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Interleukin-18 enhances breast cancer cell migration via down-regulation of claudin-12 and induction of the p38 MAPK pathway

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ABSTRACT

Interleukin-18 (IL-18) was recently reported to have a pro-tumor effect in various cancers. Increased IL-18 levels in the serum of cancer patients correlated with malignancy, and IL-18 acts a crucial factor for cell migration in gastric cancer and melanoma. Claudins, which are the most important tight junction proteins, are also linked with cancer progression and metastasis. However, the relationship between claudins and IL-18 is not well-understood. Here, we show that the migratory ability of MCF-7 cells was reduced when endogenous IL-18 expression was inhibited with IL-18 siRNA. Moreover, exogenous IL-18 enhanced breast cancer cell migration and suppressed the expression of the tight junction proteins claudin-1, claudin-3, claudin-4, and claudin-12 in MCF-7 cells. Knockdown of claudin-3, claudin-4, and claudin-12, but not claudin-1, increased breast cancer migration with maximal effects observed in claudin-12 siRNA-transfected cells. To investigate whether the mitogen-activated protein kinase (MAPK) signaling pathway is involved in IL-18-induced cell migration and claudin-12 expression, cells were pretreated with SB203580 (an inhibitor of p38 MAPK) or PD98059 (an inhibitor of ERK1/2) prior to the addition of IL-18. Although pretreatment of MCF-7 cells with SB203580 blocked both the enhanced cell migration and the decreased claudin-12 expression, PD98059 only blocked cell migration and did not affect claudin-12 expression. In addition, exogenous IL-18 induced rapid phosphorylation of p38 MAPK. These results suggest that IL-18 is an important factor inducing breast cancer cell migration through down-regulation of claudin-12 and activation of the p38 MAPK pathway.

1. Introduction

Interleukin-18 (IL-18) is a pro-inflammatory cytokine that was first isolated as an interferon (IFN)-γ-inducing factor [1]. It is produced by a variety of cell types and tissues, from immune cells to tumor cells [2–4]. In addition to its role in IFN-γ induction, IL-18 enhances NK cell cytotoxicity and the proliferation of activated T cells [3,5]. It was reported that treatment of melanoma-bearing mice with IL-18-B7.2 gene therapy and irradiation successfully induced anti-tumor effects [6]. In contrast to the anti-cancer effects of IL-18 through stimulation of the immune system, a pro-cancerous effect of IL-18 was recently suggested [7]. IL-18 stimulates the secretion of pro-angiogenic factors including vascular endothelial growth factor (VEGF) and thrombospondin (TSP)-1 in gastric cancer and acts as a growth factor for murine melanoma cells [8–10]. Moreover, IL-18 is a potent stimulator of cell migration in melanoma and gastric cancers [8,11]. IL-18 also induces Fas ligand expression and down-regulates CD70, allowing tumor cells to escape immune surveillance [9,12]. More evidence of its pro-cancerous effects include the elevated expression and secretion of IL-18 in breast and gastric cancer patients showing more rapid tumor progression, metastasis, and unfavorable outcomes [13–15].

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Like many cancers, breast cancer can spread from the primary organ to secondary areas of the body, such as the bone, lung, and liver, through immune escape. Accordingly, blocking the spread of breast cancer is linked to patient outcome because of frequent recurrence after initial treatment. Interruption of cell-to-cell junctions is a crucial step in invasion and metastasis. Tight junctions (TJs), which are responsible for controlling the paracellular ion influx and barrier function, are one type of intercellular junction, along with adherens junctions, gap junctions, and desmosomes [16]. Recently, it was shown that expression of the claudin family, which comprises 24 members that are components of TJs, are dysregulated in various cancers [17–19]. For example, dysfunction or loss of claudin-1, claudin-3, claudin-4 and claudin-12 in various cancers including breast cancers is reported to lead to increased tumor growth and metastasis [20–22]. The expression and function of the claudins are regulated by diverse mechanisms, including modulation of mitogen-activated protein (MAP) kinase signaling [23,24]. Specifically, ERK1/2 and p38 MAPK have significant effects on TJ sealing and claudin expression. In this study, we examined the role of IL-18 and claudin expression in the regulation of breast cancer cell motility.

2. Materials and methods

2.1. Cell culture and reagents

The human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from the American Type Culture Collection (ATCC). These cell lines were grown in DMEM (Gibco-BRL/Life Technologies, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS (Gibco-BRL/Life Technologies) in a 5% CO_2 incubator at 37 °C. Cells in the log phase of growth were used in experiments. In some experiments, cells were pretreated with 10 μM ERK1/2 inhibitor (PD98059) or 10 μM p38 inhibitor (SB203580) (Sigma, St Louis, MO, USA).

2.2. Migration assay

A cell migration assay was performed using Transwell plates (8 μm pore size, Costar, Corning, NY) to determine whether IL-18 regulated breast cancer cell migration. Briefly, cells were resuspended in serum-free media and treated with various concentrations of recombinant human (rh)IL-18 (50, 100, and 200 ng/ml), followed by the addition of fresh medium supplemented with mitomycin C (5 μg/ml), to inhibit proliferation. One hundred microliters of the cell suspension (3×10^5 cells/ml) was added to each Transwell. After incubation for 48 h at 37 °C, cotton swabs were used to remove the cells on the upper surfaces of the Transwells, and the migratory cells attached to the underside of the wells were stained with 0.15% crystal violet in 10% ethanol. The Transwells were rinsed with water and air-dried. The crystal violet-stained, migrated cells were counted in five random microscopic fields using the Scanscope scanning system (Aperio Scanscope), or solubilized in 100 μl of 10% acetic acid and quantitated using an ELISA reader (Molecular Devices) at a wavelength of 570 nm.

2.3. Real-time PCR

After experimental treatment, total RNA was isolated using Trizol reagent (Life Technologies). One microgram of total RNA was used for first-strand cDNA synthesis using M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. All PCR primers were synthesized and purchased from Bioneer (Daejeon, Korea) and are shown in Table S1. Real-time PCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems) with Power SYBR Green master mix (Applied Biosystems, Warrington, UK). The relative amounts of gene expression were calculated using the expression of GAPDH as an internal standard. Each sample was assayed in triplicate. For relative quantification, the cycle threshold (C_t) method (ratio $= 2^{-[Ct\ (Claudin) - Ct\ (GAPDH)]}$) was used.

2.4. siRNA transfection

Cells were transfected with IL-18, claudin-1, claudin-3, claudin-4, claudin-12, or a negative control siRNA (Darmacon Research, Korea) using Lipofectamine RNAi. Briefly, when the cells reached 60–70% confluence, siRNA at a final concentration of 100 nM was combined with Lipofectamin RNAi and allowed to complex for 20 min at room temperature. The transfection mixture was then applied and the MCF-7 cells were incubated for 6 hat 37 °C. Subsequently, cells were maintained in complete medium for 24–36 h before use in the migration experiments. The level of silencing of IL-18 or claudin-12 in MCF-7 cells was assessed using RT-PCR or Western blot analysis. The sequences of the siRNAs are shown in Table S2.

2.5. Western blot analysis

Human claudin-1, claudin-3, claudin-4, and claudin-12 antibodies were obtained from Abcam (Cambridge, UK). In some experiments, anti-phospho-p38 and anti-p38 (Cell Signaling, USA) at a final concentration of 1:500 were used. Western blot analysis was performed as previously described [4].

2.6. Statistical analysis

Statistical significance was determined using Student's t-test. The mean differences were considered to be significant when $P < 0.05$. Results are shown as the mean ± SD of three experiments.

3. Results

3.1. rhIL-18 increases the migration of breast cancer cells in a dose- and time-dependant manner

Recently, we showed that IL-18 plays an important role in melanoma and gastric cancer cell migration. To determine the function of endogenous IL-18 on breast cancer cell migration, we performed small interfering RNA (siRNA)-mediated knockdown of IL-18 in MCF-7 cells, a human breast cancer cell line. Transfection with IL-18-specific siRNA inhibited IL-18 expression and MCF-7 cell migration, whereas the level of cell migration and IL-18 expression remained unchanged in control siRNA-transfected cells (Fig. 1A and B). To further investigate whether exogenous IL-18 can regulate human breast cancer cell migration, the expression of the IL-18 receptor and the effect of IL-18 on MCF-7 cell migration were tested. Fig. 1C shows the expression of the IL-18 receptor on MCF-7 cells. Exogenous IL-18 significantly enhanced cell migration in both a dose- and time-dependent manner (Fig. 1D–F). In addition to the effect on MCF-7 cells, rhIL-18 enhanced cell migration in MDA-MB-231, MDA-MB-468 and BT-549 breast cancer cells (Fig. S1). These data suggest that IL-18 directly enhances human breast cancer cell migration.

3.2. IL-18 regulates loss of the TJ proteins claudin-1, -3, -4, and -12

Since TJs were recently shown to have a crucial role in cancer progression and metastasis [17–19], and because TJs are composed

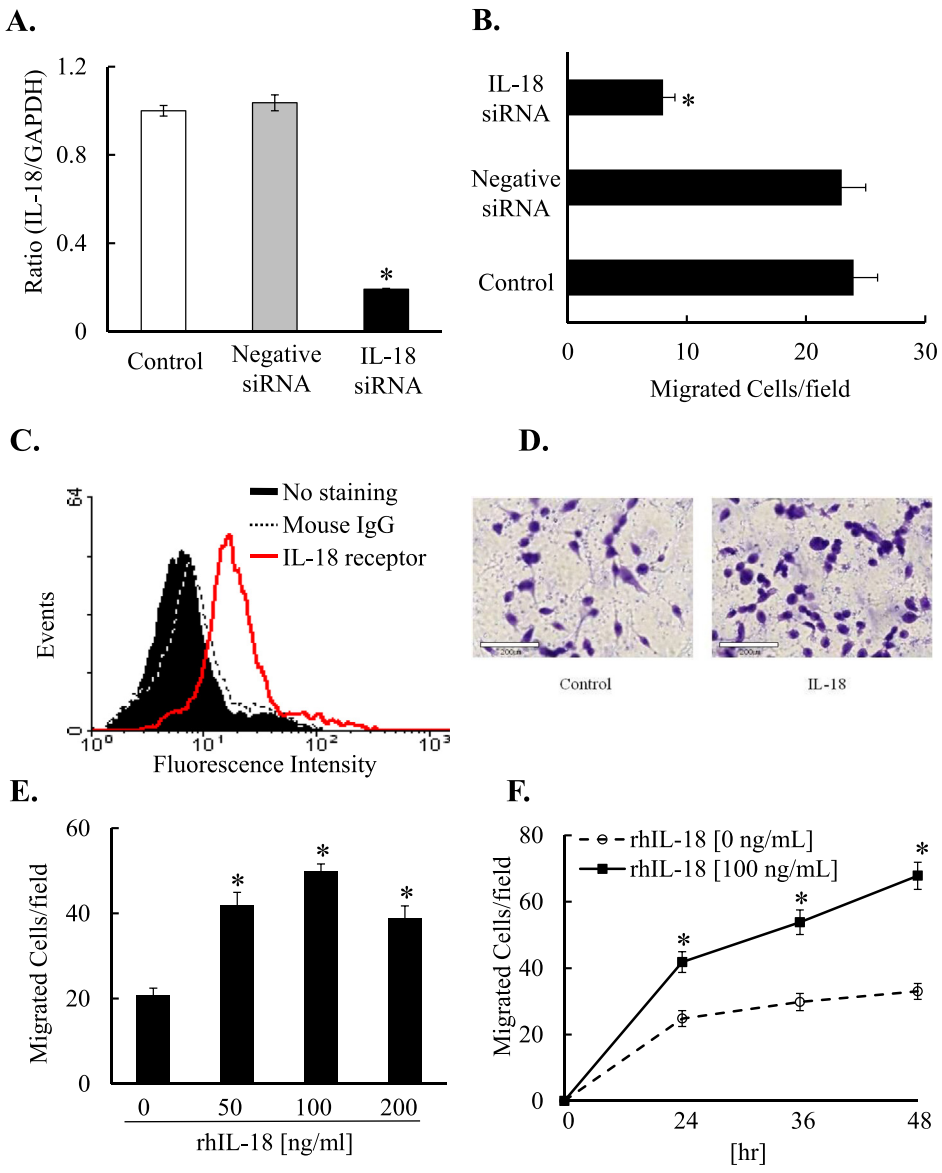
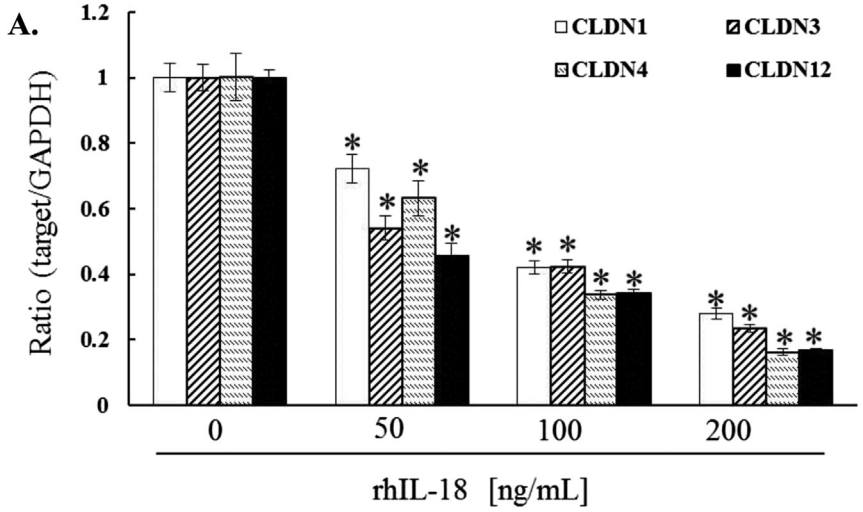
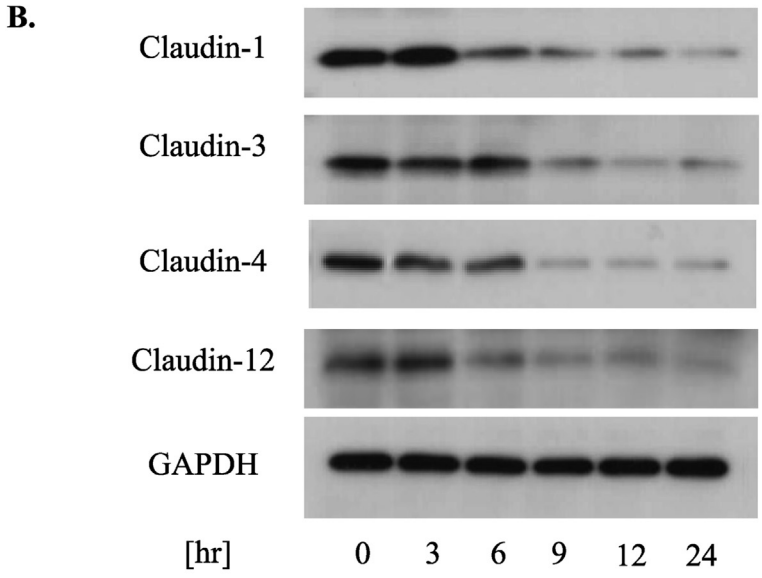


Fig. 1. IL-18 is a critical factor in the migration of MCF-7 human breast cancer cells. A. Negative control siRNA or IL-18 siRNA was transfected into MCF-7 cells. The silencing of IL-18 gene expression was confirmed by real-time PCR. A representative experiment of three independently performed experiments is shown. *Bars*, mean ± SE; *, $p < 0.05$ *versus* the negative control siRNA group (−). B. A migration assay was performed using MCF-7 control cells, negative siRNA-transfected cells, and IL-18-specific siRNA-transfected cells. A representative experiment of three independently performed experiments is shown. *Bars*, mean ± SE; *, $p < 0.05$ *versus* the negative control siRNA group (−). C. MCF-7 cells were stained with a mouse anti-human IL-18Rα antibody, and further stained with a FITC-conjugated goat anti-mouse IgG antibody (red line). The control was not stained with any antibody (filled histogram). Cells were stained with mouse IgG and the FITC-conjugated goat anti-mouse IgG antibody as an additional control (dotted line). The cells were analyzed by indirect FACS (FACSCalibur, BD, Franklin Lakes, NJ). D. MCF-7 cells were treated with 100 ng/ml of rhIL-18 for 48 h. A migration assay was performed using Transwell chambers followed by photomicrograph analysis. Scale bar: 200 μm. E. A rhIL-18 dose titration was performed. MCF-7 cells were treated with 50, 100, or 200 ng/ml of rhIL-18 for 48 h. The migrated cells were counted using the Scanscope. A representative experiment of three independently performed experiments is shown. *Bars*, mean ± SE; *, $p < 0.05$ *versus* control. F. Time kinetics was performed at 100 ng/ml of rhIL-18 for 24, 36, and 48 h. The migrated cells were counted using the Scanscope. A representative experiment of three independently performed experiments is shown. *Bars*, mean ± SE; *, $p < 0.05$ *versus* control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of claudins, we hypothesized that there may be a relationship between breast cancer cell migration and the expression of claudin family proteins. Recently, it was reported that the expression of claudin-1, -3, -4, and -12, which are the major components of TJs in breast tissue, correlate with tumor grade and aggressiveness in human breast cancer cells [21]. Therefore, we aimed to determine whether IL-18 directly regulates claudin expression as part of the mechanism underlying its role ass a migration inducer. Real-time PCR data showed that rhIL-18 treatment inhibited the expression of claudin-1, -3, -4, and -12 in a dose-dependent manner in MCF-7 cells (Fig. 2A). As shown in Fig. 2B, Western blot data showed that protein levels of claudin family members were markedly decreased by rhIL-18. The down-regulation of the TJ proteins by IL-18 was time-dependent, with maximal effects at 24 h. In addition, we further investigated claudin-1, -3, -4, and -12 expression after IL-18 siRNA transfection. Expression of claudin-12 was more significantly enhanced by knockdown of IL-18 than claudin-1, -3, or -4 in both MCF-7 (Fig. 2C) and MDA-MB-231 cells (Fig. S2), suggesting that IL-18 regulates common TJ proteins, particularly claudin-12, in breast cancer cells.





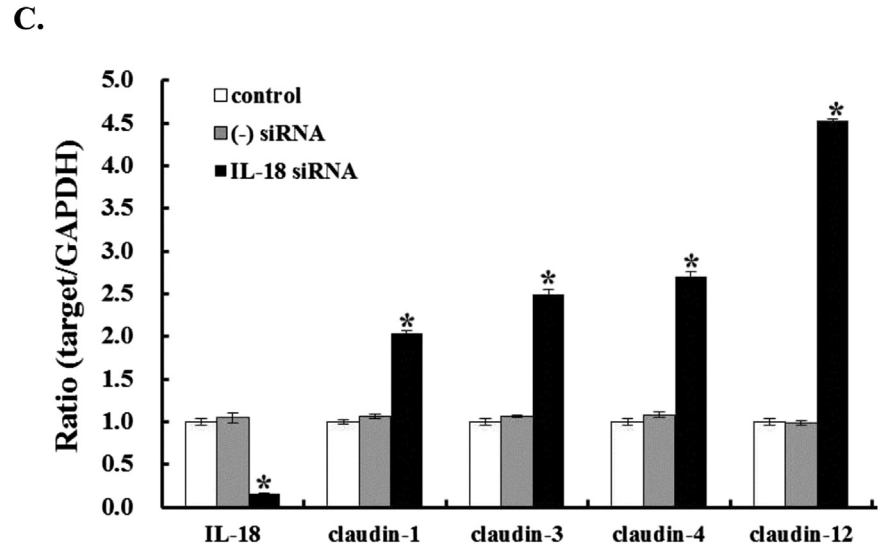


Fig. 2. Exogenous IL-18 suppresses claudins-1, -3, -4, and -12 expression. A. MCF-7 cells were stimulated with 50, 100, and 200 ng/ml of rhIL-18 for 24 h. Following incubation with rhIL-18, total RNA was isolated, and claudin-1, -3, -4, and -12 expression was analyzed by real-time PCR. A representative experiment of three independently performed experiments is shown. *Bars*, mean ± SE; *, $p < 0.05$ *versus* control. B. MCF-7 cells were treated with 100 ng/ml of rhIL-18 for 0, 3, 6, 9, 12, and 24 h. After cell lysis, the levels of claudin-1, -3, -4. and -12 were determined by Western blot analysis. GAPDH expression was used to confirm equal loading of the cell lysates. C. MCF-7 cells were transfected with siRNA targeting IL-18 or a negative control siRNA, and claudin-1, -3, -4, expression was evaluated by real-time PCR. Knockdown of IL-18 was confirmed by real-time PCR.

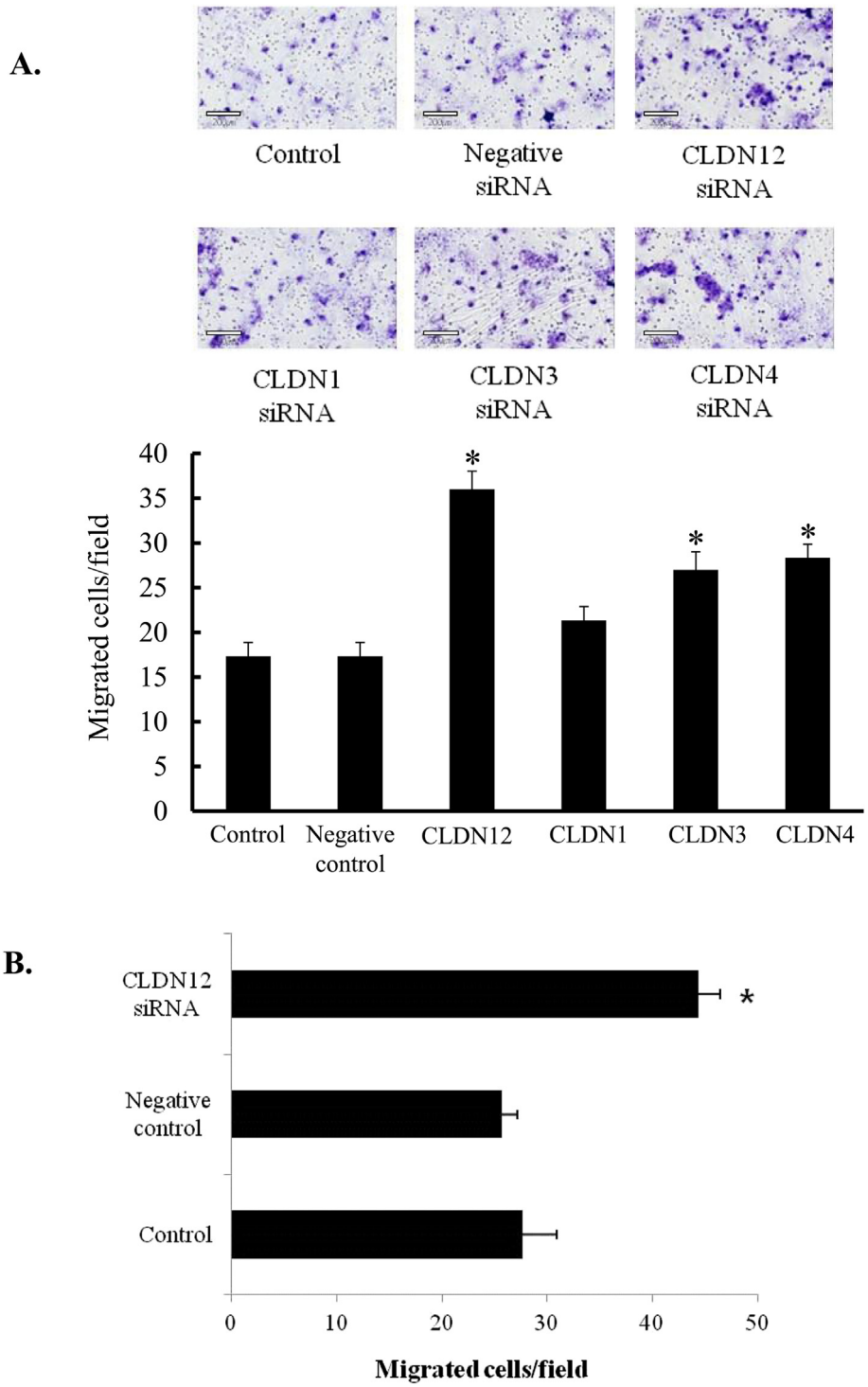


Fig. 3. Claudin-12 siRNA transfection resulted in increased the migration of both MCF-7 and MDA-MB-231 cells. A. MCF-7 cells were transfected with siRNA targeting claudin-1, -3, -4, or -12, or a negative sequence. After cell stabilization, cells were collected and claudin-1, -3, -4, and -12 siRNA transfection was confirmed using Western blot analysis. The migrating cells were imaged with a photomicrograph and then counted using the Scanscope. Results are shown as the mean ± SD of three independent experiments.*, $p < 0.05$ *versus* control. Scale bar: 200 μm. B. MDA-MB-231 cells were transfected with siRNA targeting claudin-12, or a negative sequence. Claudin-12 siRNA transfection was confirmed using RT-PCR. The migrating cells counted using the Scanscope. Results are shown as the mean ± SD of three independent experiments.*, $p < 0.05$ *versus* control.

3.3. Claudin-12 siRNA transfection increases tumor cell migration in MCF-7 and MDA-MB-231 cells.

To examine the relationship between each claudin family member and MCF-7 cell migration, cell migration assays using a Transwell chamber were performed after transfection with siRNA for claudin-1, -3, -4, or -12 (Fig. S3). As shown in Fig. 3A, knockdown of claudin-3, -4, and -12, but not claudin-1, promoted cell migration through the chamber, with the greatest effect observed after knockdown of claudin-12. Thus we next attempted to determine

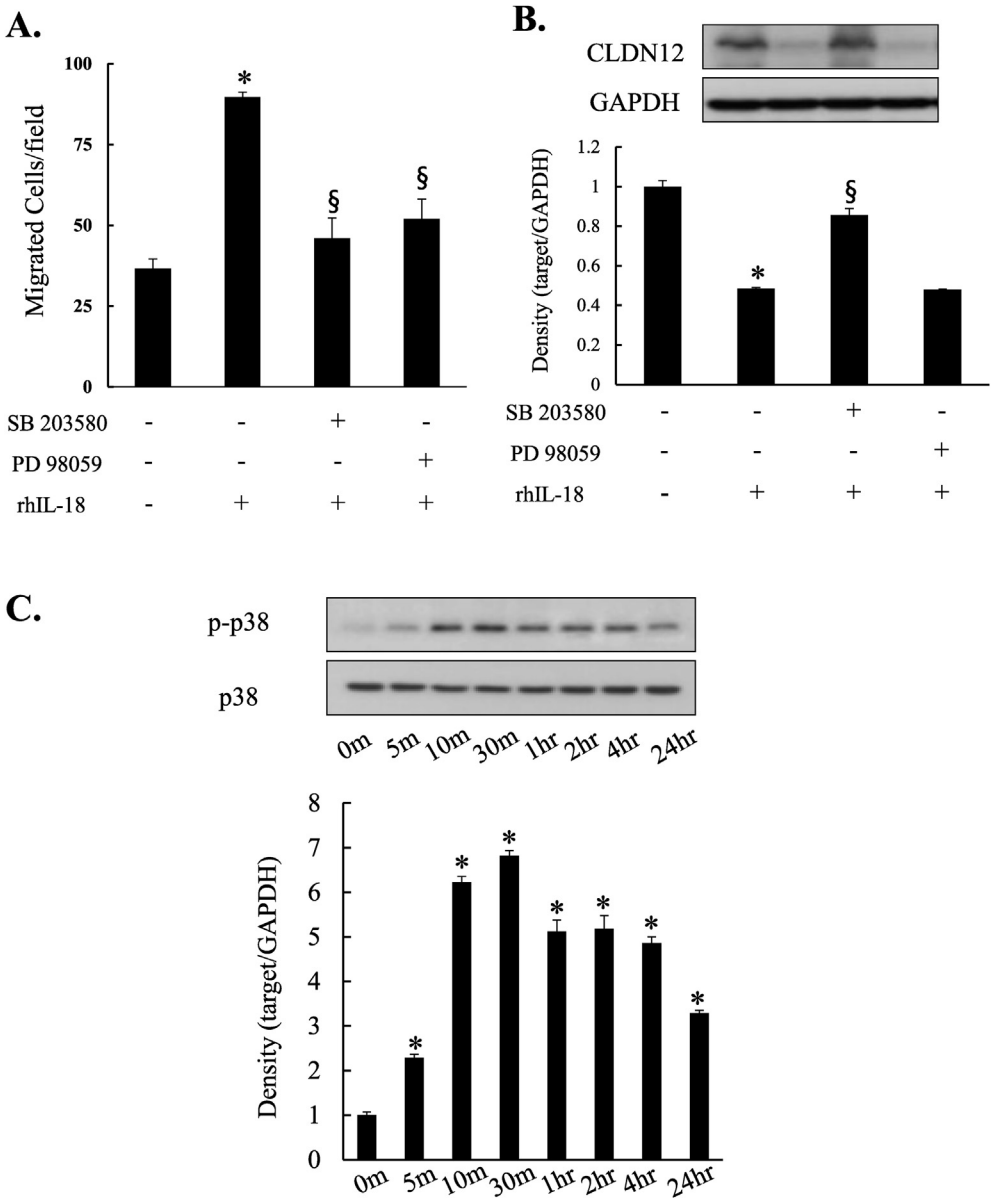


Fig. 4. Activation of the p38 MAPK pathway was involved in IL-18-induced cell migration and down-regulation of claudin-12 in MCF-7 cells. A. MCF-7 cells were pretreated with or without a specific inhibitor of ERK1/2 or p38 for 1 h and then incubated with 100 ng/ml of rhIL-18. A 48 h migration assay was performed and migrated cells were counted using the Scanscope. Results are shown as the mean ± SD of three independent experiments. *, $p < 0.05$ *versus* control. §, $p < 0.05$ *versus* IL-18-treated group. B. Cells were pretreated with or without a specific inhibitor of ERK1/2 or p38 for 1 h and then treated with 100 ng/ml of rhIL-18. Claudin-12 expression was determined by Western blot analysis. The down-regulation of claudin-12 expression induced by IL-18 was significantly inhibited by SB203580, a specific p38 MAPK pathway inhibitor, but not PD98059, a specific ERK1/2 inhibitor. A representative experiment of three experiments performed independently is shown. *Bars*, mean ± SE. *, $p < 0.05$ *versus* control. §, $p < 0.05$ *versus* IL-18-treated group. C. Cells were treated with 100 ng/ml of rhIL-18 for 5 min, 10 min, 30 min, 1 h, 2 h, 4 h, or 24 h. After cell lysis, the level of p38 MAPK phosphorylation was determined by Western blot analysis. The levels of total p38 were used to confirm equal loading of the cell lysates. The histogram represents p38 phosphorylation relative to total p38. Results are shown as the mean ± SD of three independent experiments. *, $p < 0.05$ *versus* control.

whether claudin-12 acts also important factor in the migration of other breast cancer cells. Transfection with claudin-12 siRNA in MDA-MB-231 was confirmed by RT-PCR (Fig. S4). Fig. 3B shows that knockdown of claudin-12 considerably stimulates the cell migration of MDA-MB-231 besides MCF-7. These data suggested that claudin-12 may be a critical factor in breast cancer cell migration.

3.4. Activation of the p38 MAPK pathway is required for IL-18-enhanced cell migration and down-regulation of claudin-12

Modulation of MAP kinase signaling, specifically of ERK1/2 and p38, plays a crucial role in cancer cell migration and claudin expression. To explore the potential mechanisms through which IL-18 might influence claudin-12 expression and breast cancer migration, we first investigated IL-18-enhanced cell migration using specific inhibitors of ERK1/2 (PD98059) and p38 (SB203580). As shown in Fig. 4A, both PD98059 and SB203580 significantly inhibited IL-18-enhanced cell migration. Furthermore, SB203580 dramatically prevented the down-regulation of claudin-12 expression induced by IL-18, but PD98059 had no effect (Fig. 4B). Accordingly, MCF-7 cells were incubated with 100 ng/ml of IL-18, and p38 phosphorylation was determined by Western blot analysis. Fig. 4C shows that IL-18 increased p38 phosphorylation, which reached a maximum at 30 min post-stimulation, indicating that IL-

18 stimulates p38 phosphorylation directly. These data demonstrate that IL-18 regulates claudin-12 expression and breast cancer migration via the activation of p38.

4. Discussion

Despite the immunostimulatory properties of IL-18, this cytokine also supports cancer progression and metastasis. Based on this background, this study determined the migration of recombinant IL-18-treated and IL-18 siRNA-transfected human breast cancer cells, and analyzed the role of the TJ protein claudin-12 in the promigratory mechanism of IL-18. A recent study by Gunel et al. reported that elevated serum levels of IL-18 in breast cancer patients significantly correlated with liver and bone metastases [14]. We previously demonstrated that IL-18 enhances cell migration in human gastric cancer cell lines and skin tumor cells, and increases proliferation in breast cancer cells [8,11,25]. In this study, we showed that breast cancer cell migration was enhanced by IL-18 and that siRNA-mediated knockdown of IL-18 inhibited breast cancer cell migration (Fig. 1 and Fig. S1). Taken together, these data suggest that IL-18 may be a critical factor in the metastasis and pathogenesis of breast cancer as well as gastric cancer and melanoma. Because IL-18 exerts both anti-cancer and pro-cancer effects, IL-18 treatment as a vaccine adjuvant must be carefully studied to overcome potential side effects. More extensive studies are needed to determine the general mechanism underlying the anti-cancer and pro-cancer effects of IL-18 in order to determine its utility for cancer therapy.

In this study, exogenous IL-18 promoted migration of MCF-7 cells at all concentrations tested but was most effective at 100 ng/ml (Fig. 1E). This differed from the dose-dependent relationship between IL-18 concentration and claudin expression in the MCF-7 breast cancer cell line (Fig. 2A), which suggests that, in addition to claudin, other factors and mechanisms may mediate the effect of IL-18 on the migration of MCF-7 cells. For example, IL-18 also leads to a considerable increase in N-cadherin, an epithelial-to-mesenchymal transition (EMT) gene in MCF-7 cells (unpublished data). N-cadherin promotes the migration, invasion, and metastasis of MCF-7 cells [26,27], and it may also affect proliferation and survival. Further investigation will be necessary to clarify the other mechanisms and factors involved in IL-18-induced MCF-7 cell migration.

Altered expression of several claudin proteins, in particular claudin-1, -3, and -4, is associated with tumor progression in various cancers. Their dysregulation during cancer progression suggests that the claudins play a role in the migration, invasion, and metastasis of cancer cells. Although claudin-12 is expressed constitutively in most tissues, including the breast, the exact mechanism underlying its effect on tumor cell migration remains unclear. The present study showed that IL-18 suppresses the expression of claudin-1, -3, and -4 as well as that of claudin-12 (Fig. 2A and B). This suggests that claudin-12 is not the only target of IL-18 and that the claudin-12 pathway is just one of the mechanisms underlying IL-18-enhanced cell migration. According to a recent study by Grone et al., claudin-12 is frequently overexpressed in colorectal cancer (CRC) cells, suggesting that it may be a strong prognostic indicator [20]. By contrast, our data showed that rhIL-18 enhanced breast cancer migration through down-regulation of claudin-12 (Fig. 3). A possible explanation for these results may be that claudin family proteins are abnormally regulated in human cancers in a tissue-dependent manner. For example, claudin-1 expression is frequently up-regulated in CRC, whereas claudin-1 expression is down-regulated in breast cancer compared with levels in normal tissue [28,29]. In this regard, more extensive studies are needed to demonstrate the cell type and tissue specificity of each of the claudin family members. The mechanism by which claudin family members control the migration and invasiveness of other cancers remains to be determined.

In conclusion, our data suggest that there is an inverse correlation between IL-18 and claudin-12 expression, and that IL-18 induces tumor migration in breast cancer via regulation of claudin-12 expression and the p38 MAPK pathway. Furthermore, claudin family members, particularly claudin-12, may be strong diagnostic candidates and effective gene targets for therapeutic approaches to breast cancer.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.108.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.108.

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