α-Bisabolol Inhibits Invasiveness and Motility in Pancreatic Cancer Through KISS1R Activation

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Abstract. *α-Bisabolol is a plant-derived, oily sesquiterpene alcohol that induces apoptosis of various cancer cells. We previously reported the antiproliferative effects of α-bisabolol on pancreatic cancer cell lines using in vitro and in vivo experiments. However, the effects of α-bisabolol on tumor invasiveness and motility are still unknown. In this study, demonstrated that α-bisabolol suppressed the invasiveness and motility of a pancreatic cancer cell line. Although Early growth response 1 (EGR1) was involved in antiproliferative effects of α-bisabolol, it had no relationship with the inhibitory effect of α-bisabolol on cellular invasiveness and motility. Polymerase chain reaction analysis revealed that α-bisabolol induced Kisspeptin 1 receptor (KISS1R) in pancreatic cancer cell lines. The inhibition of KISS1R weakened the inhibitory effect of α-bisabolol on invasiveness of pancreatic cancer cells. The results also implied that the inhibitory effects of α-bisabolol on tumor invasiveness and motility are at least partly associated with the activation of KISS1R. However, there is a possibility that other molecular mechanisms of α-bisabolol regulate invasiveness and motility in pancreatic cancer cells. Further investigations are necessary to clarify the precise mechanisms of α-bisabolol activity for clinical application as a novel treatment for pancreatic cancer.*

Pancreatic cancer is the fourth-leading cause of cancer-related death in developed countries (1). Even when pancreatic cancer is completely removed by surgical operation, the prognosis is still poor because of metastatic or local recurrence (2). The 5-year survival rate is less than 20% (3, 4). Although chemotherapy and radiotherapy have shown some benefit for the prognosis of patients with pancreatic cancer, the impact of these therapies remains unsatisfactory (5). Therefore, novel treatments are urgently needed to improve the prognosis of the metastasis of pancreatic cancer.

α-Bisabolol is a plant-derived, oily sesquiterpene alcohol. It has several functions, such as being gastric-protective, antimicrobial, and anti-inflammatory. α-Bisabolol also induces apoptosis of glioma cells, acute leukemia cells, and liver carcinoma cells (6-8). We previously reported an antiproliferative effect of α-bisabolol against pancreatic cancer (9). However, the precise role of α-bisabolol concerning the invasiveness and motility of pancreatic cancer is still not fully understood. Furthermore, the key regulatory mechanism of α-bisabolol for invasiveness and motility of cancer cells has never been investigated.

In this study, we investigated the effect of α-bisabolol on invasiveness and motility of pancreatic cancer cells and the mechanism involved.

Materials and Methods

Materials. α-Bisabolol and antibody to β-actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies to early growth response 1 (EGR1) and anti-Kisspeptin 1 receptor (KISS1R) were obtained from Cell Signaling Technology (Danvers, MA, USA).

Cell culture. The human pancreatic cancer cell lines (KLM1, KP4 and Panc1) were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Japan. The cell lines were cultured in RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. All cell lines were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Establishment of shRNA-transfected cells. *EGR1* shRNA-transfected KLM1 cells in which *EGR1* is constitutively suppressed were established by retrovirus infection. To produce the *EGR1* shRNA-transfected KLM1 cell line, oligonucleotides encoding an shRNA specific for human *EGR1*: 5'-CCAACGACAGCAGUCCCAUTT-3' (sense) and 5'-AUGGGACUGCUGUCGUUGGTT-3' (antisense) were cloned into the pSIREN-RetroQ retroviral vector (Clontech,

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Mountain View, CA, USA). Recombinant retrovirus was produced, and infected KLM1 cells were selected using 1 μg/ml puromycin for 3 days. Control shRNA-transfected KLM1 cells, using the following oligonucleotides: 5'-UUCUCCGAACGUGUCACGUTT-3' (sense) and 5'-ACGUGACACGUUCGGAGA-3' (antisense), were also established using the same protocol.

Transfection of siRNAs. The human *KISS1R* siRNA were chemically synthesized by Sigma-Aldrich (St. Louis, MO). The sequences of the strands were as follows: *KISS1R* siRNA, sense: 5'- CACUCU GACCGCCAUGAGUTT -3', antisense: 5'- ACUCAUGGCGGUCAG AGUGTT -3'. Cancer cell lines were transfected with 25 nM siRNA using a CUY21EDIT Ver2.10 electroporation system (BEX, Tokyo, Japan) according to the manufacturer's instructions.

Invasion assay. Invasion assays were performed using Boyden chambers containing a polycarbonate membrane (8.0 μm pore size; Corning Incorporated, NY, USA) that was coated on the upper side with Matrigel (BD, Franklin Lakes, NJ, USA). 24-Well plates were placed into the chambers. Cancer cells (2×10^5) were placed in the upper chamber with fresh medium containing different concentrations (0 or 1.56 μM) of α-bisabolol (diluted from a 5 mg/ml stock solution using ethanol). The lower chamber was filled with 600 μl of RPMI-1640 without FBS and antibiotics and then incubated at 37˚C in 5% CO_2. After 24 h of incubation, the cells that passed through the membrane and invaded the lower chamber were fixed in methanol for 60 seconds, and this was followed by staining in hematoxylin and eosin for 3 min and 30 s, respectively. Cells remaining in the upper chambers were completely removed by gentle swabbing, and these membranes were mounted on slides. On each membrane, the invaded areas were measured in six randomly selected visual fields (×5 objective), and the mean dimension was determined.

Cell-migration assay. The motility of the cells was assessed using a cell-migration assay. Cancer cells were plated overnight to achieve a subconfluent cell layer in 6-well plates. A scratch was made on the cell layer with a micropipette tip, and the cultures were washed twice with phosphate-buffered saline to remove floating cells. Cells were incubated in RPMI-1640 with 10% FBS and different concentrations of α-bisabolol that were dissolved in fresh medium at 37˚C in 5% CO_2. Wound healing was visualized using photographs at 0, 12, 24 and 36 h. The distance traveled by the cells was measured at the same six points, and the mean distance was determined for 36 h.

PCR array analysis. cDNA samples extracted from pancreatic cancer cell lines were analyzed using the RT^2 Profiler™ PCR Array Human Tumor Metastasis (SA Biosciences Corp., Frederick, MD, USA), which comprises a panel of 84 primer sets for genes involved in tumor metastasis. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was chosen as the internal control gene. PCR arrays were analyzed using the 7300 Real Time PCR System (Applied Biosystems, Waltham, MA, USA). The amplification protocol consisted of an initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 s, and annealing and extension at 60˚C for 1 min. The expression of each gene was quantified based on its Ct (cycle threshold), the number of cycles at which the linear phase crossed the threshold level.

Western blot analysis. Whole-cell extracts were prepared by lysing cells in Laemmli sample buffer. An equivalent amount of cell lysate was electrophoresed on sodium dodecyl sulfate-polyacrylamide gels, transferred to Polyvinylidene difluoride membranes (Immobilon; Millipore Corporation, Billerica, MA, USA), and probed with antibodies to EGR1 (1:1000; Cell Signaling Technology, Danvers, MA, USA), KISS1R (1:1000; Cell Signaling Technology), and β-actin (1:10000; Sigma-Aldrich, St Louis, MO, USA) were applied as primary antibodies. Anti-rabbit IgG, horse radish peroxidase (HRP) linked antibody (1:1000; Cell Signaling Technology) and anti-mouse IgG, HRP linked antibody (1:1000; Cell Signaling Technology) were applied as the secondary antibodies. Signals were detected using an enhanced chemiluminescence system (GE Healthcare Life Sciences, Buckinghamshire, UK).

Statistical analysis. Data are presented as the mean±SEM. The significance of differences between experimental values was assessed using Student's *t*-test, and a value of $p<0.05$ was considered significant.

Results

α-Bisabolol suppressed the invasiveness and motility of pancreatic cancer cell lines. The effects of α-bisabolol were evaluated using invasion and motility assays in KLM1, KP4, and Panc1 cells. Invasiveness of pancreatic cancer cell lines KLM1, KP4 and Panc1 was significantly suppressed when cells were treated with 1.56 μM of α-bisabolol for 24 h (Figure 1a). The cell-migration assays also showed that α-bisabolol significantly inhibited motility of pancreatic cancer cells (Figure 1b).

EGR1 has no influences in the inhibitory effect of α-bisabolol for invasiveness and motility. *EGR1* is a known tumor-suppressor gene of the zinc finger transcription factor family. We previously reported that EGR1 activation was one of the antitumor mechanisms of α-bisabolol against pancreatic cancer (9). To examine the effects of EGR1 on invasiveness and motility in α-bisabolol-treated pancreatic cancer cells, an *EGR1* shRNA-transfected KLM1 cell line was established. Western blot analysis demonstrated that EGR1 expression was strongly attenuated in *EGR1* shRNA-transfected KLM1 cells after exposure to α-bisabolol 6.25 μM (Figure 2a).

To investigate the effects of α-bisabolol on invasiveness, control shRNA-transfected, and Egr1 shRNA-transfected KLM1 cell lines were treated with 1.56 μM α-bisabolol for 24 h. In spite of the suppression of EGR1 expression, the invasive capacity was not affected in the *EGR1* shRNA-transfected cells, compared to that in control shRNA-transfected cells (Figure 2b). Next, cell-migration was evaluated by comparing the effect of α-bisabolol between *EGR1* shRNA-transfected cells and control shRNA-transfected cells. Although α-bisabolol suppressed cell motility in both cell lines, inhibition of *EGR1* also had no influence on cell motility (Figure 2c). These data suggest that *EGR1* has no relationship to the inhibitory effect of α-bisabolol on cellular invasiveness and motility.

Gene profiling concerning tumor metastasis in α-bisabolol-treated pancreatic cancer cell. To further investigate the key molecules that are related to the inhibitory effects of α-

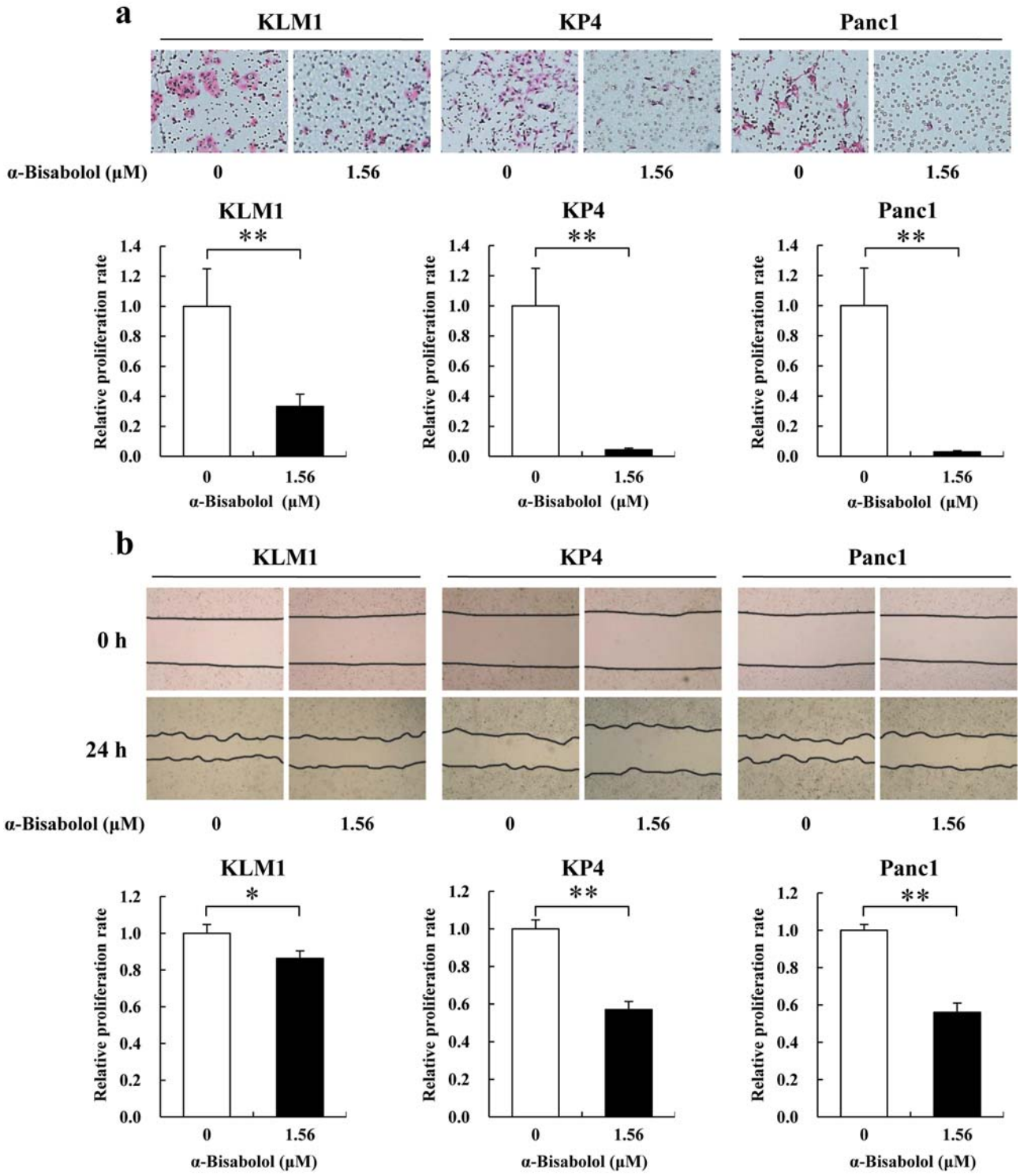

Figure 1. *α-Bisabolol suppressed the invasiveness and motility in pancreatic cancer cell lines. a: The invasiveness was assessed in α-bisabolol-treated pancreatic cancer cell lines KLM1 (left), KP4 (center), and Panc1 (right) using a Boyden chamber method. The cells were treated with 1.56 μM of α-bisabolol for 24 h. On each membrane, the invaded areas were measured at six randomly selected visual fields (×5 objective). Data represent the relative invasion rate compared to that of the untreated control. b: Cell motility was determined in α-bisabolol-treated pancreatic cancer cell lines KLM1 (left), KP4 (center), and Panc1 (right) using a cell migration assay. The cells were treated with 1.56 μM of α-bisabolol for 24 h. The distance traveled by the cells was measured at the same six points, and the mean distance was determined 0 and 24 h after treatment with α-bisabolol. The relative migration rate is shown relative to the migration distance in control cells. *p<0.05, **p<0.01.*

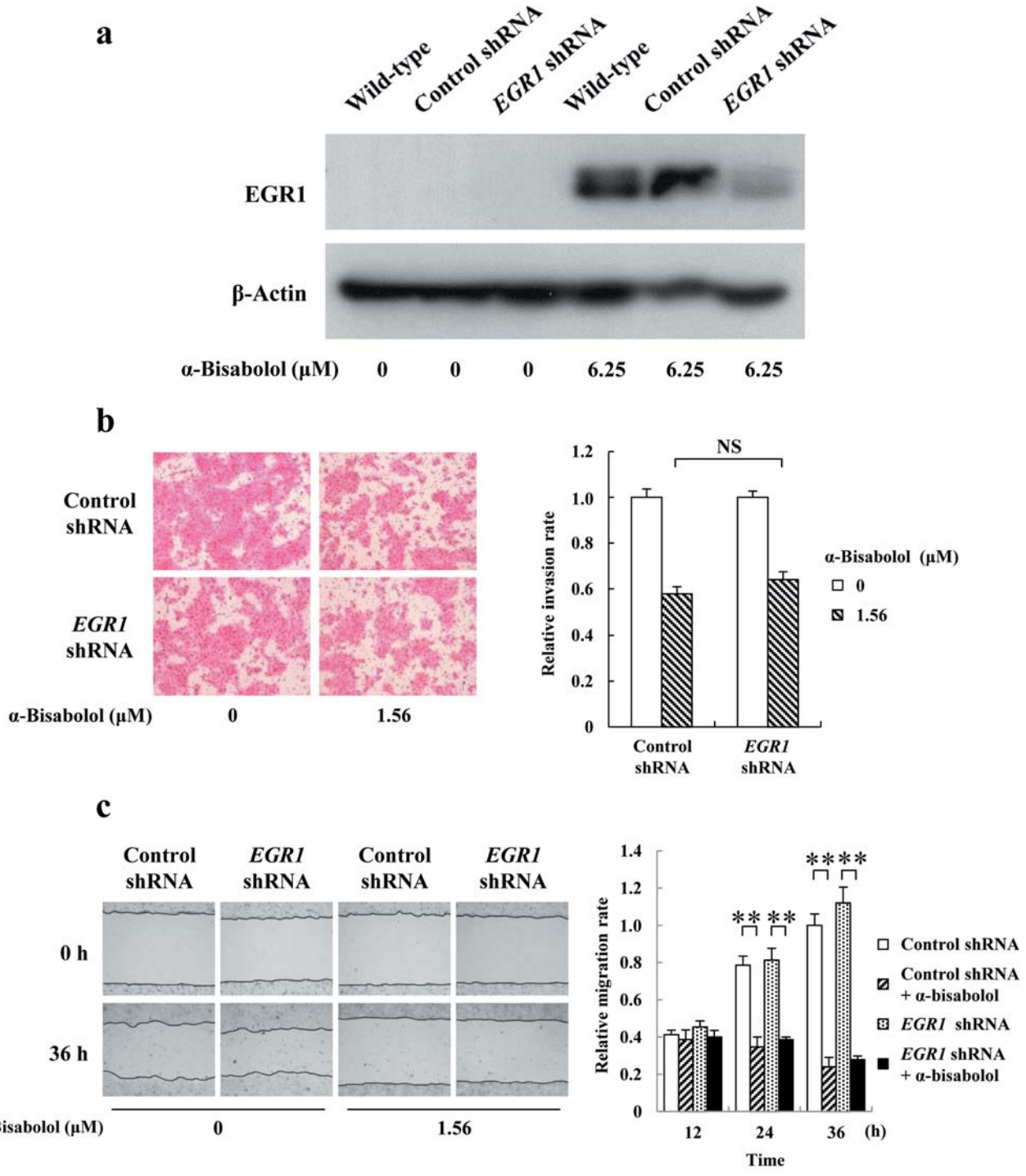

Figure 2. *Early growth response 1 (EGR1) has no influence on the inhibitory effect of α-bisabolol on invasiveness and motility. a: EGR1 expression was detected in wild-type KLM1, control shRNA-transfected KLM1 cells and EGR1 shRNA-transfected KLM1 cells after a 6-h treatment with 6.25 μM of α-bisabolol by western blot analysis. β-Actin was used as an internal control. b: Cell invasiveness was assessed in control shRNA-transfected KLM1 cells and EGR1 shRNA-transfected KLM1 cells using Boyden chamber method. The cells were treated with 1.56 μM of α-bisabolol for 6 h. On each membrane, the invaded areas were measured at six randomly selected visual fields (×5 objective). Data represent the relative invasion rate compared to that of the control shRNA-transfected KLM1 cells. Cell invasiveness was not significantly altered by suppression of EGR1 expression. c: Cell motility of EGR1 shRNA-transfected KLM1 cells and control shRNA-transfected KLM1 cells was assessed using a cell-migration assay. The distance traveled by the cells was measured at the same six points, and the mean distance was determined at 0, 12, 24, and 36 h after treatment with 1.56 μM α-bisabolol. The relative migration rate is shown relative to the migration distance in control shRNA-transfected KLM1 cells; **p<0.01. NS: Not significant.*

bisabolol on invasiveness and motility of pancreatic cancer cells, PCR array analysis concerning tumor metastasis were performed on α-bisabolol-treated pancreatic cancer cell lines. Several genes, such as *KISS1R*, metastasis suppressor 1 (*MTSS1*), and tissue inhibitor of metalloproteinase 2 (*TIMP2*), were up-regulated in α-bisabolol-treated pancreatic cancer cells (Figure 3a). Among these genes, we focused on *KISS1R*. KISS1R and its ligand KISS1 are known as metastasis suppressors, inhibiting cancer cell migration and invasion (10). The data suggest that KISS1R might be one of the key molecules related to the inhibitory effect of α-bisabolol on cellular invasiveness and motility.

α-Bisabolol suppressed invasiveness through induction of KISS1R in pancreatic cancer cells. To examine whether KISS1R directly affects the inhibitory effect of α-bisabolol or not, invasiveness was analyzed in *KISS1R*-suppressed pancreatic cancer cell lines. Invasiveness was enhanced in KLM1 and KP4 cells upon treatment with *KISS1R* siRNA, but not sufficiently in Panc1 cells (Figure 4). The inhibitory effect of α-bisabolol was weakened in *KISS1R*-suppressed KLM1 and KP4 cells.

Discussion

We previously reported that α-bisabolol inhibited cell proliferation and induced apoptosis of pancreatic cancer in *in vitro* and *in vivo* experiments. Metastasis is defined as the secondary tumor at different organs from the primary tumor. Although complete surgical resection provides the only potential cure, the surgical outcome in pancreatic cancer remains unsatisfactory because of metastasis, peritoneal dissemination and local recurrence (10). Therefore, a novel treatment is urgently required to improve the prognosis of metastasis of pancreatic cancer. However, no studies on the efficiency of α-bisabolol on tumor invasiveness and motility, that are related to the metastatic potential of tumor cells, have been reported. This study first demonstrated that α-bisabolol suppressed the invasiveness and motility of a pancreatic cancer cell line.

Cavalieri *et al*. reported that α-bisabolol rapidly induced apoptosis of human malignant glioma cell lines through the mitochondrial pathway without toxicity to normal glial cells (7). In our previous report, we demonstrated that EGR1 was involved in the antiproliferative and apoptosis-inducing potentials of α-bisabolol in a pancreatic cancer cell line (9). In addition to our previous report, many reports demonstrated that EGR1 plays an important role in inhibiting cell-migration, proliferation, and angiogenesis (11-13). Therefore, we first investigated whether the induction of *EGR1* by α-bisabolol had some impact on the invasiveness and motility of a pancreatic cancer cell line. Unexpectedly, EGR1 expression did not impact the inhibitory effects of tumor invasiveness and motility.

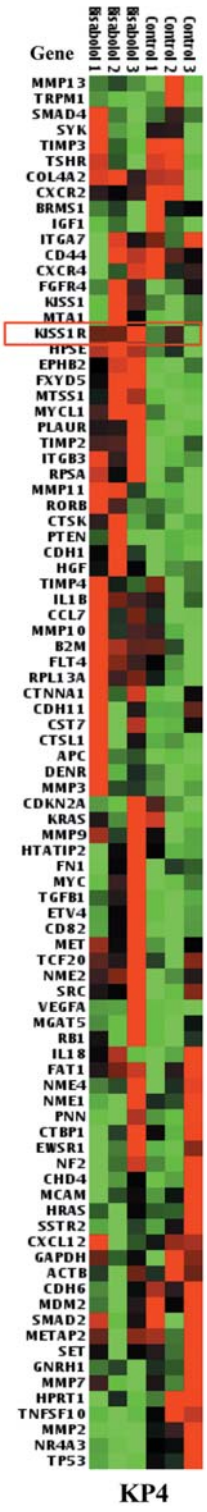

Figure 3. *Gene-profiling heatmap concerning tumor metastasis in α-bisabolol-treated pancreatic cancer cells. Polymerase chain reaction array analysis for tumor metastasis-related genes was performed using KP4 cells treated with 1.56 μM of α-bisabolol for 24 h. The data show one-dimensional hierarchical clustering of 84 genes between the α-bisabolol-treated (n=3) and control KP4 (n=3) cells.*

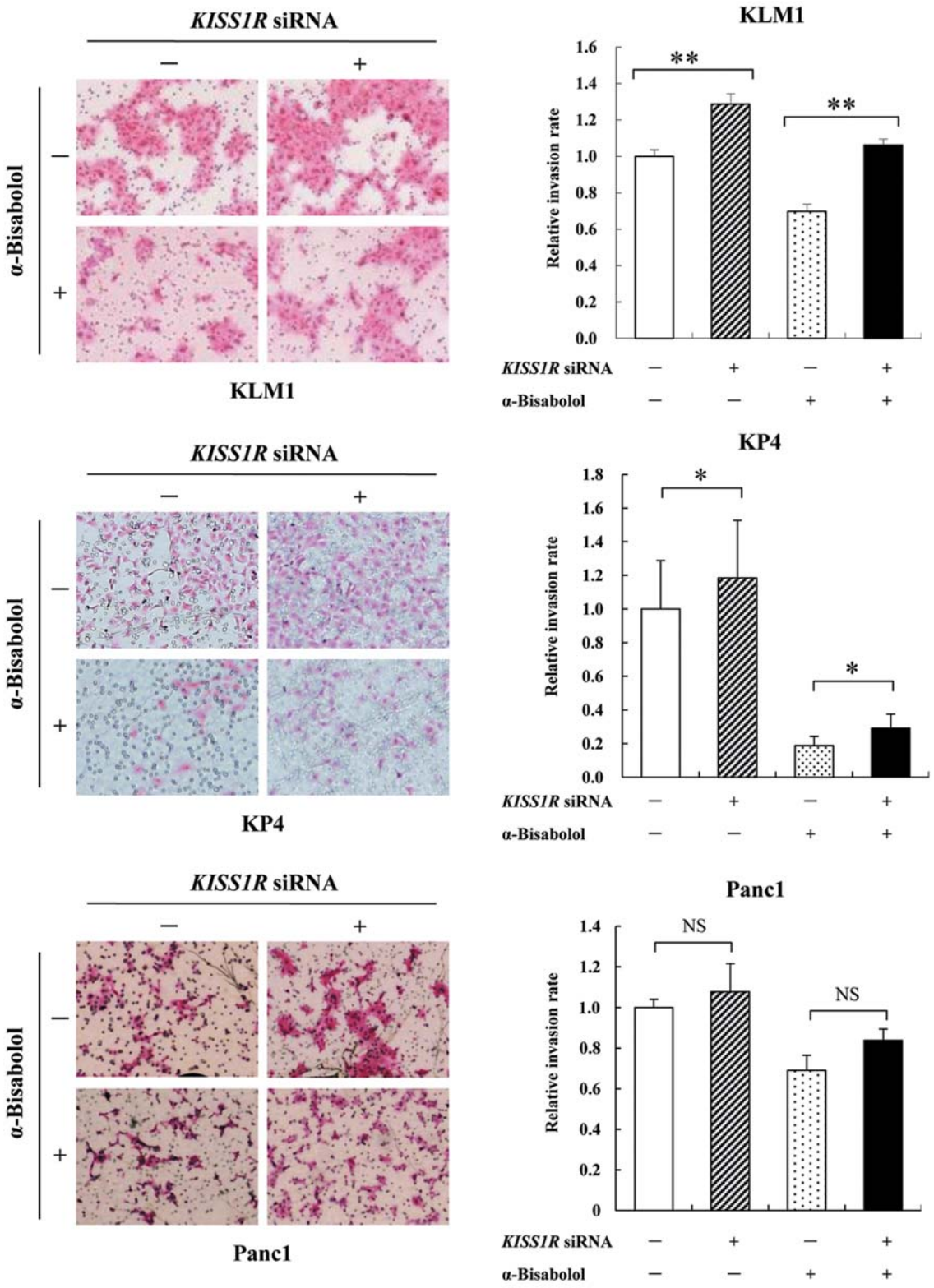

Figure 4. *α-Bisabolol suppressed invasiveness through induction of Kisspeptin 1 receptor (KISS1R) in pancreatic cancer cells. Invasiveness was analyzed in KISS1R-suppressed pancreatic cancer cell lines. The cells were transfected with KISS1R siRNA or control siRNA, and then treated with 1.56 μM of α-bisabolol for 24 h. On each membrane, the invaded areas were measured at six randomly selected visual fields (×5 objective). Data represent the relative invasion rate compared to that of the untreated control siRNA-transfected cells; *p<0.05, **p<0.01. NS: Not significant.*

KISS1R, also called G-protein coupled receptor 54 (GPR54), is a receptor for metastin (kisspeptin-54 or kp-54), which is a *C*-terminally amidated peptide of KISS1. Several reports showed that KISS1R suppressed the metastasis of various cancer types, such as melanoma, breast carcinoma, and gastrointestinal carcinoma by inhibiting cancer cell migration and invasion (14, 15). Masui *et al*. reported that KISS1/KISS1R signaling activated extracellular signal-regulated kinase (ERK1), but inhibited cell-migration in pancreatic cancer (16). Nagai *et al*. reported that high expression of KISS1 and KISS1R was associated with longer survival of patients with pancreatic cancer (17). KISS1R is also known as a metastasis-suppressor, inhibiting metastasis without affecting the growth rate of tumor (18, 19). Several genes, such as Kangai 1 (*KAI1*) and metastasis suppressor (*MTSS*), were identified as metastasis-suppressing genes in association with the progression of metastasis. Our data demonstrated that activation of *KISS1R* is a novel mechanism of α-bisabolol against pancreatic cancer. We consider that the inhibitory effects of α-bisabolol on tumor invasiveness and motility are, at least partly, associated with the activation of *KISS1R*. There is a possibility that other molecular mechanisms of α-bisabolol exist in regulating invasiveness and motility of pancreatic cancer cells.

In summary, our study demonstrated, as far as we are aware for the first time, that α-bisabolol can be a potent therapeutic drug against tumor invasiveness and motility in pancreatic cancer. These effects are associated with up-regulation of *KISS1R*. Further investigations are necessary to clarify the precise mechanisms of α-bisabolol action for clinical application as a novel treatment for pancreatic cancer.

Disclosure Statement

The Authors have no conflict of interest with regard to this study.

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