAK1/2, STAT3, FAK, ERK, and GSK3αβ signalings are involved in enhanced ICAM-1 expression induced by leptin.

Furthermore, we attempted to reveal the upstream and downstream signalings of leptin-induced ICAM-1 expression. After pretreatment of indicated inhibitors for 30 min, leptin was administered for another 5 min for evaluation of FAK phosphorylation, and 10 min for evaluation of ERK and GSK3αβ phosphorylation. As shown in Figure 6(a–c), by inhibiting JAK1/2 by JAKi, JAK2 by AG490, and STAT3 by Stattic, leptin-induced FAK, ERK, and GSK3αβ phosphorylation were all decreased dose dependently. Moreover, by inhibiting FAK by PF573228, leptin-increased ERK and GSK3αβ phosphorylation were both reversed in a dose-dependent manner [Figure 6(d, e)]. Furthermore, by inhibiting ERK by U0126, leptin-induced GSK3αβ phosphorylation was also abolished [Figure 6(f)]. These results indicate that leptin-enhanced ICAM-1 expression was mediated through the JAK1/2/STAT3/FAK/ERK/GSK3αβ axis.

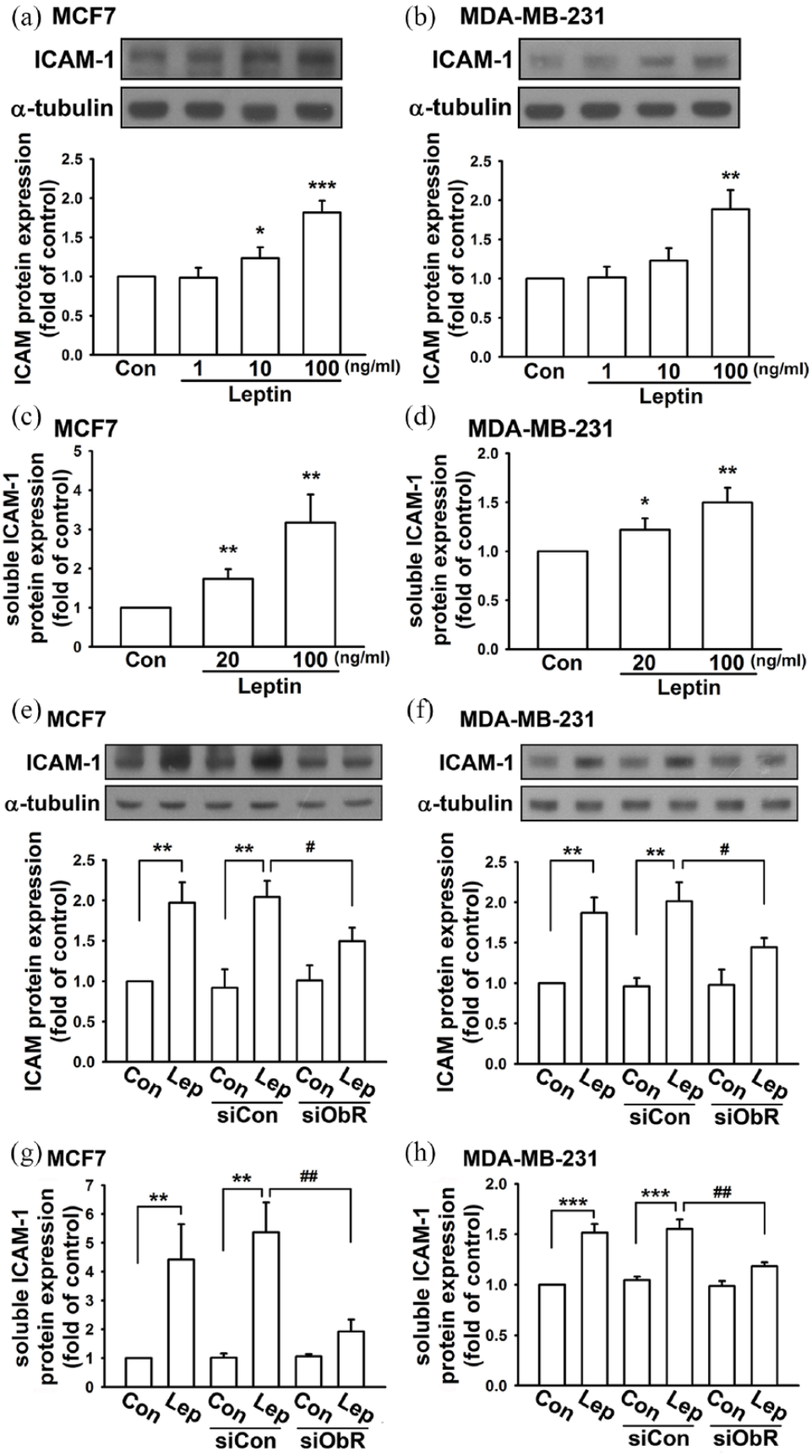

Figure 3. Leptin-induced ICAM-1 expression is mediated through ObR in breast cancer cells.

Figure 3. (Continued)

Leptin dose dependently induced ICAM-1 protein expression examined by Western blot in MCF7 (a) and MDA-MB-231 breast cancer cells (b); measured by ELISA, sICAM-1 secreted by MCF7 (c) and MDA-MB-231 cells (d) were elevated in a dose-dependent manner; leptin-induced increase of ICAM-1 protein expression was decreased when ObR was silenced in MCF7 (e) and MDA-MB-231 breast cancer cells (f); secreted ICAM-1 enhanced by leptin (100 ng/ml) was also reduced by silencing ObR in MCF7 (g) and MDA-MB-231 cells (h). Graphs show mean ± SD of at least three independent experiments. Control values were used as baseline to normalize the treatment group values.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with the control group.
$^{\#}p < 0.05$; $^{\#\#}p < 0.01$ compared with the siCon-transfected leptin-treated group.
Con, control; ELISA, enzyme-linked immunosorbent assay; ICAM-1, intercellular adhesion molecule-1; Lep, leptin; ObR, leptin receptor; SD, standard deviation; sICAM-1, soluble ICAM-1; siRNA, small interfering ribonucleic acid; siCon, scrambled control siRNA; siObR, siRNA against ObR.

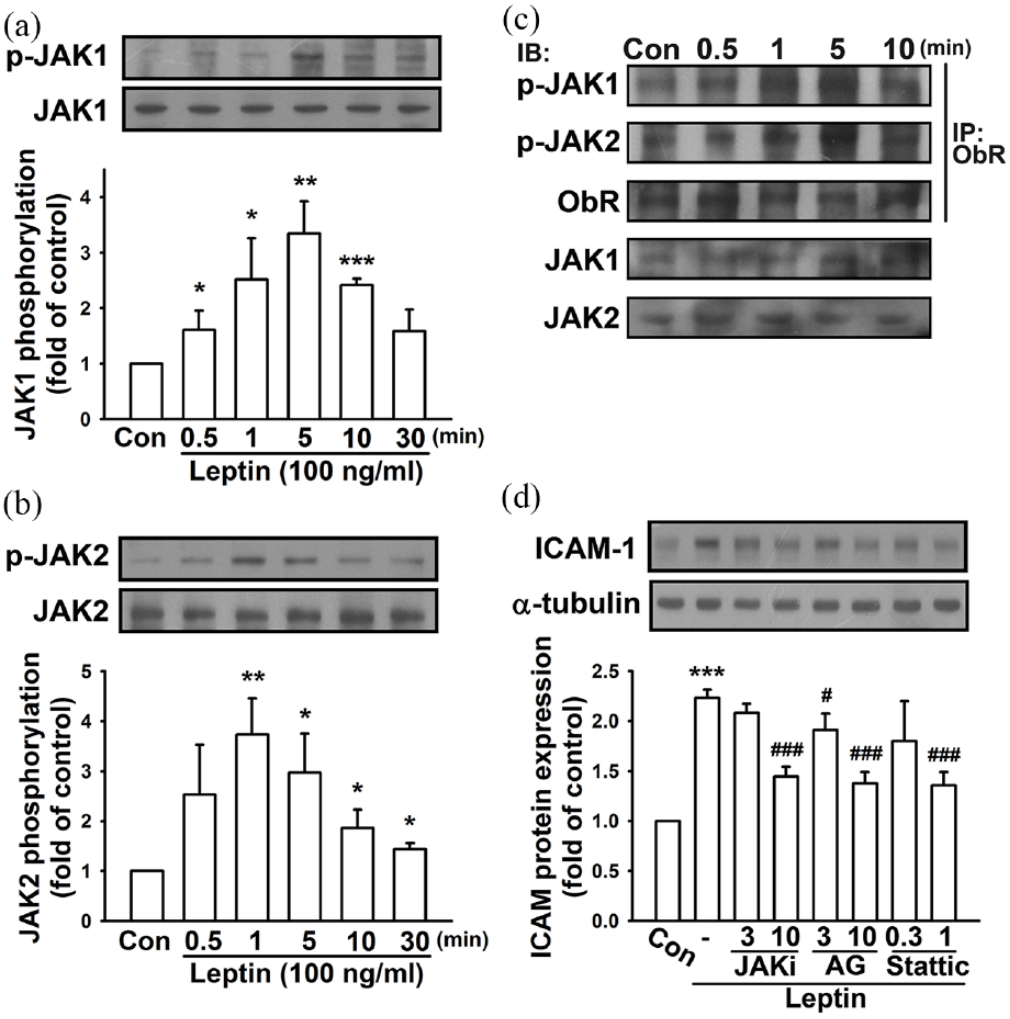

Figure 4. Leptin-induced increased of ICAM-1 expression is regulated by JAK1/2 and STAT3 in lung cancer cells.
The expression of p-JAK1 (a) and p-JAK2 (b) reached peak within 5 min of leptin (100 ng/ml) treatment; (c) whole-cell lysates were immunoprecipitated (IP) using antibody against ObR. The IPs were then immunoblotted (IB) using antibodies against p-JAK1, p-JAK2, and ObR. Leptin time dependently enhanced the interaction of ObR and p-JAK1 and p-JAK2. (d) By inhibiting JAK1/2 by JAK inhibitor I, JAK2 by AG490, and STAT3 by Stattic, leptin-elevated ICAM-1 expression was reversed. Graphs show mean ± SD of at least three independent experiments. Control values were used as baseline to normalize the treatment group values.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with the control group.
$^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$ compared with the leptin-treated group.
Con, control; AG, AG490; ICAM-1, intercellular adhesion molecule-1; JAKi, Janus kinase inhibitor I; ObR, leptin receptor; p-, phospho; SD, standard deviation; STAT, signal transducer of activators of transcription.

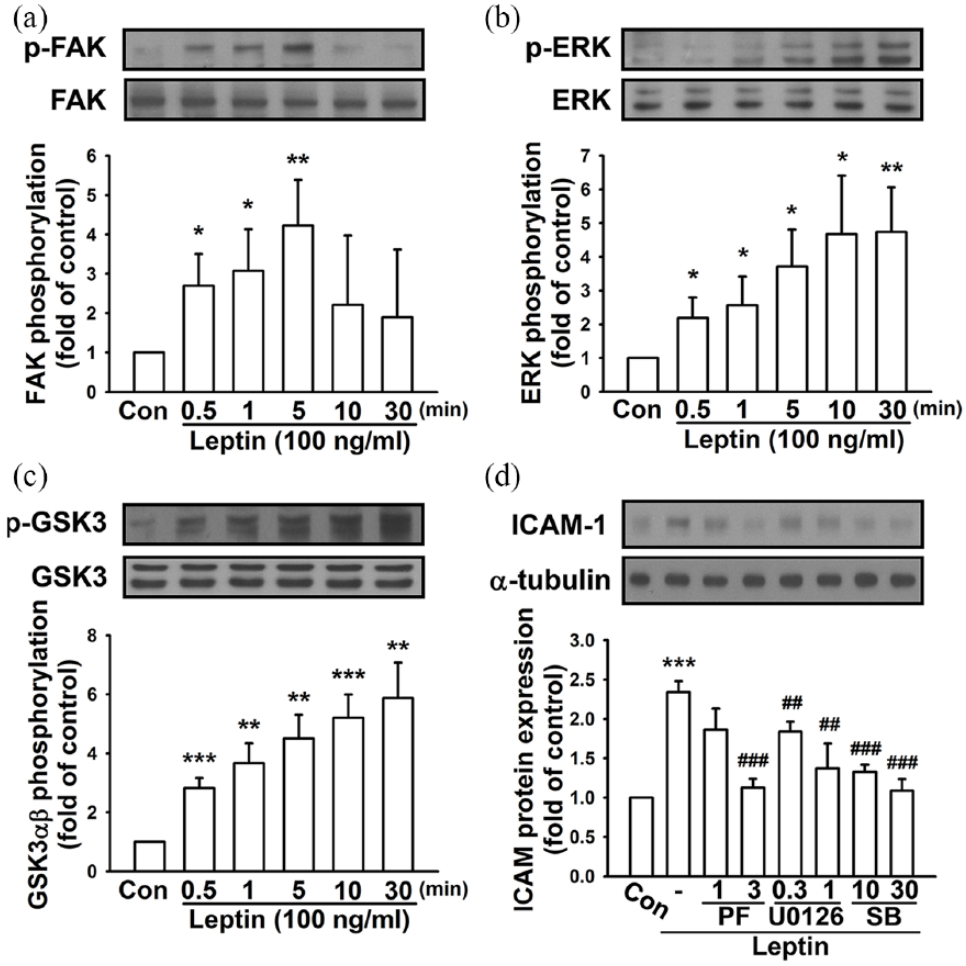

Figure 5. Leptin-induced increase of ICAM-1 expression is regulated by FAK, ERK, and GSK3αβ in lung cancer cells.

The expression of p-FAK (a), p-ERK (b) and p-GKS3αβ (c) were increased under leptin (100 ng/ml) treatment. (d) By inhibiting FAK by PF573228, ERK by U0126, and GSK3αβ by SB216763, leptin-increased ICAM-1 expression was decreased. Graphs show mean ± SD of at least three independent experiments. Control values were used as baseline to normalize the treatment group values.

$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with the control group.

$^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ compared with the leptin-treated group.

Con, control; ICAM-1, intercellular adhesion molecule-1; ERK, extracellular signal-regulated kinase; FAK, focal-adhesion kinase; GSK, glycogen synthase kinase; intercellular adhesion molecule-1; p-, phospho; PF, PF573228; SB, SB216763; SD, standard deviation.

Leptin-induced soluble ICAM-1 promotes osteoclast-like cells formation

Osteolytic bone metastasis is a common occurrence in late-stage lung, breast, and many other cancers.[42] Aberrant expression of signaling molecules by bone-metastatic-prone cancer cells have been shown to recruit preosteoclasts to the site of osteolytic metastasis and induce their differentiation, leading to degradation of the bone and cancer bone metastasis.[43] Previous studies have shown that murine macrophage RAW264.7 cells can be induced to differentiate into mature multinucleated osteoclasts under the stimulation of 50 ng/ml receptor activator of nuclear factor kappa-B ligand (RANKL), and hence RAW264.7 is also considered as a preosteoclast cell.[44,45] To investigate the potential for tumor-secreted sICAM-1 to induce osteoclast differentiation and the expression of osteoclast-specific markers,[46,47] nuclear factor of activated T cells c1 (NFATc1) and TRAP, RAW264.7 cells were stimulated with CM collected from H292 lung cancer cells or MCF7 breast cancer cells which were treated with or without 100 ng/ml leptin previously. As shown in both Figure 7(a, b), 20 ng/ml RANKL alone did not promote osteoclast formation significantly;

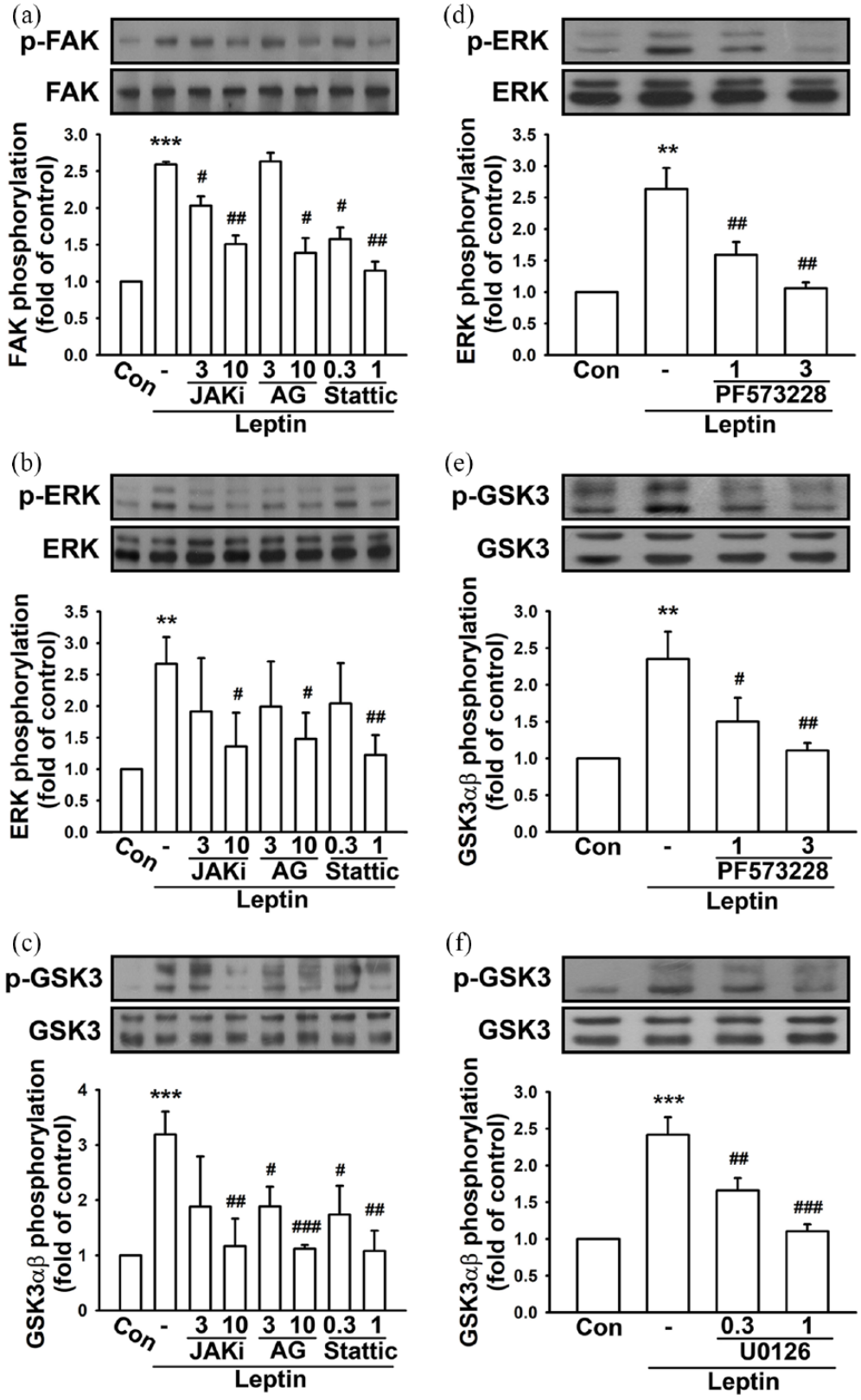

Figure 6. Leptin-induced phosphorylation of the signaling cascade is mediated by the JAK1/2/STAT3/FAK/ERK/GSK3αβ axis in lung cancer cells.
By inhibiting JAK1/2 by JAKi, JAK2 by AG490, and STAT3 by Stattic, leptin-induced phosphorylation of FAK (a), ERK (b), and GSK3αβ (c) were all reversed. By inhibiting FAK by PF573228, leptin-induced phosphorylation of ERK (d) and GSK3αβ (e)

Figure 6. (Continued)

were both decreased. (f) By inhibiting ERK by U0126, leptin-induced GSK3αβ phosphorylation was decreased. Graphs show mean ± SD of at least three independent experiments. Control values were used as baseline to normalize the treatment group values.
$^{**}p < 0.01$; $^{***}p < 0.001$ compared with the control group.
$^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ compared with the leptin-treated group.
Con, control; AG, AG490; ERK, extracellular signal-regulated kinase; FAK, focal-adhesion kinase; GSK, glycogen synthase kinase; JAK, Janus kinase; JAKi, JAK inhibitor I; p-, phospho; PF, PF573228; SB, SB216763; SD, standard deviation.

however, higher concentration of 50 ng/ml RANKL markedly induced multinucleated osteoclast formation and the mRNA expression of NFATc1 and TRAP. In addition, 10 ng/ml recombinant sICAM alone did not enhance osteoclast formation; nevertheless, sICAM, with the presence of low-dose RANKL (20 ng/ml), markedly promoted osteoclast formation, and NFATc1 and TRAP expression. Furthermore, both control CM and activated CM collected from H292 lung cancer cells [Figure 7(a, c)] did not enhance osteoclast formation; however, activated CM, along with 20 ng/ml RANKL significantly promoted multinucleated osteoclast formation and the mRNA expression of NFATc1 and TRAP [Figure 7(e, f)], indicating that additional factors in the CM, other than RANKL, play important roles in inducing osteoclast formation. By adding ICAM-1-neutralizing antibody, osteoclast formation was inhibited, and NFATc1 and TRAP mRNA expression were antagonized, whereas normal IgG served as a negative control. Similar effects were also observed by treating CM collected from MCF7 breast cancer cells [Figure 7(b, d)]. These findings suggest that sICAM-1 secreted by cancer cells played synergistic effect with low-concentration RANKL to enhance multinucleated osteoclast formation, while CM or low-concentration RANKL alone did not induce osteoclast formation.

Soluble ICAM-1 enhances bone osteolysis on tumor-bearing mice

The interaction between cancer cells and bone suggests that factors regulating osteoclast-mediated bone erosion are crucial.[48] Hence, we examined whether sICAM plays important role in tumor-induced osteolysis in the bone microenvironment. 4T1-Luc cells were injected into the bone marrow cavity of tibia, and saline, recombinant sICAM-1, control IgG, or ICAM-neutralizing antibody was intratibially injected twice a week from day 7. At day 21, the luminescence intensity showed that tumor growth was enhanced by sICAM-1 and reduced by ICAM-neutralizing antibody analyzed by the IVIS system [Figure 8(a)]. Some mice with sICAM administration also experienced tumor-cell outburst from the tibia, possibly explaining the phenomenon of larger tumor growth. Micro-CT scans showed that tumor-induced osteolysis was also enhanced by sICAM-1 and reduced in the tibias from mice treated with ICAM-neutralizing antibody [Figure 8(b)]. As shown in Figure 8(c–e), quantitative data also revealed that administration of sICAM-1 significantly induced osteolysis by reducing bone volume and trabecular thickness, and increasing trabecular pattern factor compared with control tumor-bearing mice. As compared with control tumor-bearing mice or IgG-treated mice, treatment of ICAM-neutralizing antibody noticeably reduced osteolysis by increasing bone volume and trabecular thickness and decreasing trabecular pattern factor. Since serum TRAP is reported to be elevated in bone metastasis and serves as a biomarker for monitoring treatment,[49,50] we also examined TRAP activity by analyzing blood sera. As shown in Figure 8(f), we observed that administration of sICAM elevated serum TRAP activity compared with control tumor-bearing mice. On the other hand, treatment of ICAM-neutralizing antibody markedly decreased serum TRAP activity as compared with control tumor-bearing mice or IgG-treated mice. These results provide evidence that sICAM-1 in the bone microenvironment enhances tumor-induced osteolysis, and neutralizing ICAM-1 protects against osteolysis in mouse bone.

Discussion

Obesity increases the risk of a variety of cancers in both males and females in general; however, the role of leptin is crucial. In leptin-deficient (ob/ob) or leptin receptor-deficient (db/db) obese mice, reduced spontaneous tumor development was observed as compared with wildtype mice.[51,52] However, in a diet-induced obese mice model, the increase in body weight is observed with increased body fat and elevated serum leptin

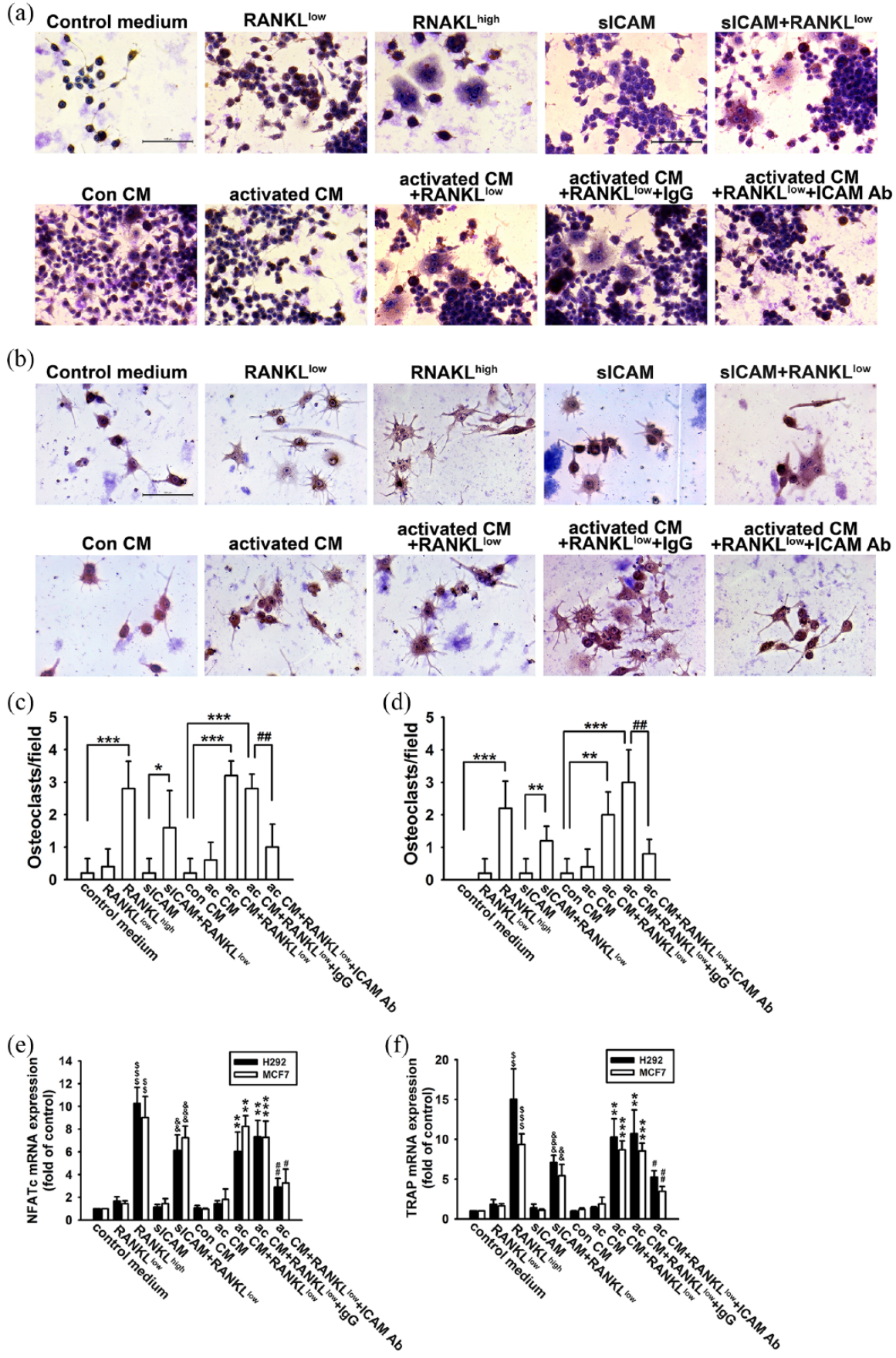
(a)
Control medium
RANKL low
RNAKL high
sICAM
sICAM+RANKL low
Con CM
activated CM
activated CM +RANKL low
activated CM +RANKL low+IgG
activated CM +RANKL low+ICAM Ab
(b)
Control medium
RANKL low
RNAKL high
sICAM
sICAM+RANKL low
Con CM
activated CM
activated CM +RANKL low
activated CM +RANKL low+IgG
activated CM +RANKL low+ICAM Ab
(c)
Osteoclasts/field
control medium
RANKL low
RANKL high
sICAM
sICAM+RANKL low
con CM
ac CM
ac CM+RANKL low
ac CM+RANKL low+IgG
ac CM+RANKL low+ICAM Ab
(d)
Osteoclasts/field
control medium
RANKL low
RANKL high
sICAM
sICAM+RANKL low
con CM
ac CM
ac CM+RANKL low
ac CM+RANKL low+IgG
ac CM+RANKL low+ICAM Ab
(e)
NFATc mRNA expression (fold of control)
H292
MCF7
control medium
RANKL low
RANKL high
sICAM
sICAM+RANKL low
con CM
ac CM
ac CM+RANKL low
ac CM+RANKL low+IgG
ac CM+RANKL low+ICAM Ab
(f)
TRAP mRNA expression (fold of control)
H292
MCF7
control medium
RANKL low
RANKL high
sICAM
sICAM+RANKL low
con CM
ac CM
ac CM+RANKL low
ac CM+RANKL low+IgG
ac CM+RANKL low+ICAM Ab

Figure 7. (Continued)

Figure 7. Soluble ICAM-1 in cancer-cell conditioned medium promote osteoclast-like cell formation.
RAW264.7 cells were stained for TRAP after treatment for 6 days with 20 ng/ml RANKL [RANKL^{low}], or 50 ng/ml RANKL [RANKL^{high}], or conditioned medium from indicated cell lines, or conditioned medium plus 20 ng/ml RANKL with ICAM-1-neutralizing antibody (ICAM-1 Ab) or control IgG, or recombinant sICAM 10 ng/ml with or without 20 ng/ml RANKL. DMEM medium was used as control medium. Control conditioned medium (conCM) was collected from indicated cancer cells without leptin treatment and activated conditioned medium (acCM) was collected from cells previously subjected to 100 ng/ml leptin treatment. Detailed method of collecting conditioned medium was describe in Materials and Methods. (a, c) Representative images and quantification of TRAP-positive osteoclast-like cells from RAW264.7 cells treated with conditioned medium collected from H292 lung cancer cells; (b, d) representative images and quantification of TRAP-positive osteoclast-like cells from RAW264.7 cells treated with conditioned medium collected from MCF7 breast cancer cells. Scale bar represents 100 μm. Graphs show mean ± SD of at least three independent experiments.
$^{**}p < 0.01$; $^{***}p < 0.001$ compared with the indicated group.
$^{\#\#}p < 0.01$ compared with the indicated group.
The mRNA expression of osteoclast-specific markers NFATC (e) and TRAP (f) were also evaluated. Graphs showed mean ± SD of at least three independent experiments.
$^{\$\$}p < 0.01$; $^{\$\$\$}p < 0.001$ compared with the 'control medium' group.
$^{\&\&}p < 0.01$; $^{\&\&\&}p < 0.001$ compared with the sICAM-treated group.
$^{**}p < 0.01$; $^{***}p < 0.001$ compared with the conCM-treated group.
$^{\#}p < 0.05$; $^{\#\#}p < 0.01$ compared with the 'acCM + [RANKL^{low}] + immunoglobulin' group.
acCM, activated conditioned medium; conCM, control conditioned medium; DMEM, Dulbecco's Modified Eagle Medium; ICAM-1, intercellular adhesion molecule-1; ICAM-1 Ab, ICAM-1-neutralizing antibody; IgG, normal immunoglobulin G isotype; mRNA, messenger ribonucleic acid; NFATC, nuclear factor of activated T cells; sICAM, recombinant soluble ICAM; RANKL, receptor activator of nuclear factor kappa-B ligand; [^{high}], high concentration; [^{low}], low concentration; SD, standard deviation; TRAP, tartrate-resistance acid phosphatase.

level, and tumor growth rate is also enhanced in obese mice compared with control mice.[53] It has also been reported that diet-induced obese mice have shortened latency of tumorigenesis.[54,55] Moreover, diet-induced obese mice demonstrate increased distant metastasis formation due to the obese tumor microenvironment stimulating the expansion of metastasis-initiating cells.[56] Another study also showed that diet-induced obese mice with higher serum leptin level experience metabolic alterations potentiating tumor metastasis.[57] Taken together, leptin exerts crucial impact on tumorigenesis and tumor progression in obese patients.[53,58]

The multifunctional role of leptin in cancer biology and pathophysiology has been widely studied in the past few years. Although leptin is mainly regulated by food intake and secreted by adipose tissue, leptin affects both innate and adaptive immunity by activating neutrophils, macrophages, and dendritic cells, and the production of pro-inflammatory cytokines, which induce chronic inflammation and carcinogenesis.[59] Evidence has shown that a functional polymorphism of the leptin gene is associated with earlier onset of non-small cell lung cancer (NSCLC), suggesting that leptin may accelerate cancer initiation.[60] Leptin also induces epithelial–mesenchymal transition (EMT) and promotes metastasis in lung cancer cells. Downregulation of leptin is found to inhibit tumor growth and induce apoptosis in NSCLC.[61] In breast cancers, leptin promotes angiogenesis, cell proliferation, and migration in an autocrine and paracrine manner.[5] It has also been reported that leptin mediates breast cancer stem-cell activity and upregulates the expression of cancer stem-cell markers in breast cancers.[4]

Recent meta-analysis demonstrated that serum and tissue leptin may be involved in pathogenesis of lung and breast cancer metastasis.[12,62] A meta-analysis indicates that the leptin level plays an important role in breast cancer progression and has potential for development as a diagnosis and prognosis biomarker.[12] Another meta-analysis revealed evidence that there is a significant association between leptin expression in lung cancer tissue, and leptin levels are statistically different between a lymph-node metastasis group and non-lymph-node metastasis group.[62] In addition, by gathering clinical data, the association of leptin/leptin receptor expression and bone metastasis was observed in pulmonary adenocarcinoma patients.[63] Another report regarding the mechanism of leptin in promoting EMT, leading to metastasis of lung cancer cells, also revealed that leptin was present at a higher level in bone metastatic lung cancer than in nonmetastatic lung cancer tissue.[14]

The leptin receptor is a member of the class I cytokine receptor super-family, and the JAK/STAT pathway is recognized as the primary

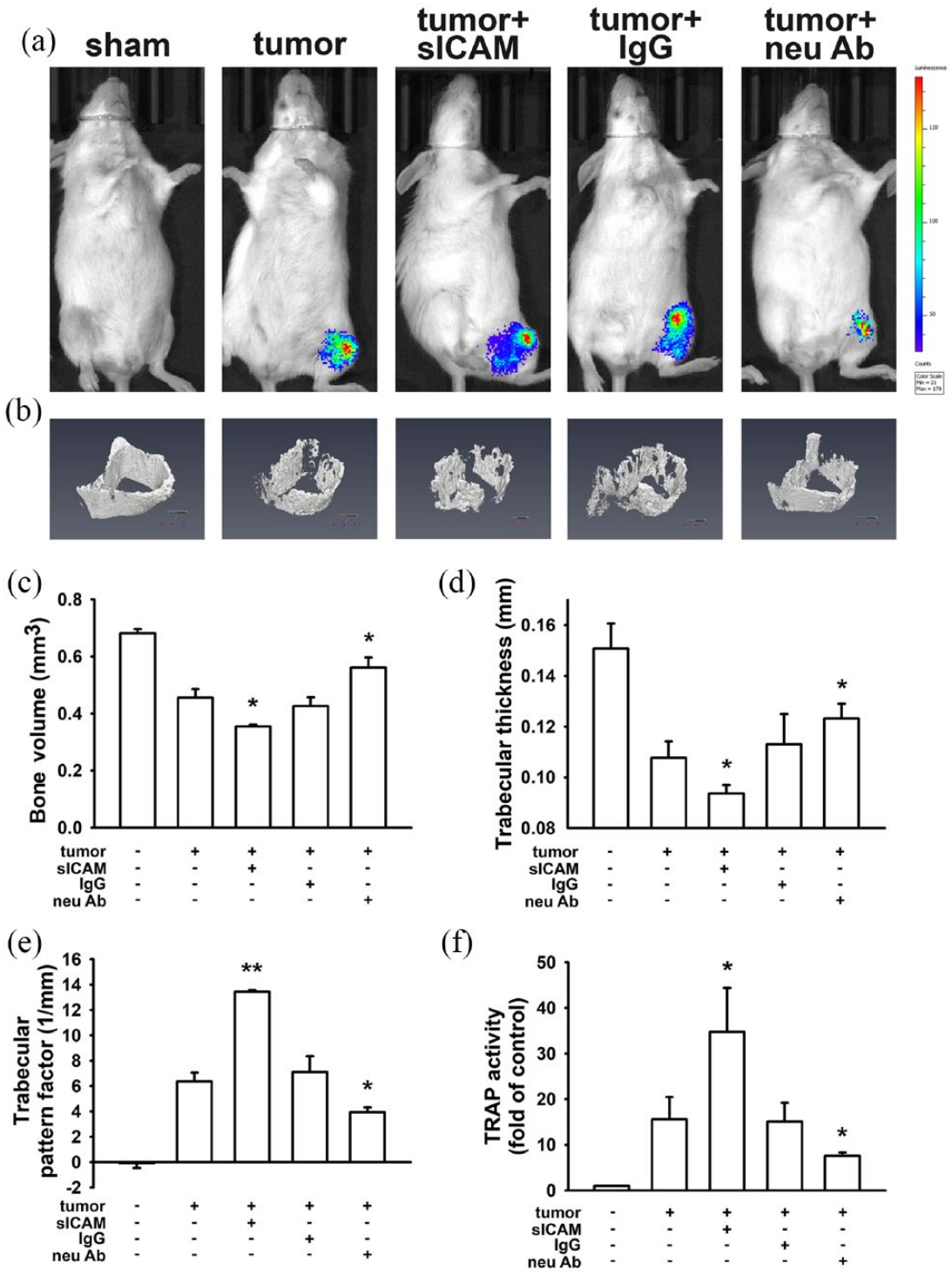

Figure 8. ICAM-1 neutralizing antibody protects against bone loss in intratibial 4T1-injected osteolytic mice.
4T1-Luc cells were injected into the bone marrow cavity of tibia, and intratibial injection of saline, soluble ICAM, IgG, or ICAM-neutralizing antibody were started from 7 days later, twice a week. (a) Evaluation of tumor growth was performed on day 21 by measuring luciferase activity; after sacrificing, tibias were analyzed by micro-CT scans (b), and bone volume (c), trabecular thickness (d), and trabecular pattern factor (e) were evaluated. Blood sera were also collected for evaluating TRAP activity (f). Representative pictures are shown for each group. Scale bar = 0.5 mm. Graphs show mean ± SD of at least three independent experiments.
$^{*}p < 0.05$; $^{**}p < 0.01$ compared with tumor-bearing mice.
CT, computerized tomography; IgG, control immunoglobulin G antibody; ICAM-1, intercellular adhesion molecule-1; neu Ab, ICAM-1 neutralizing antibody; SD, standard deviation; sICAM, recombinant soluble ICAM.

signaling route.[38] Moreover, leptin-induced JAK/STAT signaling is reported to be important in cancer growth.[39] Other than this, alternative pathways, including the MAPK cascade, PI3K, and AMPK are also reported as induced by leptin.[64] On the other hand, ICAM-1 is reported to be upregulated by FAK-driven ERK activation in Toll-like receptor signaling in eosinophils, and is central in initiation of the inflammatory response.[40] Upon FAK activation, Tyr397 is autophosphorylated.[65] In addition, it has been reported that GSK3β activation is essential for inflammation stimulator-induced ICAM-1 expression.[41] Inhibition of GSK3 ameliorates ICAM-1 upregulation induced by the glycolysis metabolite, methyglyoxal.[66] In our study, we demonstrated that leptin activates JAK/STAT signaling, leading to the phosphorylation of FAK/ERK, followed by GSK3β activation, resulting in increased ICAM-1 production.

The expression of ICAM-1 and sICAM-1 in cancer has gained interest in recent years. The sICAM-1 can be derived from the mRNA transcript lacking a transmembrane domain, or generated by proteolytic cleavage of the membrane-bound form by neutrophil elastase or cathepsin G.[67,68] Early studies have investigated the possible differences of sICAM-1 expression in lung cancer patients, patients with benign lung diseases, healthy smokers, and nonsmokers.[69–71] Lung cancer patients exhibit elevated sICAM-1 production, and it has been implicated in tumor progression, metastasis, and therapeutic response.[30,72,73] In other types of tumor, such as breast, liver, and colorectal cancer, increased serum sICAM-1 expression is also associated with extensive tumor burden and poor outcome.[74–76] It has been reported that sICAM-1 binds to cytotoxic lymphocytes, allowing cancer cells to escape immune recognition.[68,77,78] Moreover, sICAM-1 has a substantial role in angiogenesis and promotes tumor growth.[34,79]

About 30–40% of patients with lung cancer developed bone metastases during the course of their disease; the median survival time of patients with this secondary lesion is 7 months.[80] Similarly, bone is the most frequent site for breast cancer metastases and is involved in about 70% of all metastatic patients.[81] Metastatic-cancer-related bone destruction occurs when osteoclasts are presented in higher levels or in cellular activity.[82] Under the regulation of macrophage colony-stimulating factor and RANKL, macrophage lineage cells undergo differentiation into multinucleated osteoclasts.[83] As shown in Figure 7, while a low level of RANKL (20 ng/ml) was insufficient to induce osteoclast formation, RANKL (20 ng/ml) along with CM collected from cancer cells previously treated by leptin significantly enhanced osteoclast formation. Nevertheless, CM alone also did not affect the differentiation process. These results suggest that additional factor(s) were secreted by leptin-stimulated cancer cells which synergized with RANKL to enhance osteoclast formation. When sICAM-1 in activated CM was neutralized by ICAM-1-neutralizing antibody, the synergistic effect was abolished and less osteoclasts were observed. Similar results were observed in both H292 lung cancer CM and MCF7 breast cancer CM. These findings indicate that sICAM-1 secreted by leptin-treated cancer cells synergize with RANKL to promote osteoclast formation and is possibly involved in bone metastasis.

The present study provides evidence to support the role of sICAM-1 in osteoclast differentiation; and leptin-induced sICAM-1 expression in lung and breast cancers suggests multiple avenues for translational application of the findings. Cancer cells releasing leptin and expressing leptin receptor exert potential autocrine or paracrine effects on cancer progression/metastasis on many types of tumor, experimentally and clinically. In particular, we identified sICAM-1 as a downstream effector of leptin, which is insufficient for independently activating osteoclast differentiation but able to induce osteoclastogenesis synergized with low, physiological concentration of RANKL.[45] The essentiality of osteoclasts in bone metastasis is underscored by treatment efficacy targeting osteoclast differentiation and activity.[42,43,84] Our *in vivo* study also showed that sICAM in the local bone microenvironment enhanced bone erosion in tumor-induced osteolysis. Moreover, injection of ICAM-neutralizing antibody noticeably reduced osteolysis in tumor-bearing mice. The effects were seen in reduced decrement of bone volume, trabecular thickness, and lowering trabecular pattern factor and serum TRAP activity. Taken together, these findings establish sICAM as a potential therapeutic target or diagnostic tool of lung and breast cancer bone metastasis.

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Author Contribution

WLY conceived and designed the experiments. SLW and WLY performed the experiments. WLY, CFT, and JHC analyzed the data. WLY, CFT, JHC, CTW, and PCC contributed to writing and editing of the manuscript.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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