Acidic microenvironment induction of interleukin-8 expression and matrix metalloproteinase-2/-9 activation via acid-sensing ion channel 1 promotes breast cancer cell progression

MASAKO NAKANISHI, AYAKA KORECHIKA, HARUKA YAMAKAWA, NAOKO KAWABE, KAZUMA NAKAI and YASUTERU MURAGAKI

Department of Pathology, Wakayama Medical University, Wakayama 641-8509, Japan

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Abstract. The cancer microenvironment exhibits local acidosis compared with the surrounding normal tissue. Many reports have shown that acidosis accelerates the invasiveness and metastasis of cancer, yet the underlying molecular mechanisms remain unclear. In the present study, we focused on acid-induced functional changes through acid receptors in breast cancer cells. Acidic treatment induced interleukin (IL)-8 expression in MDA-MB-231 cells and promoted cell migration and invasion. The acidic microenvironment elevated matrix metalloproteinase (MMP)-2 and MMP-9 activity, and addition of IL-8 had similar effects. However, inhibition of IL-8 suppressed the acid-induced migration and invasion of MDA-MB-231 cells. MDA-MB-231 cells express various acid receptors including ion channels and G protein-coupled receptors. Interestingly, acidic stimulation increased the expression of acid-sensing ion channel 1 (ASIC1), and acid-induced IL-8 was significantly decreased by *ASIC1* knockdown. Moreover, phosphorylation of nuclear factor (NF)-κB was induced by acidic treatment, and inhibition of NF-κB activation reduced acid-induced IL-8 expression. These results suggest that IL-8 induction by an acidic microenvironment promotes breast cancer development and that ASIC1 might be a novel therapeutic target for breast cancer metastasis.

Introduction

An acidic microenvironment is a characteristic feature of tumors resulting from cancer metabolism, inflammation, regional hypoxia, and poor vascular perfusion. Previous studies have reported that local acidosis promotes cancer progression via accelerated cell proliferation and induces the expression of proteinases such as cathepsin K, matrix metalloproteinase (MMP)-2 and MMP-9, resulting in local invasion (1-5). Rofstad *et al* showed that acidic conditions promoted the metastasis of human melanoma cells in athymic nude mice *in vivo*, which was accompanied by increased secretion of protease and pro-angiogenic factors (6). In addition, Nishisho *et al* demonstrated that expression of a3 isoform vacuolar type H^+-ATPase, which regulates proton transport and creates extracellular acidosis, promotes distant metastasis of B16 mouse melanoma cells (7). However, the regulatory factors that function between the acidic microenvironment and cancer cells are not fully understood.

Interleukin (IL)-8, also known as CXCL8, is a pro-inflammatory CXC chemokine that activates multiple intracellular signaling pathways through binding to receptors (CXCR1 and CXCR2). During inflammation, IL-8 is produced in macrophages, epithelial cells, and fibroblasts following stimulation of IL-1 and/or tumor necrosis factor (TNF)-α. Secreted IL-8 induces the recruitment and activation of neutrophils and lymphocytes. Increased expression of IL-8 and/or its receptors in cancer cells has been observed (8,9), which suggests that IL-8 is a critical molecule in cancer development. IL-8 signaling promotes the activation of the primary effectors, phosphatidylinositol-3 kinase or phospholipase C, which in turn activate AKT, PKC, and the MAPK signaling cascade (8). These pathways stimulate the proliferation, survival, chemoresistance, adhesion, and migration of cancer cells. Srivastava *et al* demonstrated that IL-8 inhibition drastically decreased the proliferation, migration, and invasiveness of melanoma cells. Furthermore, IL-8 depletion inhibited endothelial cell proliferation and the formation of capillary-like structures (10). We previously demonstrated that IL-8 accelerated the proliferation of lymphatic endothelial cells, and inhibition of IL-8 diminished tube formation and cell migration (11). While various roles for IL-8 in cancer progression have been reported, the mechanism of IL-8 upregulation and its association with the acidic microenvironment remain to be elucidated.

Extracellular acidosis stimulates cells via ion channels such as transient receptor potential vanilloid subtype 1 (TRPV1), acid-sensing ion channel 1-4 (ASIC1-4), and proton-sensing

Correspondence to: Dr Masako Nakanishi, Department of Pathology, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8509, Japan
E-mail: n-masako@wakayama-med.ac.jp

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G-protein coupled receptors (GPCRs) including GPR4, GPR65 (also known as TDAG8), GPR68 (OGR1), and GPR132 (G2A). TRPV1 and ASICs are non-selective cation channels that are activated by extracellular protons and mainly expressed in peripheral sensory and central nervous system neurons. TRPV1 is activated by capsaicin, noxious heat, and protons (12), and an acidic microenvironment is thought to be responsible for TRPV1-mediated pain signals in patients with inflammation and cancer (13,14). In the peripheral nervous system, ASICs are involved in nociception such as pain signaling and mechanosensation. In the central nervous system, ASICs have various roles; ASIC1a is involved in synaptic plasticity, learning, and memory, and ASIC2a is required for the maintenance of retinal integrity and the survival of neurons in ischemia (15).

Previous studies have reported that acid-sensing receptors are expressed in tumor cells and are involved in cell proliferation, apoptosis, angiogenesis, and metastasis (1,3,16,17). Studies have demonstrated higher expression of ASIC1 in high-grade glioma cells compared with normal astrocytes (18,19). Berdiev *et al* demonstrated that ASIC1 and ASIC2 are involved in the proliferation and migration of glioblastoma cells (20). Furthermore, pharmacological block or knockdown of ASIC1 inhibited acid-induced currents and cell migration in glioblastoma cells (18,21). These data suggest the importance of the interactions between the acidic microenvironment and tumor cell-expressing ASICs in cancer development. However, the expression of acid-sensing receptors in breast cancer cells and the role of these receptors in cancer progression remain unclear.

In the present study, we investigated the morphological and functional changes in breast cancer cells stimulated by acidic conditions. We found that acidosis induced the migration and invasion of MDA-MB-231 cells, and acid-induced IL-8 expression was responsible for these phenomena. We also found that the expression of ASIC1 was upregulated by acidic stimulation and ASIC1 was responsible for the induction of IL-8. Our findings will increase the understanding of the mechanism of acid-mediated progression in breast cancer.

Materials and methods

Cell culture. The MDA-MB-231 human cell line, which is derived from triple-negative breast cancer, was purchased from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA) with 10% fetal bovine serum (FBS; Biowest) and 1% penicillin-streptomycin solution (Thermo Fisher Scientific K.K.) at 37˚C in a humidified 5% CO_2 incubator.

Acidic stimulation. For the acidic stimulation, pH 6.4 medium was prepared using lactic acid (FujiFilm Wako Pure Chemical Corp.). Many studies have shown that the extracellular pH ranges from 6.2 to 6.9 in malignant tumors (22-24). Furthermore, the pH sensitivity (pH_{50}) of ASIC1a is pH 5.8-6.8 (25). We first investigated the IL-8 mRNA expression in MDA-MB-231 cells under pH 5.5, 6.0, 6.4, and 6.8. We observed the most prominent expression at pH 6.0 and 6.4 (data not shown). In addition, we previously performed studies on lymphatic endothelial cells cultured under pH 6.4 conditions (11). Therefore, we selected a condition of pH 6.4 for the present study.

Cell staining. For visualization of cellular morphology, cells were fixed with 4% paraformaldehyde and incubated with rhodamine-phalloidin (Cytoskeleton, Inc.) for 30 min at room temperature. After washing with PBS, the cells were counterstained with DAPI (Vector Laboratories, Inc.).

Cell proliferation assay. Cell growth was measured using a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc., Japan) according to the manufacturer's instructions. MDA-MB-231 cells were seeded in 96-well plates and treated with control, pH 6.4, or control medium containing IL-8 (Peprotech, Inc.). After incubation, the CCK-8 reagent was added to each well and cells were incubated for another 2 h. Absorbance was measured using a microplate reader.

In vitro wound healing assay. MDA-MB-231 cells ($5x10^5$/well) were plated in 6-well plates and incubated for 24 h. After a complete monolayer had formed, cells were then cultured with serum-free DMEM for 8 h. The monolayers were scratched using a 200-μl plastic tip, and then the media were replaced with fresh control (pH 7.4) or acidic (pH 6.4) DMEM without serum. Migration and cell movement throughout the wound area was observed (at 12, 24, 30, and 48 h after scratching) with a phase-contrast microscope and images were obtained (x100 magnification). The percentage of the remaining wounded area was measured on the images.

Transwell migration and invasion assays. For the migration and invasion assays, 24-well Transwell plates (Corning Incorporated) were used. First, sub-confluent MDA-MB-231 cells were incubated with serum-free media (control or pH 6.4) in 6-cm dishes for 8 h. Cells ($2x10^4$/well) were then resuspended in the appropriate media and placed in the polycarbonate membrane inserts (8-μm pore size) coated without or with Matrigel (Corning Incorporated) for the migration or invasion assays, respectively. The bottom wells contained DMEM with 10% FBS. After incubation for 18 h (for migration assays) or 24 h (for invasion assays), cells on the upper surface of the membrane were removed. The migrated or invaded cells on the lower side were fixed with 4% paraformaldehyde and stained with Giemsa. The number of cells that passed to the lower side was counted in five fields per membrane using a light microscope (x100 magnification) (Nikon Solutions Co., Ltd.).

Gelatin zymography. MDA-MB-231 cells were cultured in 6-well plates and grown to 70% confluence. Cells were washed twice with serum-free DMEM and then cultured for 48 h with control or pH 6.4 serum-free media. Conditioned media were concentrated 30-fold on a Vivaspin 6 (10 kDa cut-off; Cytiva). MMP activity was assessed by gelatin zymography (Gelatin-zymography kit, Cosmobio Co., Ltd.) according to the manufacturer's protocol.

RNA preparation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using a NucleoSpin RNA kit (Macherey-Nagel) and single-stranded cDNA was synthesized using PrimeScript RT Master Mix (Takara Bio Inc.) according to the manufacturer's instructions. Real-time PCR was performed using the SYBR Green detection protocol and Thermal Cycler Dice Real Time System (Takara Bio Inc.).

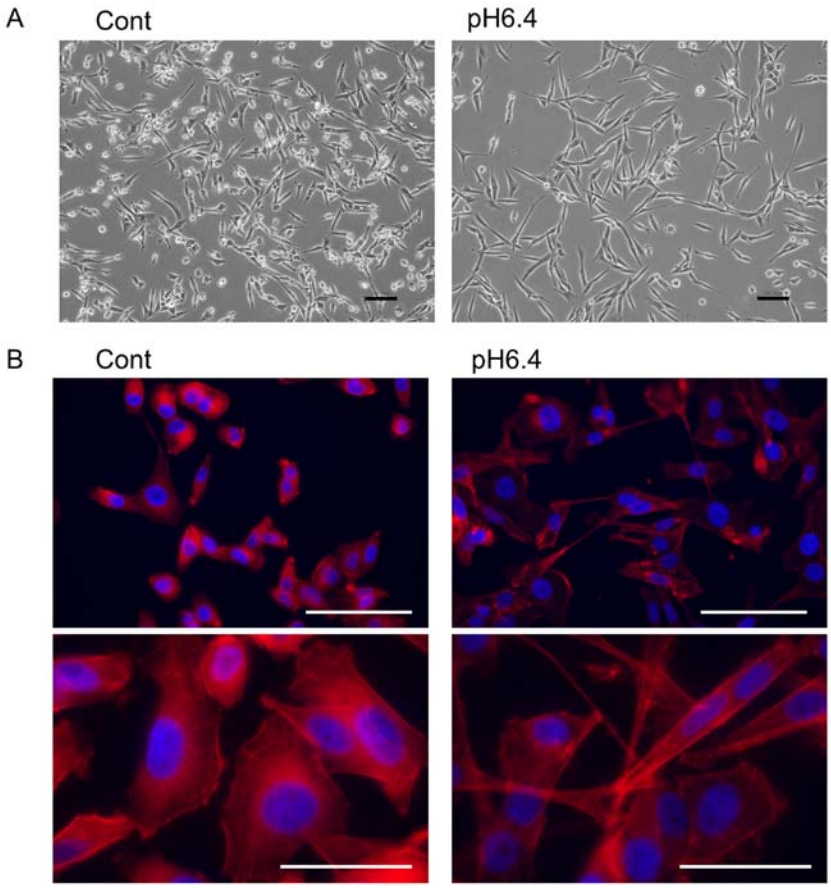

Figure 1. Morphological characteristics of MDA-MB-231 cells cultured in acidic conditions (pH 6.4). (A) Compared with controls (left), MDA-MB-231 cells cultured in acidic medium for 24 h (right) changed into spindle-shaped cells with prominent processes. Scale bar, 100 μm. (B) MDA-MB-231 cells were cultured in control or low pH medium. Actin filaments were visualized by rhodamine-phalloidin staining (red) and nuclei were stained with DAPI (blue). Scale bar, 50 μm.

The following conditions were used for quantitative PCR: initial denaturation for 30 sec at 95˚C, followed by 40 cycles of denaturation for 5 sec at 95˚C and annealing for 30 sec at 60˚C. The primers used for amplification were as follows: IL-1β forward, 5'-AGGCACAAGGCACAACAGGCT-3' and reverse, 5'-AACAACTGACGCGGCCTGCC-3'; IL-6 forward, 5'-TGGCTGCAGGACATGACAA-3' and reverse, 5'-TGAGGTGCCCATGCTACATTT-3', IL-8 forward, 5'-CGTGGCTCTCTTGGCAGCCTTC-3' and reverse, 5'-TTCCTTGGGGTCCAGACAGAGCTC-3'; ASIC1 forward, 5'-GGATGGAGGTCTACCCTCGA-3' and reverse, 5'-GACCTCAGCTTCTGCCTGTCA-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-CCCTTCATTGACCTCAACTACATGGT-3' and reverse, 5'-TGATGACAAGCTTCCCGTTCTCAG-3'. Relative gene expression was determined and normalized to GAPDH mRNA expression.

RT-PCR. Total RNA from MDA-MB-231 cells was extracted using a NucleoSpin RNA kit (Macherey-Nagel) and single-stranded cDNA was synthesized using PrimeScript RT Master Mix (Takara Bio Inc.) according to the manufacturer's instructions. PCR amplifications were performed using DNA polymerase kit (Takara Ex Taq; Takara Bio Inc.) and the following primer pairs: TRPV1 forward, 5'-CTCCTACAACAGCCTGTAC-3' and reverse, 5'-AAGGCCTTCCTCATGCACT-3'; ASIC1 forward, 5'-GGATGGAGGTCTACCCTCGA-3' and reverse, 5'-GACCTCAGCTTCTGCCTGTCA-3'; ASIC2 forward, 5'-GCAACCTAACCCGCTACAAC-3' and reverse, 5'-AGCAGGCAATCTCCTCCAAG-3'; ASIC3 forward, 5'-TATGAGACCGTGGAGCAG-3' and reverse, 5'-TGTGTGACAAGGTAGCAGG-3'; OGR1 forward, 5'-ACTTCGGCTACCTGCAGATCAA-3' and reverse, 5'-AGCCCACGCTGATGTAGATGTT-3'; TDAG8 forward, 5'-TGCCGTTGATCGGTATTTGGCT-3' and reverse, 5'-TTGCATAGCCTGTACACGTCCT-3'; GPR4 forward, 5'-ATACCACAGCTCACTGGCTTTC-3' and reverse, 5'-TCATGGCTTTGGCTGTGCTGTT-3'; G2A forward, 5'-TGCAACATCTACGTCAGCATCC-3' and reverse, 5'-ATCTGCAGCATGTCAAAGCAGG-3'; GAPDH forward, 5'-CCCTTCATTGACCTCAACTACATGGT-3' and reverse, 5'-TGATGACAAGCTTCCCGTTCTCAG-3'. The following thermocycling conditions were used: initial denaturation for 5 min at 95˚C, followed by 30 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec, and elongation at 72˚C for 30 sec. The PCR products were separated on 2% agarose gels containing ethidium bromide and visualized under ultraviolet light.

Enzyme-linked immunosorbent assay (ELISA). MDA-MB-231 cells ($1x10^5$/well) were seeded in 24-well plates and incubated with serum-free medium for 12 h. The medium was changed

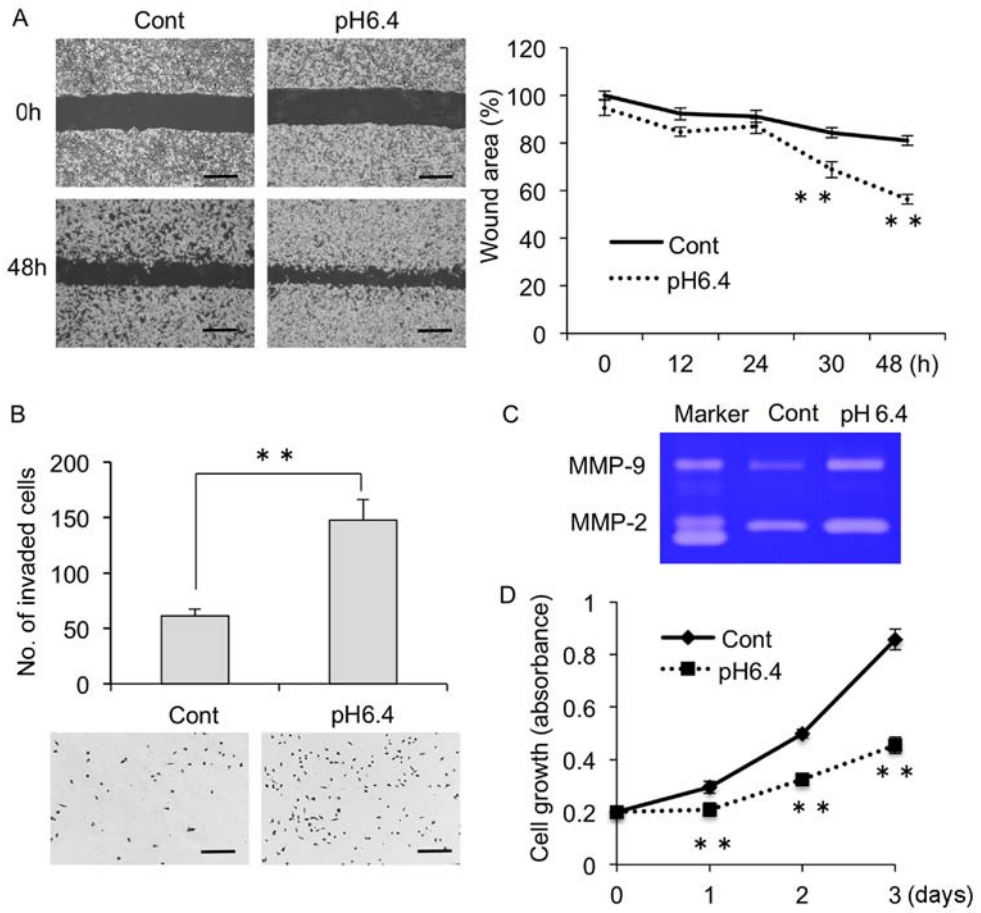

Figure 2. Acidic conditions induce migration and invasion abilities in MDA-MB-231 cells. (A) Cell migration assays were performed in MDA-MB-231 cells cultured in the indicated medium using a wound-healing assay. Scale bar, 500 μm. At various time points, the remaining wound area was determined. Data are expressed as mean ± SD. **P<0.01 vs. the control. (B) Invasion activity was evaluated using a Transwell assay. Cells that invaded into Matrigel and migrated through the polycarbonate membrane to the lower surface of the membrane were stained (lower figures) and quantified (top graph). Scale bar, 500 μm. Data are expressed as mean ± SD. **P<0.01 vs. the control. (C) Gelatin zymography of conditioned media of MDA-MB-231 cells cultured in control or low pH media. (D) Cell proliferation analysis of MDA-MB-231 cells cultured in control or low pH media. Absorbance data are expressed as mean ± SD. **P<0.01 vs. the control. MMP, matrix metalloproteinase.

to fresh control or pH 6.4 serum-free media and then cells were incubated for 24, 48, or 72 h. Culture supernatants were collected and analyzed for IL-8 levels using a human IL-8 ELISA kit (Quantikine ELISA; R&D Systems) according to the manufacturer's instructions.

Knockdown of IL-8 and ASIC1. Small interfering RNA (siRNA) targeting IL-8 (siIL-8), ASIC1 (siASIC1) and negative control siRNA (siNC) were purchased from Thermo Fisher Scientific K.K. MDA-MB-231 cells were cultured in DMEM containing 10% FBS without antibiotics, and siIL-8 was transfected using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific K.K.) according to the manufacturer's protocol. After incubation for 12 h, the transfected cells were pre-incubated with serum-free control or pH 6.4 media for 8 h and then used for migration or invasion assays. siASIC1 was transfected by electroporation using the Neon Transfection System (Thermo Fisher Scientific K.K.) according to the manufacturer's protocol. Knockdown of IL-8 and ASIC1 were examined by real-time PCR after 24 h.

Anti-IL-8 treatment. Anti-human IL-8/CXCL8 purified polyclonal goat IgG and normal goat IgG control were purchased from R&D Systems, Inc. For migration and invasion assays, MDA-MB-231 cells were suspended in serum-free control or low pH media with anti-IL-8 antibody (100 ng/ml) or control IgG, and seeded in the inserts. After incubation for 18 h (for migration assays) or 24 h (for invasion assays) at 37˚C, migrated or invaded cells on the lower side were investigated.

Treatment with NF-κB inhibitors. The NF-κB inhibitor BAY11-7082 and dexamethasone were purchased from FujiFilm Wako Pure Chemical Corporation. MDA-MB-231 cells were preincubated for 10 min with BAY11-7082 (10 μM) or dexamethasone (1 μM), and treated with control or low pH media containing inhibitors or vehicle (ethanol) for 24 h at 37˚C.

Western blotting. MDA-MB-231 cells were rinsed with PBS and lysed in lysis buffer (FujiFilm Wako Pure Chemical Industries Ltd.). The lysates were centrifuged at 15,000 x g for 20 min at 4˚C and then boiled in SDS sample buffer for 5 min. The proteins were separated by 4-20% SDS-polyacrylamide gel electrophoresis (Bio-Rad Laboratories) and transferred to polyvinylidene difluoride membranes. After blocking in 5% BSA for 2 h at room temperature, the membranes

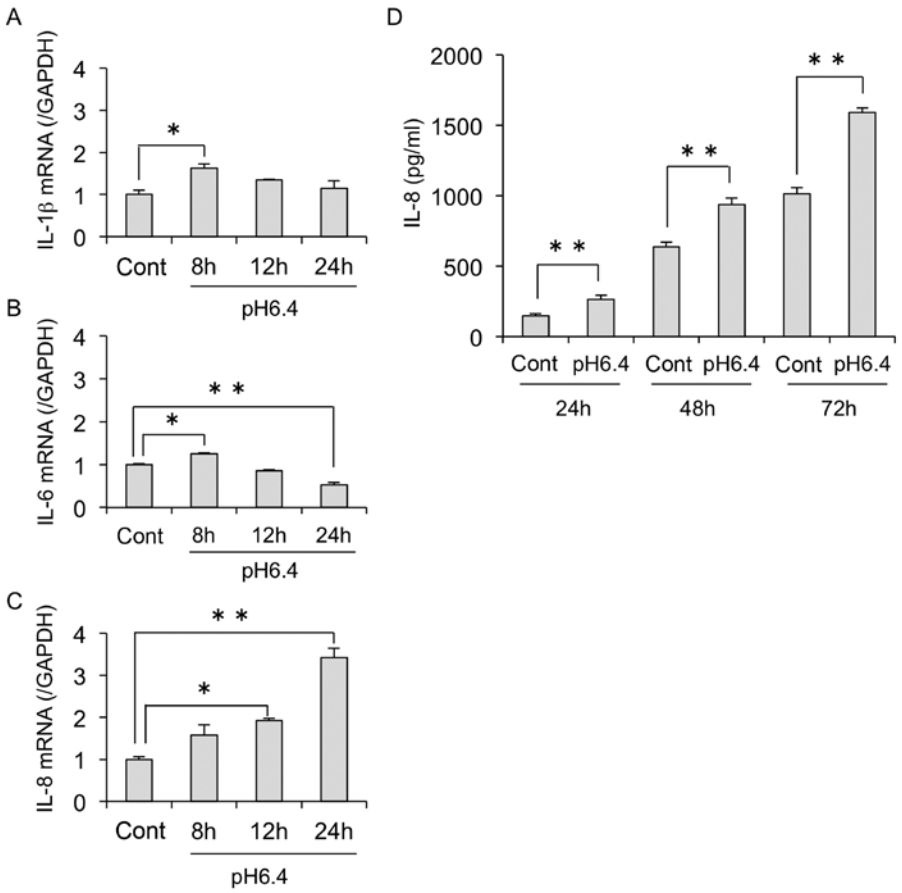

Figure 3. Upregulation of IL-8 expression in MDA-MB-231 cells by acidic stimulation. MDA-MB-231 cells were treated with pH 6.4 medium for the indicated times. Total RNA isolated from cells was used for real-time PCR analysis of *IL-1B* (A), *IL-6* (B), and *IL-8* (C). Data are shown as the fold expression normalized to the controls (mean ± SD). *P<0.05, **P<0.01 vs. the control. (D) IL-8 protein levels in the culture supernatant of MDA-MB-231 cells were measured by ELISA. Data are shown as mean ± SD. **P<0.01 vs. the respective control group. IL, interleukin; MMP, matrix metalloproteinase.

were incubated with primary antibodies, followed by incubation with horseradish peroxidase-coupled anti-rabbit IgG antibodies, and then bands were visualized using the ECL detection kit (Amersham ECL Prime, Cytiva). Primary antibodies included rabbit anti-nuclear factor (NF)-κB p65 (cat. no. 8242; 1:1,000 dilution, Cell Signaling Technology Inc.), rabbit-anti-phospho-NF-κB p65 (cat. no. 3033; 1:1,000 dilution, Cell Signaling Technology), and mouse anti-β-actin (product no. A1978; 1:4,000 dilution, Sigma-Aldrich; Merck KGaA). β-actin was used for reference protein. Secondary antibodies were as follows: HRP-conjugated goat anti-rabbit IgG (H+L) (product code 111-036-003, 1:5,000 dilution, Jackson Immuno Research Laboratories Inc.) and HRP-conjugated goat anti-mouse IgG (H+L) (product code 115-036-003, 1:10,000, Jackson Immuno Research Laboratories Inc.). The bands were analyzed using Densitograph software CS Analyzer ver 3.0 (Atto Corporation).

Statistical analysis. Data are presented as the mean ± SD. Statistical analyses were performed using data obtained from three independent experiments. Student's t-test was used to compare data between two groups. For more than three groups, we used a one-way ANOVA followed by the Tukey-Kramer test or Dunnett test (JMP Pro software version 14.1, SAS Institute Inc.). P-values of <0.05 were considered to indicate statistical significance.

Results

Acidic conditions induce morphological changes, migration activity, and invasion activity of MDA-MB-231 cells. As shown in Fig. 1A, the culture of MDA-MB-231 cells in low pH medium (pH 6.4) induced morphological changes to spindle-shaped cells with processes. These structures were also detected by Phalloidin staining (Fig. 1B). Wound healing assay revealed that the migration activity was markedly increased in the MDA-MB-231 cells cultured in pH 6.4 medium (Fig. 2A). We also found that the invasion activity was increased in cells cultured in acidic conditions, as determined by invasion assays using a Transwell plate coated with Matrigel (Fig. 2B). In addition, conditioned medium from cells cultured in acidic medium showed increased activity of gelatinases such as MMP-2 and MMP-9 compared with medium from cells cultured in normal medium (Fig. 2C). However, proliferation was suppressed in cells cultured under acidic conditions compared with the control cells (Fig. 2D).

Increased IL-8 expression in MDA-MB-231 cells under acidic conditions. To identify the potential factors involved in the altered activity of cells under acidic conditions, we analyzed changes in the expression of several cytokines induced by acidic stimulation. The results showed that acidic conditions upregulated the mRNA expressions of *IL-8* but not *IL-1β*

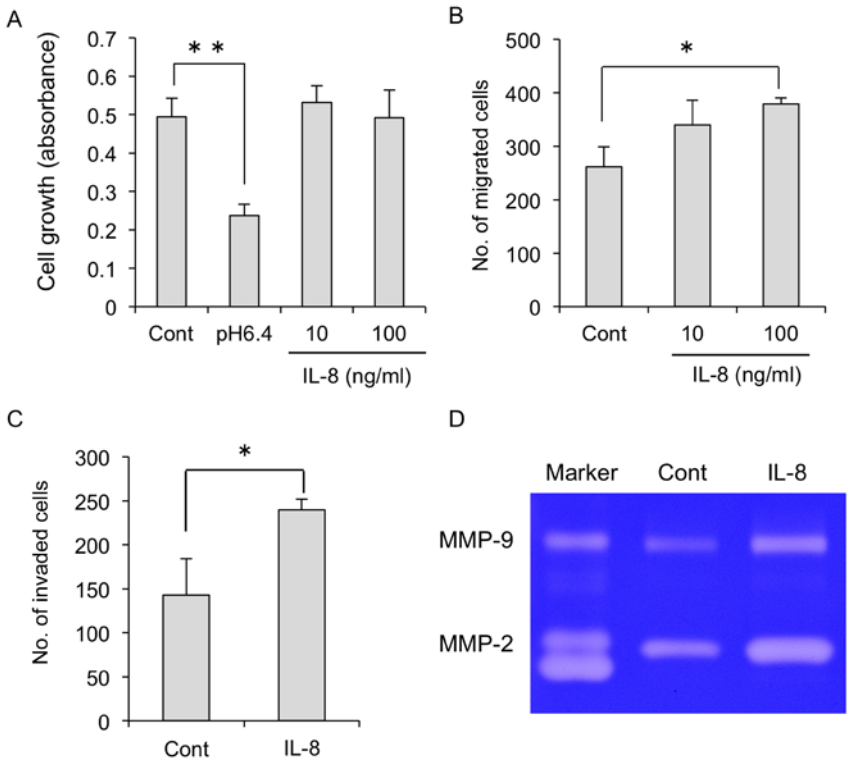

Figure 4. IL-8 directly increases migration, invasion and MMP activity in MDA-MB-231 cells. (A) Cells were treated with low pH medium or IL-8 (10 or 100 ng/ml) for 3 days, and cell proliferation was measured using a CCK-8 kit. The absorbance data are expressed as mean ± SD. **P<0.01 vs. the control. The effects of IL-8 on migration (B) and invasion (C) were evaluated by Transwell assay. *P<0.05 vs. the control. (D) Gelatin zymography for supernatants of MDA-MB-231 cells cultured in serum-free medium with or without IL-8 (100 ng/ml). IL, interleukin; MMP, matrix metalloproteinase.

and *IL-6* in the MDA-MB-231 cells (Fig. 3A-C). In addition, we found that IL-8 secretion was also increased in a time-dependent manner in acidic conditions, as determined by ELISA (Fig. 3D).

IL-8 is critical for migration and invasion of MDA-MB-231 cells. We next verified the direct effects of IL-8 on the migration and invasion of MDA-MB-231 cells. As shown in Fig. 4A, IL-8 had no effects on cell proliferation. In contrast, migration and invasion were significantly increased by IL-8 addition (Fig. 4B and C). Of note, gelatinase activity was also increased in MDA-MB-231 cells treated with IL-8 (Fig. 4D).

Therefore, we next confirmed the role of IL-8 by knockdown experiments using siRNA targeting human *IL-8* in MDA-MB-231 cells. We confirmed no significant differences in *IL-8* mRNA expression between the untransfected cells and those transfected with the negative control siRNA (siNC). The expression of *IL-8* mRNA was clearly decreased in cells transfected with *IL-8* siRNA (siIL-8 #1, #2, #3) compared with the siNC-transfected cells (Fig. 5A). As shown in Fig. 5B and C, acidic conditions increased the migration and invasion of the MDA-MB-231 cells, while knockdown of *IL-8* inhibited these effects. Furthermore, similar results were confirmed when cells were treated with an anti-IL-8 antibody (Fig. 5D and E). Treatment with the anti-IL-8 antibody markedly suppressed acid-induced migration; however, no significant difference was observed with invasion due to the large SD. Therefore, these results suggest that IL-8 induced by acidic stimulation is important for cell motility and invasion.

The NF-kB pathway is involved in acid-induced IL-8 expression. Our results showed that acidic stimulation upregulated IL-8 expression in MDA-MB-231 cells. Previous studies have shown that NF-κB signaling regulates IL-8 induction in various cell types (8,10,11,31). To investigate the potential factors involved in acidic stimulation of IL-8, we next examined whether acidic stimulation affects NF-κB activation. Western blot analysis revealed that acidic conditions increased the phosphorylation of NF-κB p65 (Fig. 6A). Moreover, treatment with the NF-κB inhibitor BAY11-7082 decreased the expression of *IL-8* mRNA induced by acidic conditions (Fig. 6B). Acid-induced *IL-8* mRNA was also markedly decreased by treatment with dexamethasone, another inhibitor of NF-κB signaling (Fig. 6C).

ASIC1 is involved in acid-induced changes in MDA-MB-231 cells. To elucidate the mechanism of the acid-induced effects on in MDA-MB-231 cells, we next investigated the expression of acid-sensing receptors. Previous studies have shown that extracellular acidity activates acid receptors including ion channels such as TRPV1 and ASICs (ASIC1–4), and GPCRs (OGR1, TDAG8, GPR4, G2A). RT-PCR analysis revealed that MDA-MB-231 cells expressed various pH-sensing receptors including *TRPV1, ASIC1, ASIC3, OGR1, TDAG8* and *G2A* (Fig. 7A). Furthermore, qPCR analysis revealed that *ASIC1* and *ASIC3* mRNA expression was significantly increased by acidic stimulation, whereas other receptors were unchanged (Fig. 7B). Therefore, we focused on ASIC1. Unexpectedly, acid stimulation partially restored *ASIC1* mRNA expression even when *ASIC1* was knocked down (Fig. 7C). Furthermore, we

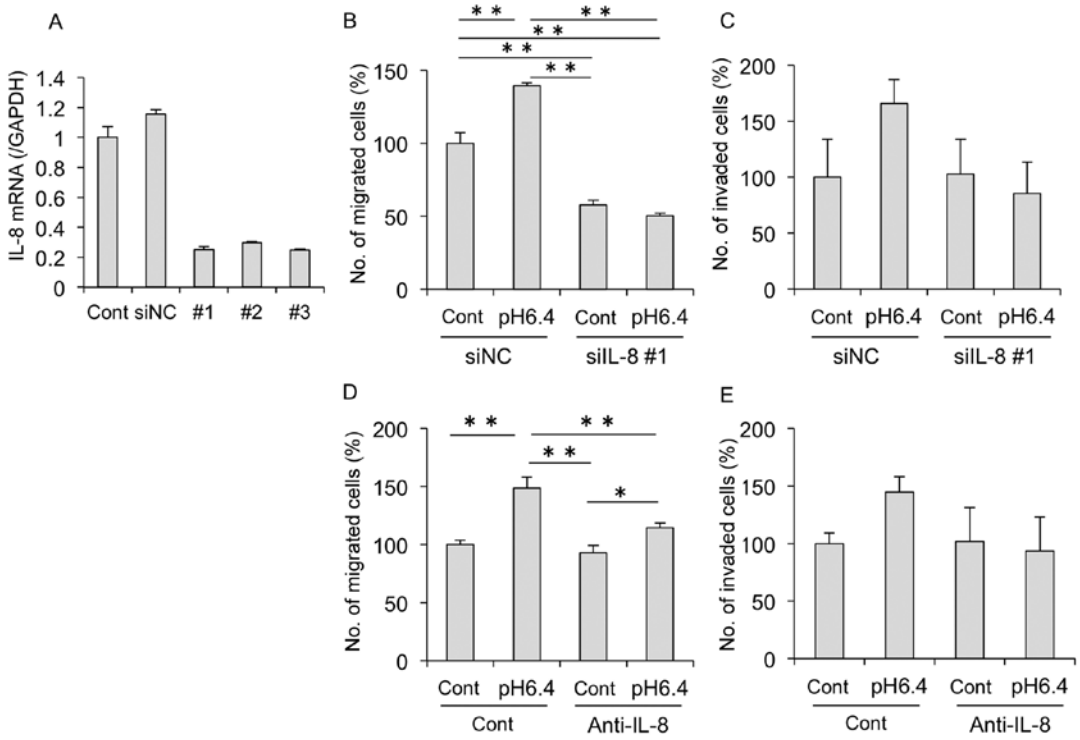

Figure 5. Effects of *IL-8* knockdown and inhibition on acid-induced migration and invasion of MDA-MB-231 cells. (A) MDA-MB-231 cells were transfected with negative control siRNA (siNC) or siRNA against *IL-8* (siIL-8 #1, #2, #3). IL-8 mRNA expression was analyzed by real-time PCR. Data were normalized to *GAPDH* levels and shown as fold expression relative to levels in cells transfected with siNC. Migration (B and D) and invasion (C and E) were examined using a Transwell assay. Cells were seeded into inserts with control or pH 6.4 serum-free media at 24 h after transfection of siNC or siIL-8 (B and C) or the anti-IL-8 antibody (100 ng/ml) was added to serum-free medium in inserts (D and E). Cells that had migrated to the lower surface of the membrane were stained and then counted. Data are expressed as mean ± SD. *P<0.05, **P<0.01. IL, interleukin.

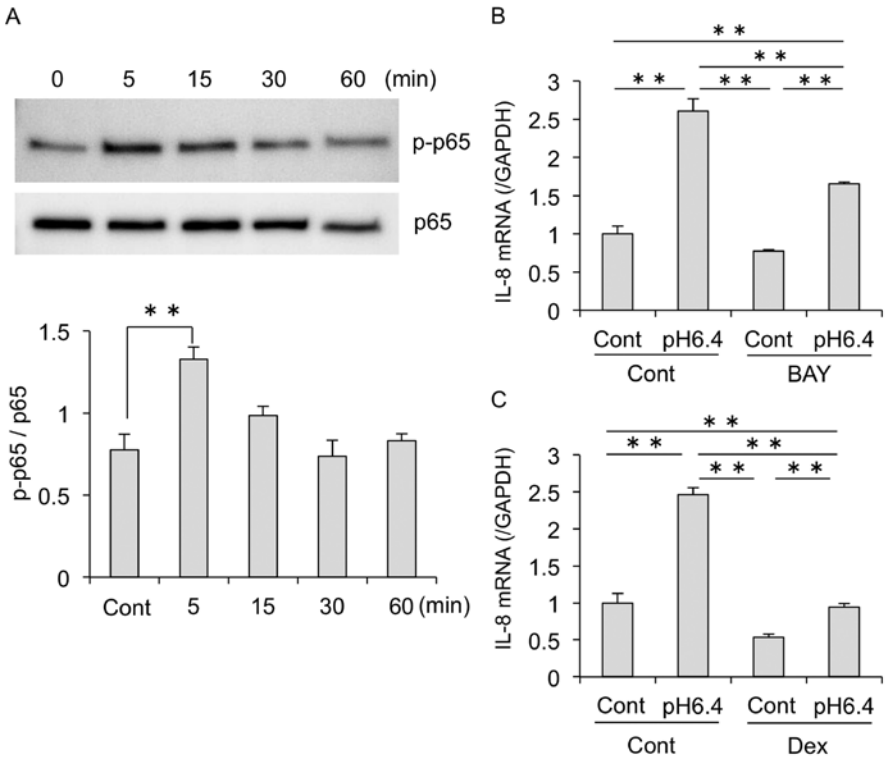

Figure 6. NF-κB is activated by acidic stimulation in MDA-MB-231 cells and is critical for IL-8 expression. (A) Activation of the NF-κB pathway in MDA-MB-231 cells by acidic stimulation was examined by western blotting. Bottom histogram: densitometric intensities of the signals are expressed as the ratio of p-p65 to p65. Data are expressed as mean ± SD. **P<0.01 vs. the control. (B) MDA-MB-231 cells were stimulated by acidic medium with or without the NF-κB inhibitor BAY11-7082 (BAY) (10 μM) for 24 h. (C) MDA-MB-231 cells were stimulated by acidic medium with or without dexamethasone (Dex), an NF-κB inhibitor. *IL-8* mRNA expression was analyzed by real-time PCR. Data are shown as the fold change normalized to the control (mean ± SD). **P<0.01. NF-κB, nuclear factor-κB; IL, interleukin.

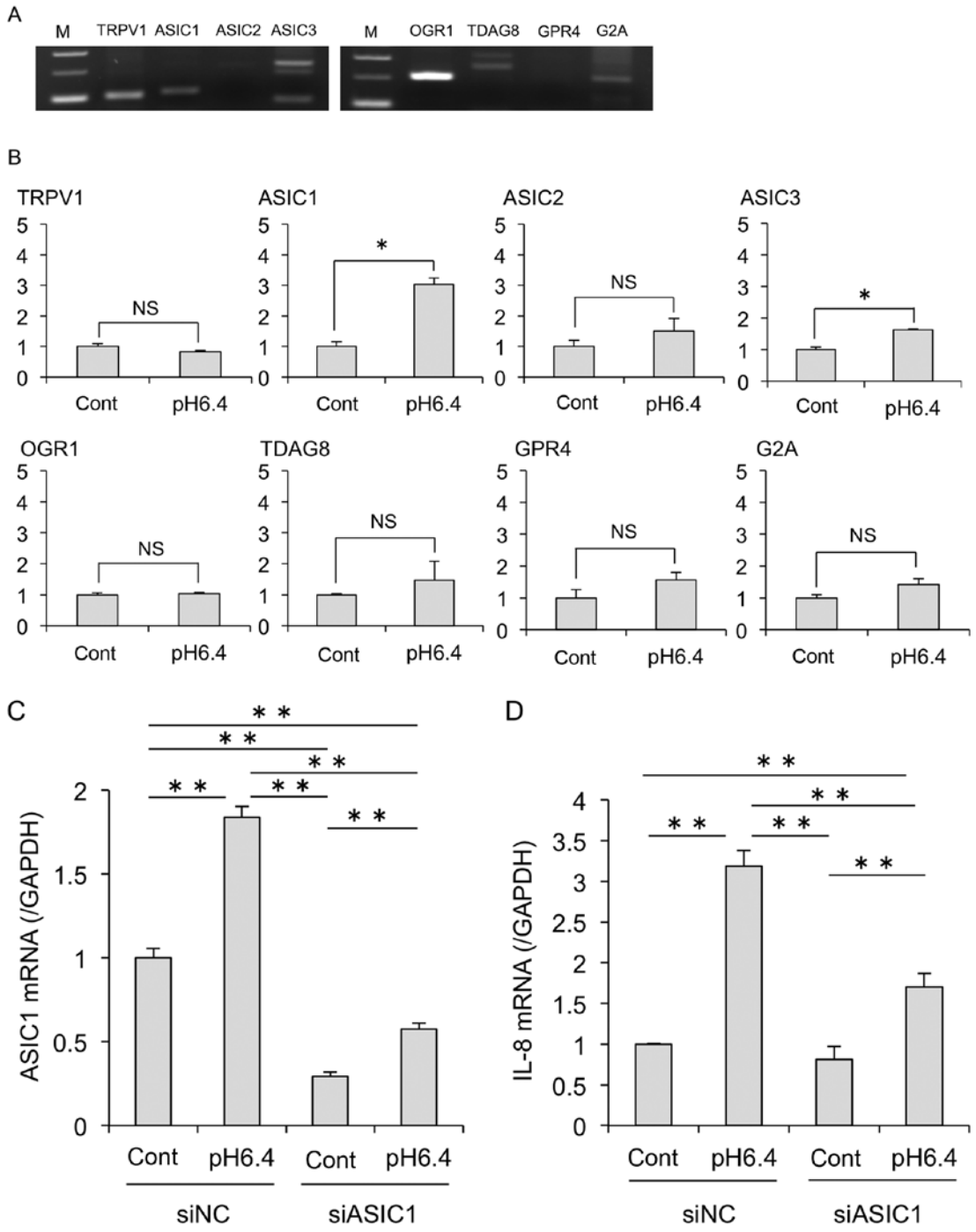

Figure 7. Acid-sensing receptor ASIC1 is critical to acid-induced IL-8 expression. (A) RT-PCR analysis revealed that MDA-MB-231 cells express genes encoding various acid receptors including ion channels and GPCRs. M, marker. (B) The effect of acidic stimulation on the gene expression of acid receptors was evaluated by real-time PCR. *P<0.05; NS, not significant. (C and D) At 24 h after transfection of siRNA (siNC or siASIC1) by electroporation, MDA-MB-231 cells were treated with control or acidic medium. After 24 h, mRNA expression levels of *ASIC1* (C) and *IL-8* (D) were analyzed by real-time PCR. Data are shown as the fold expression normalized to the control transfected with siNC (mean ± SD). *P<0.05, **P<0.01. IL, interleukin; ASIC1, acid-sensing ion channel 1; GPCRs, G-protein coupled receptors; TRPV1, transient receptor potential vanilloid subtype 1; OGR1, also known as GPR68; G2A, also known as GPR132; TDAG8, also known asGPR4.

also found that knockdown of *ASIC1* decreased *IL-8* mRNA expression induced by acidic stimulation (Fig. 7D). We also performed experiments using siRNA against *OGR1*, which is strongly expressed in MDA-MB-231; however, no inhibitory effect was observed on the acid-induced expression of *IL-8* mRNA (data not shown). Together these results suggest that ASIC1 plays an important role in acid-induced IL-8 expression.

Discussion

Local acidosis is a characteristic feature of cancer. The Warburg effect is a phenomenon in which tumors consume significant amounts of glucose and produce lactate via the anaerobic glycolytic pathway (4,26,27). Because of the increased lactate, the tumor microenvironment exhibits acidification, with the extracellular pH ranging between 6.0 and 6.5. This acidosis

promotes processes such as metastasis, angiogenesis, and immunosuppression, which have been associated with a poor clinical prognosis (1-6).

We found that acidic stimulation induced morphological changes in MDA-MB-231 cells to more spindle-shaped cells with protrusions. Recently, Chen *et al* showed that the acidic microenvironment induced invasion of prostate cancer cells through induction of Snail *(SNAI1)* and Twist *(TWIST1)* mRNA (28). We also examined the expression of EMT-related genes, such as *CDH1, CDH2, VIM, SNAI1,* and *TWIST1*, but there were no significant changes in MDA-MB-231 cells following acidic treatment (data not shown). Because MDA-MB-231 cells originally exhibit a mesenchymal phenotype with higher levels of *VIM* and lower levels of *CDH1*, EMT-related genes may have no relationship with the morphological changes in this cell line. Therefore, we focused on protease activity as a cause of migration and invasion. Under acidic conditions, there were no significant changes in *MMP2* and *MMP9* mRNA expression (data not shown). However, we observed that cells cultured in acidic medium exhibited increased secretion of gelatinase compared with cells in normal medium.

In this study, the proliferation was suppressed in cells under acidic conditions. We initially expected that acidic conditions promote cell growth. In fact, we found that the growth of other cell lines was promoted by acidic conditions (data not shown). We speculated that acidic stimulation of MDA-MB-231 cells would preferentially induce cell survival pathways rather than proliferation, although we did not examine apoptosis and cell death. A recent study showed that extracellular acidosis induced upregulation of p21, G1/G0 cell cycle arrest, and a reduction in proliferation in human melanoma cells (29). In addition, the authors detected no induction of cellular apoptosis but an increase in senescence characteristics. Wojtkowiak *et al* reported that acute exposure to acidic medium (pH 6.7 for 3 days) reduced the proliferation of cells and promoted autophagy as a survival adaptation in MDA-MB-231 cells (30). Furthermore, the authors showed that long-term exposure (3 months) to low pH restored cellular proliferative activity. We believe that changes in cellular function associated with the acidic microenvironment need to be investigated in more detail.

To determine the factors involved in acid-induced migration and invasion, we investigated several cytokines that have been implicated in the expression of the malignant phenotype in cancer cells. We found that acidic stimulation upregulated the mRNA expression and secretion of IL-8 in MDA-MB-231 cells. Végran *et al* revealed that lactic acid from tumor cells stimulates the IL-8 pathway in endothelial cells, resulting in angiogenesis and tumor growth (31). In addition, we previously showed that the acidic microenvironment induced IL-8 production in lymphatic endothelial cells and promoted cellular proliferation and tube formation (11). These results suggest that increased IL-8 expression in the tumor microenvironment has an important role in cancer progression.

Therefore, we next investigated the direct effects of IL-8 on several activities of MDA-MB-231 cells. Although a previous study showed that the secretion of IL-8 from cancer cells enhances the proliferation and survival of cancer cells via the autocrine pathway (8), the proliferative activity of MDA-MB-231 cells was not altered by IL-8 treatment in this study. In contrast, we found that the migration and invasion of MDA-MB-231 cells were markedly increased by IL-8 treatment. Moreover, MMP-2/-9 activity was upregulated following IL-8 treatment, which suggests a relationship between IL-8 and acid-induced functional changes in MDA-MB-231 cells. Noteworthy, *IL-8* knockdown markedly inhibited these acid-induced effects. These results showed that the acid-induced IL-8 production accelerated migration and invasion via the autocrine pathway in MDA-MB-231 cells.

The expression of IL-8 has been shown to be regulated by many stimuli, such as inflammatory signals, chemical and environmental stresses, and steroid hormones, and is primarily induced via activator proteins and/or NF-κB-mediated transcriptional activity (8,32). We observed that acidic stimulation induced the phosphorylation of p65 within 15 min in MDA-MB-231 cells. In addition, acid-induced IL-8 expression was markedly decreased by treatment with NF-κB inhibitors. Recently, Chen *et al* showed that acidosis induced NF-κB activation through ERK signaling in prostate cancer cells (28). However, even in the presence of inhibitors, IL-8 expression levels in acidic conditions were higher compared with the control. These results suggest that signaling pathways other than NF-κB may also be involved in the acid-induced IL-8 expression.

We demonstrated that MDA-MB-231 cells express various acid-sensing receptors and that ASIC1 has an important function in the regulation of IL-8 expression. Several reports have described a relationship between breast cancer and the acid-sensing ion channel TRPV1 (33-36). Weber *et al* showed that breast cancer cells express functional TRPV1 and that TRPV1 activation inhibited cell proliferation and induced apoptosis and necrosis (34). In addition, Lozano *et al* showed that intracellular aggregated TRPV1 is associated with a shorter survival in breast cancer patients (35). These reports indicate that TRPV1 has antitumor activity in breast cancer cells. In this study, we also observed TRPV1 expression in MDA-MB-231 cells. However, whether TRPV1 was involved in acid-induced IL-8 expression is unknown. In addition to TRPV1, MDA-MB-231 cells also highly express OGR1. Several studies suggest that OGR1 expressed in breast cancer cells also functions as a tumor suppressor, promoting apoptosis and inhibiting cell proliferation and migration (37-39). In the present study, we also observed that acid-induced IL-8 expression was not altered by *OGR1* knockdown (data not shown). Further studies are needed to clarify the role of acid-sensing GPCRs in breast cancer development.

Of note, we observed that acidic conditions induced the expression of *ASIC1* mRNA, and acid-induced IL-8 expression was markedly inhibited by *ASIC1* knockdown. Unexpectedly, *ASIC1* mRNA was partially restored by acidic stimulation, despite the siRNA knockdown. The mechanism by which acid stimulation induces *ASIC* mRNA expression is not yet known, but we speculate that this phenomenon is the cause of the increased *IL-8* expression in the siASIC1-transfected group. Several studies suggest that ASIC1 expression is involved in breast cancer progression (16,40). Chen *et al* reported that ASIC1 expression is upregulated in prostate cancer cases, and knockdown of *ASIC1* significantly suppressed cell proliferation and invasion *in vitro* and *in vivo* (28). In addition, some reports

have indicated a relationship between ASIC1 expression and the development of glioma (18,20). Collectively, these findings suggest that ASIC1 has unique functions that differ from other acid receptors and that there are important implications of ASIC1 expression in cancer progression in the acidic microenvironment.

Acidosis in the cancer microenvironment is formed by protons and lactic acid produced by cancer cells and the surrounding stromal cells (2,27,41). In this study, we focused on proton-sensing receptors, but it is also necessary to study the effects of lactate via the lactate receptors or transporters. Kolesnik *et al* revealed that lactic acidosis promoted the survival and proliferation of Lewis lung cancer cells through inhibition of autophagy and apoptosis (42). In addition, Romero-Garcia *et al* showed that lung cancer cells consume lactate and induce mitochondrial biogenesis to support survival and proliferation in lactic acidosis conditions (43). Monocarboxylate transporter (MCT)1-4 are lactate transporters, and overexpression of MCTs is a common feature of some cancers with high metabolic rates (27,44). Furthermore, some studies have reported that several GPCRs function as sensors of lactate, and the expression levels of these GPCRs correlate with tumor growth and metastasis (45,46). Further investigation of the role of lactate receptors in IL-8 expression and cellular functions in breast cancer cells is required.

In summary, we showed that an acidic microenvironment induced IL-8 expression and invasion activity through the activation of ASIC1 in breast cancer cells. These findings suggest a critical role for ASIC1 in the cancer microenvironment and indicate that ASIC1 and IL-8 may be novel therapeutic targets for cancer progression.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MN designed the study. MN, AK, HY, NK and KN performed the experiments. MN and YM analyzed and interpreted the data, and wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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