Sphingosine 1-phosphate regulates matrix metalloproteinase-9 expression and breast cell invasion through S1P$_3$–G$_{\alpha q}$ coupling

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Summary

Recent evidence suggests that inflammation is involved in malignant progression of breast cancer. Sphingosine 1-phosphate (S1P), acting on the G-protein-coupled receptors, is known as a potent inflammatory mediator. In this study, the effect of the inflammatory lipid S1P on the regulation of invasive/migratory phenotypes of MCF10A human breast epithelial cells was investigated to elucidate a causal relationship between inflammation and the control of invasiveness of breast cells. We show that S1P causes induction of matrix metalloproteinase-9 (MMP-9) in vitro and in vivo, and thus enhances invasion and migration. We also show that fos plays a crucial role in the transcriptional activation of MMP-9 by S1P. In addition, activation of extracellular-signal-regulated kinases 1 and 2 (ERK1/2), p38 and alpha serine/threonine-protein kinase (Akt) are involved in the process of S1P-mediated induction of MMP-9 expression and invasion. Activation of the S1P receptor S1P$_3$ and G$_{\alpha q}$ are required for S1P-induced invasive/migratory responses, suggesting that the enhancement of S1P-mediated invasiveness is triggered by the specific coupling of S1P$_3$ to the heterotrimeric G$_{\alpha q}$ subunit. Activation of phospholipase C-β_4 and intracellular Ca^{2+} release are required for S1P-induced MMP-9 upregulation. Taken together, this study demonstrated that S1P regulates MMP-9 induction and invasiveness through coupling of S1P$_3$ and G$_{\alpha q}$ in MCF10A cells, thus providing a molecular basis for the crucial role of S1P in promoting breast cell invasion.

Key words: S1P, Breast cell invasion, MMP-9

Introduction

Breast cancer is one of the main causes of cancer deaths among women (Jemal et al., 2008) and cancer metastasis is an important cause of poor prognosis in breast cancer patients. Recent evidence suggests that inflammation is involved in the malignant progression of breast cancer. Invasion, migration and metastasis of breast cancer cells are mediated through NFκB and cytokines such as interleukin 6 (Wu et al., 2009; Barbieri et al., 2010). Inflammatory breast cancer in a human xenograft model displays the phenotype of lymphovascular invasion (Alpaugh et al., 1999). Inflammation has been associated with poor prognosis of breast cancer (Hojilla et al., 2008) and chronic inflammation is a risk factor for breast cancer recurrence (Cole, 2009).

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid metabolite, which acts as a ligand for specific G-protein-coupled receptors (GPCRs), namely S1P$_{1-5}$ (van Brocklyn et al., 1998) (also known as S1PR1–5). Investigations into the roles of S1P have provided direct evidence for its importance in inflammation (Ammit et al., 2001; Hait et al., 2006; Tauseef et al., 2008) (reviewed by Nixon, 2009). Responses triggered by S1P are often mediated by coupling of GPCRs to different heterotrimeric G proteins, such as G$_{12}$ and G$_{13}$, (G$_{12/13}$) G$_q$ or G$_i$ (Katoh et al., 1998). The S1P$_1$ receptor is known to couple to G$_i$, S1P$_2$ and S1P$_3$ couple to G$_{12/13}$, G$_q$ and G$_i$, whereas S1P$_4$ and S1P$_5$ couple to G$_{12/13}$ and G$_i$ (Rosen et al., 2009). S1P$_2$ has been shown to inhibit migration, whereas S1P$_1$ and S1P$_3$ induce migration in most cell types (Takuwa 2002; Taha et al., 2004). S1P induces cell migration and invasion in epithelial ovarian cancer and breast carcinomas via S1P$_1$ or S1P$_3$ (Smicun et al., 2006; Shida et al., 2008), but can inhibit invasion or migration in B16 melanoma and human ovarian surface epithelial cells via S1P$_2$ receptor (Arikawa et al., 2003; Yamaguchi et al., 2003).

S1P differentially regulates activation or inhibition of Rho-family small GTPases (Rho and Rac) through S1P receptor-dependent mechanisms: S1P–S1P$_2$ leads to the activation of Rho and inhibition of Rac in vascular smooth muscle cells (Ryu et al., 2002), whereas S1P–S1P$_3$ stimulates Rho and Rac in Chinese hamster ovary cells and human umbilical vein endothelial cells (Siehler and Manning, 2002; Paik et al., 2001). S1P-induced cell motility is regulated by the p38 MAPK, ERK and Rho pathways in human primary fibroblast-like synoviocytes (Zhao et al., 2008). S1P can also stimulate the activation of PI3K–Akt and ERKs in human dermal MVECs cells (Hsieh et al., 2008; Heller et al., 2008).

Tumor invasion is often associated with the enhanced synthesis of matrix metalloproteinases (MMPs), among which high levels of MMP-2 and MMP-9 have been correlated with breast cell invasion (Stetler-Stevenson, 1999; Sun et al., 2009). We have previously shown that MMP-2 plays a major role in MCF10A human breast epithelial cell invasion induced by oncogenic H-Ras (Moon et al., 2000; Kim et al., 2003; Song et al., 2006) and by G$_{\alpha 12/13}$ (Kim et al., 2010). Recently, we showed that breast cell invasion induced by G$_{\alpha 12/13}$ is mediated by MMP-2 in MCF10A cells (Kim et al., 2010). The effects of S1P on MMPs have been reported in various

cell systems. S1P induces invasion of human endothelial cells and HT1080 fibrosarcoma cells by regulating MMP-2 or MT1–MMP (Wu et al., 2005; Fisher et al., 2006). In contrast, the inhibitory role of S1P in IL-β-induced MMP-9 expression has been shown in mesangial cells (Xin et al., 2004).

In an attempt to elucidate the molecular link between inflammation and breast cancer progression, the present study investigated the effect of an inflammatory lipid S1P on the invasive program of breast epithelial cells. Although the induction of mammary epithelial cell invasion occurs as a normal physiological process during pregnancy when the gland expands in preparation for lactation (Traurig, 1967), an invasive phenotype has been shown to induce the expression of MMPs, thus accelerating extracellular matrix degradation and metastasis in experimental models of tumorigenesis. In the present study, we focused on the role of S1P in the induction of cell invasion in a pathological condition where S1P promotes inflammation, cell proliferation and tumor aggressiveness. We show that S1P induces MMP-9 upregulation, invasive and migratory phenotypes through S1P$_3$–G$_{\alpha q}$ coupling in human breast epithelial cells. Furthermore, we show the involvement of phospholipase C-β$_4$ (PLC-β$_4$) and Ca^{2+} in S1P-induced MMP-9 upregulation.
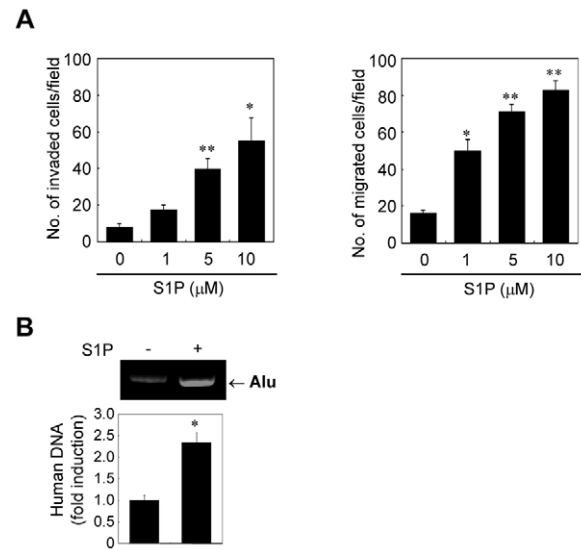
Results
S1P induces invasive and migratory phenotypes in MCF10A cells
To determine the effect of S1P on invasive and migratory phenotypes of MCF10A cells, in vitro invasion and migration assays were conducted on cells treated with various concentrations of S1P. Physiological concentration of S1P in human blood is in the range 200–500 pmol/ml (Yatomi et al., 1997). However, to observe the cellular responses upon S1P treatment in vitro, cells have previously been treated with 0.01–10 μM S1P (Siehler et al., 2001; Radeke et al., 2005; Takashima et al., 2008). We used the concentration range of 1–10 μM for our in vitro experiments. As shown in Fig. 1A, invasion and migration were significantly induced by S1P treatment in a dose-dependent manner, demonstrating that S1P confers invasive and migratory phenotypes on MCF10A cells.

In vivo cell invasion was evaluated using a chick embryo invasion model in which cells were examined for their ability to degrade the embryo chorioallantoic membrane (CAM) and penetrate into the bloodstream (Mira et al., 2002). As shown in Fig. 1B, intravasation of MCF10A cells treated with S1P in the chick CAM model was enhanced up to 2.3-fold compared with controls, indicating that S1P significantly induced an invasive capacity in MCF10A cells in vivo.

S1P upregulates MMP-9 by transcriptional activation
Because tumor invasion and metastasis are often associated with enhanced synthesis of MMP-2 and/or MMP-9, we examined the effect of S1P on the expression of these proteins. A marked induction of MMP-9 was observed in S1P-treated MCF10A cells using a gelatin zymogram assay and immunoblot analysis (Fig. 2A, left and center, respectively). We further assessed the level of active MMP-9 in the conditioned media using a Fluorokine E enzyme activity assay. Although the active MMP-9 band was not detected by the gelatin zymogram assay, the increased level of active MMP-9 was detected in conditioned media of S1P-treated MCF10A cells by the Fluorokine E enzyme activity assay (Fig. 2A, right). By contrast, S1P had no effect on MMP-2 expression,

Fig. 1. S1P induces invasive and migratory phenotypes in MCF10A cells. (**A**) In vitro invasion and Transwell migration assays were conducted on MCF10A cells treated with the indicated concentrations of S1P for 17 hours. Values are means + s.e.m. of triplicate samples (*$P<0.05$ and **$P<0.01$ compared with control). (**B**) In vivo invasion of MCF10A cells treated with 10 μM S1P for 48 hours was examined by inoculating them onto the upper side of the CAM of a chick embryo. The Alu-PCR bands from three experiments were quantified and the results were expressed as the percentage change in band intensity compared with the control. Values are means + s.e.m. for triplicate samples (*$P<0.01$ compared with the control).

indicating that S1P selectively upregulates MMP-9, but not MMP-2, in MCF10A cells.

To investigate gene transcription as a potential mechanism for S1P-induced MMP-9 upregulation, RT-PCR and a luciferase reporter assay were conducted. The mRNA level of MMP-9 was increased by S1P, as evidenced by RT-PCR (Fig. 2B, left), indicating that S1P induces MMP-9 at the transcription level. A prominent activation of the MMP-9 promoter was observed in S1P-treated MCF10A cells, whereas the activity of the MMP-2 promoter was not significantly altered (Fig. 2B, center and right). These results demonstrate that S1P induces upregulation of MMP-9 by transcriptional activation in MCF10A cells.

To address the role of MMP-9 in the S1P-induced invasive/migratory phenotypic changes, we knocked-down MMP-9 by short interfering RNA (siRNA). Decreased expression of MMP-9 by siRNA was confirmed by gelatin zymography (Fig. 2C, left). Invasion and migration of MCF10A cells treated with S1P were significantly inhibited by knockdown of MMP-9 (Fig. 2C, center and right), indicating the essential role of MMP-9 in S1P-induced invasive and migratory phenotypic changes.

fos is a potential transcription factor responsible for S1P-induced transcriptional activation of MMP-9
To identify the potential transcriptional element(s) in the MMP-9 promoter responsible for transcriptional regulation by S1P, we examined the DNA binding activities of transcription factors AP-1 and NFκB, which are known to regulate the expression of MMP-9 (Sato et al., 1993; Kim et al., 2006). We performed an electrophoretic mobility shift assay (EMSA) using a probe

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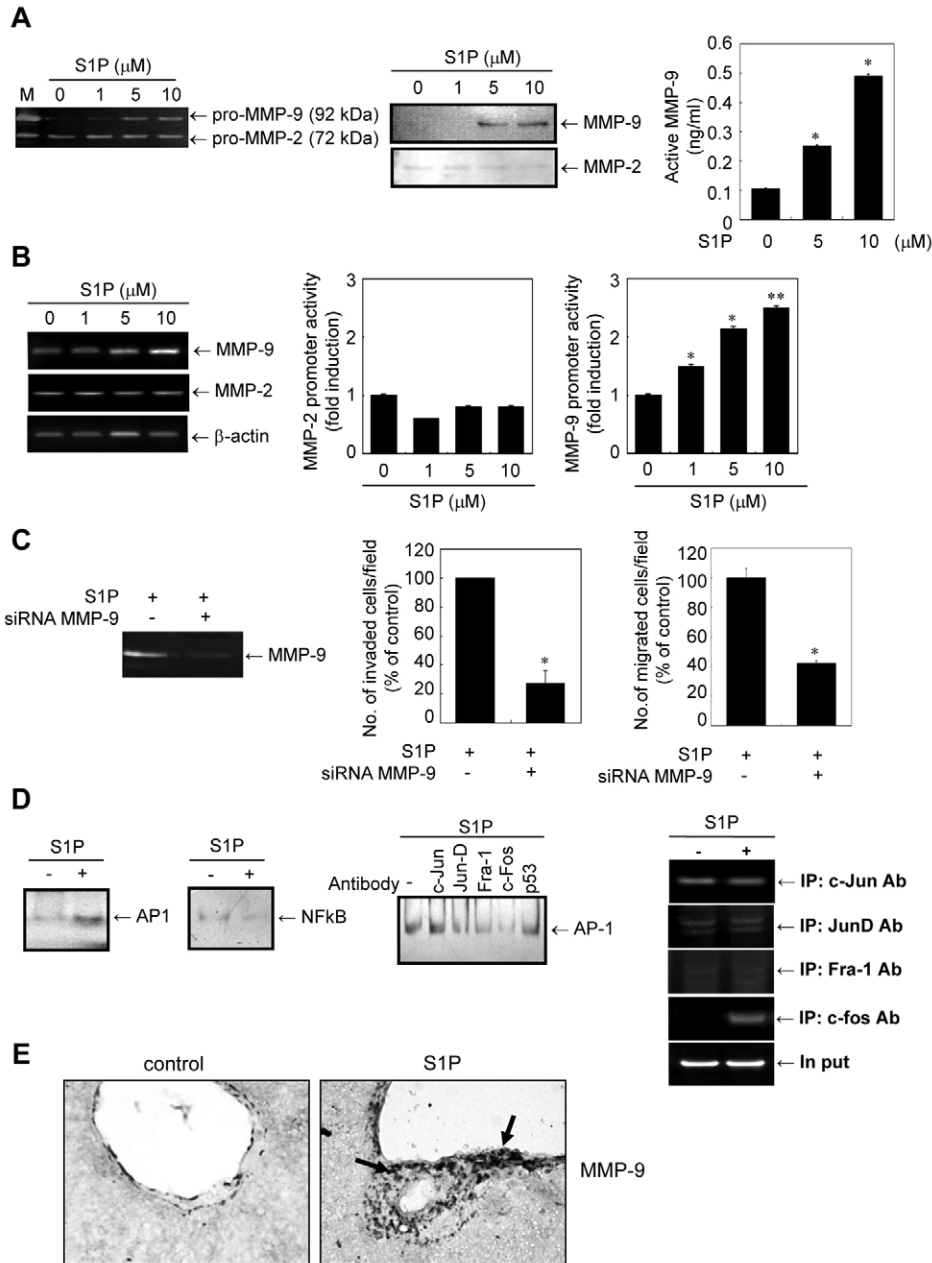
Fig. 2. S1P upregulates MMP-9 by transcriptional activation. (A) Gelatin zymogram assay (left) and immunoblot analysis (center) were conducted on cells treated with S1P for 48 hours (right: the level of active MMP-9 was measured in the conditioned media). The conditioned media of HT1080 human fibrosarcoma cells were used as molecular markers for MMP-2 and MMP-9. **(B)** Left: RT-PCR was conducted in cells treated with S1P for 48 hours. Right: luciferase assays were performed to detect promoter activities of MMP-2 and MMP-9. The luciferase activity in 1 μg of cell lysate was normalized to β-galactosidase activity. Values are means + s.e.m. for triplicate samples (*$P<0.05$ and **$P<0.01$ compared with control). **(C)** Cells were transfected with control siRNA or siRNA targeting MMP-9. Knockdown of MMP-9 was confirmed by gelatin zymography. The transfected cells were subjected to in vitro invasion and transwell migration assays (*$P<0.05$). **(D)** Left: nuclear extracts were subjected to EMSA with a probe for AP-1 or NFκB. Center: nuclear extracts were analyzed by EMSA for DNA binding activity of AP-1 with Jun (c-Jun), JunD, Fra-1, fos (c-fos) and p53 antibodies (immunoinhibition assay). Right: ChIP assay was performed on the DNA–protein complexes. The samples were PCR-amplified using specific primers of the AP-1 site in the MMP-9 promoter. The 357 bp PCR product was detected. A quarter of the total input was loaded as a control. IP, immunoprecipitation. **(E)** Immunohistochemical staining for MMP-9 in liver of the mice treated with vehicle (control) or S1P (S1P). Infiltration of MMP-9-positive macrophages (arrows) was increased in the periportal area of the rats treated with S1P. Original magnification was 400×.

containing the binding site of AP-1 or NFκB. Increased AP-1 binding activity was observed in nuclear extracts from MCF10A cells treated with S1P compared with control, untreated cells (Fig. 2D, left), suggesting the induction of DNA binding activity of AP-1 by S1P. By contrast, the level of DNA binding activity of NFκB was not altered by S1P treatment.

To elucidate the identity and specificity of the binding protein to the AP-1 site, an immunoinhibition study was conducted with specific antibodies directed against AP-1 family members, including Jun, JunD, Fra-1 and fos. p53 antibody was used as a negative control. A marked depletion of the band was observed by addition of an antibody against fos (Fig. 2D, center). To confirm the binding of fos to the AP-1 binding site of the MMP-9 promoter region, a chromatin immunoprecipitation (ChIP) assay was performed using an anti-fos antibody in combination with primers amplifying two AP-1 binding sites (–533 bp site and –79

bp site) in the MMP-9 promoter region. A 357 bp band representing the AP-1 binding site at the –533 bp site in the MMP-9 promoter was detected in the S1P-treated cells using a fos antibody, but no DNA amplification was observed using antibodies against Jun, JunD or Fra-1 (Fig. 2C, right). The data demonstrate that upon S1P treatment, fos binds to an AP-1 binding site in the MMP-9 promoter region, and thus increases MMP-9 gene transcription. It remains to be elucidated, however, whether S1P increased the amount of fos or the affinity of AP-1 containing fos for the cis-element of MMP-9 promoter. Consistent with our results, the activation of AP-1 in response to GPCR signaling (Oyesanya et al., 2010) and the involvement of fos in MMP-9 transcriptional regulation have been reported in other cell systems (Crowe and Brown, 1999; Han et al., 2006). Of note, the data show that Jun is associated with the promoter region, regardless of the stimulation by S1P. These results suggest that Jun may be

constantly bound to the promoter region of MMP-9 and regulate the basal level of MMP-9 transcription, whereas fos binds to the MMP-9 promoter upon S1P treatment.

To determine whether S1P induces MMP-9 upregulation in vivo, an immunohistochemical analysis was performed on liver sections of mice that had been given a single dose of S1P (0.1 mg/kg) as an infusion into a femoral vein over 30 minutes. As shown in Fig. 2E, the cells expressing MMP-9 were increased in liver tissue

infused with S1P compared with the control. The data demonstrate that S1P induces MMP-9 expression in vivo.

S1P activates Rac1, ERK1/2, p38 and Akt signaling pathways

We next investigated the signaling pathways involved in S1P-induced invasive/migratory phenotypic changes. A kinetic study showed that Rho was not activated by S1P for up to 60 minutes,
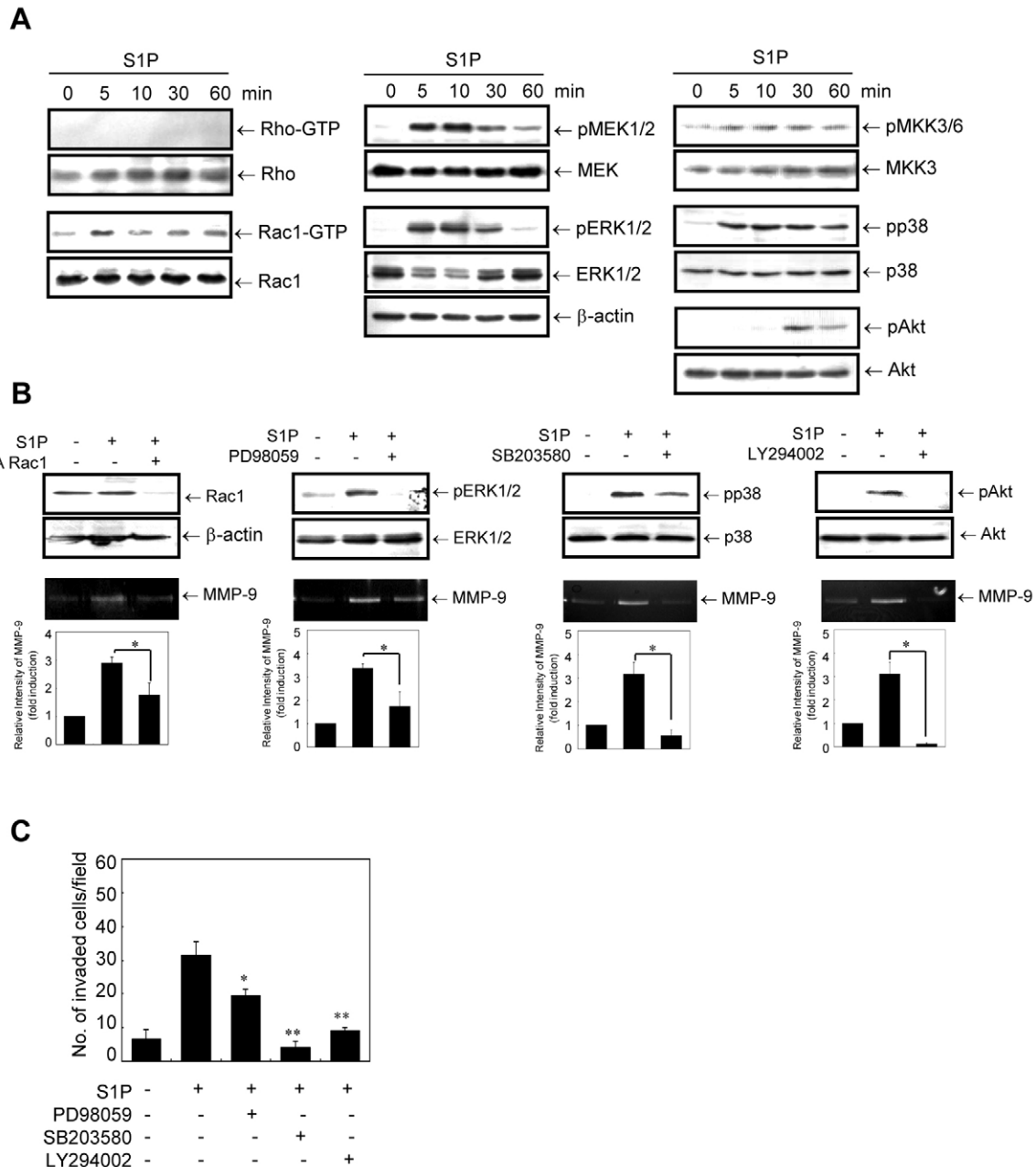
Fig. 3. p38 MAPK and Akt signaling pathways are essential for the S1P-induced MMP-9 upregulation and invasion of MCF10A cells. (A) Cells were treated with 10 μM S1P for the indicated time. Rac1 and Rho activities were determined by a pull-down assay. The levels of activated MEK1/2, ERK1/2, MKK3/6, p38 MAPK and Akt were determined by immunoblot analyses using phospho-specific antibodies (pMEK1/2, pERKs, pMKK3/6, pp38 and pAkt, respectively). (B) Left: cells were transfected with control siRNA or siRNA targeting Rac1. The siRNA-transfected cells were treated with 10 μM S1P for 5 minutes. Knockdown of Rac1 was confirmed by immunoblot analysis. Gelatin zymogram assay was performed to detect MMP-9. Relative band intensities were measured using an image analyzer. Center and right: cells were treated with S1P (10 μM) for 48 hours in the absence or presence of each inhibitor (50 μM). Inhibition of ERK1/2, p38 and Akt were confirmed by immunoblot analyses. A gelatin zymogram assay was performed on cells treated with inhibitors for 48 hours. (C) An in vitro invasion assay was conducted on MCF10A cells treated with 10 μM S1P for 17 hours in the absence or presence of each inhibitor (50 μM). Values are means + s.e.m. for triplicate samples (*$P<0.05$ and **$P<0.01$ compared with control).

indicating that S1P did not activate the Rho-dependent pathway in MCF10A cells (Fig. 3A). Treatment with S1P rapidly activated Rac1. The levels of the phosphorylated forms of MAPK kinase 1 and 2 (MEK1/2) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) were markedly increased 5 minutes after S1P treatment and decreased after 10 minutes. Activation of mitogen-activated protein kinase kinase 3 and 6 (MKK3/6) and p38 was observed 5 minutes after S1P treatment and was maintained until 30 minutes. A late induction of alpha serine/threonine-protein kinase (Akt) activation was detected 30 minutes after S1P treatment. The data indicate that S1P induced activation of Rac1, MEK1/2, ERK1/2, MKK3/6, p38 and Akt at different time points.

To determine the role of Rac1 in S1P-induced MMP-9 upregulation, we knocked-down Rac1 by an siRNA targeting Rac1. Rac1 siRNA decreased Rac1 expression, as confirmed by immunoblot analysis. S1P-induced upregulation of MMP-9 was significantly inhibited by knockdown of Rac1 (Fig. 3B, left). The results demonstrate that Rac1 mediates S1P-induced MMP-9 upregulation in MCF10A cells. To elucidate the functional role of these signaling molecules in S1P-induced MMP-9 upregulation and induction of the invasive phenotype, a gelatin zymogram and in vitro invasion assays were conducted on cells treated with pharmacological inhibitors of ERK1/2, p38 and Akt. Treatment with PD98059 (an inhibitor of MEK1), SB203580 (an inhibitor of p38) and LY294002 (an inhibitor of PI3K) effectively inhibited the activation of ERK1/2, p38 and Akt, respectively (Fig. 3B). As shown in Fig. 3B, PD98059, SB203580 and LY294002 reduced the level of MMP-9. The S1P-induced invasive ability of MCF10A

cells was also significantly inhibited by PD98059, SB203580 or LY294002 (Fig. 3C). The data demonstrate that activation of the ERKs, p38 and Akt signaling pathways play important roles in S1P-induced upregulation of MMP-9 and the invasive phenotype of MCF10A cells.

$G_{\alpha q}$ is crucial for S1P-induced invasive/migratory phenotypes

S1P binds to GPCR, which in turn activates the heterotrimeric G-proteins, including $G_{\alpha 12/13}$ and/or $G_{\alpha q}$. Because we observed that S1P did not activate Rho (Fig. 3A), which has been shown to be activated by $G_{\alpha 12/13}$ (Suzuki et al., 2003; Wells et al., 2002), we hypothesized that $G_{\alpha q}$, rather than $G_{\alpha 12/13}$, would mediate S1P-induced MMP-9 upregulation and invasive/migratory phenotypic changes in MCF10A cells. To address this issue, the cells were transfected with an siRNA targeting $G_{\alpha q}$. Knockdown of $G_{\alpha q}$ was confirmed by immunoblot analysis (Fig. 4A). As shown in Fig. 4B, S1P-upregulated MMP-9 was almost completely blocked by $G_{\alpha q}$ siRNA. The invasive/migratory abilities of MCF10A cells treated with S1P were significantly reduced by knockdown of $G_{\alpha q}$ (Fig. 4C). Knockdown of $G_{\alpha q}$ significantly decreased the activation of ERKs, p38 and Akt by S1P (Fig. 4D). Taken together, these data demonstrate that $G_{\alpha q}$ mediates S1P-induced MMP-9 upregulation, invasion/migration and activation of signaling molecules in MCF10A cells.

We then determined if activation of $G_{\alpha q}$ induced invasion and migration of MCF10A cells. To this end, we established a stable cell line of MCF10A transfected with $G_{\alpha q}$QL (Vara Prasad et al.,
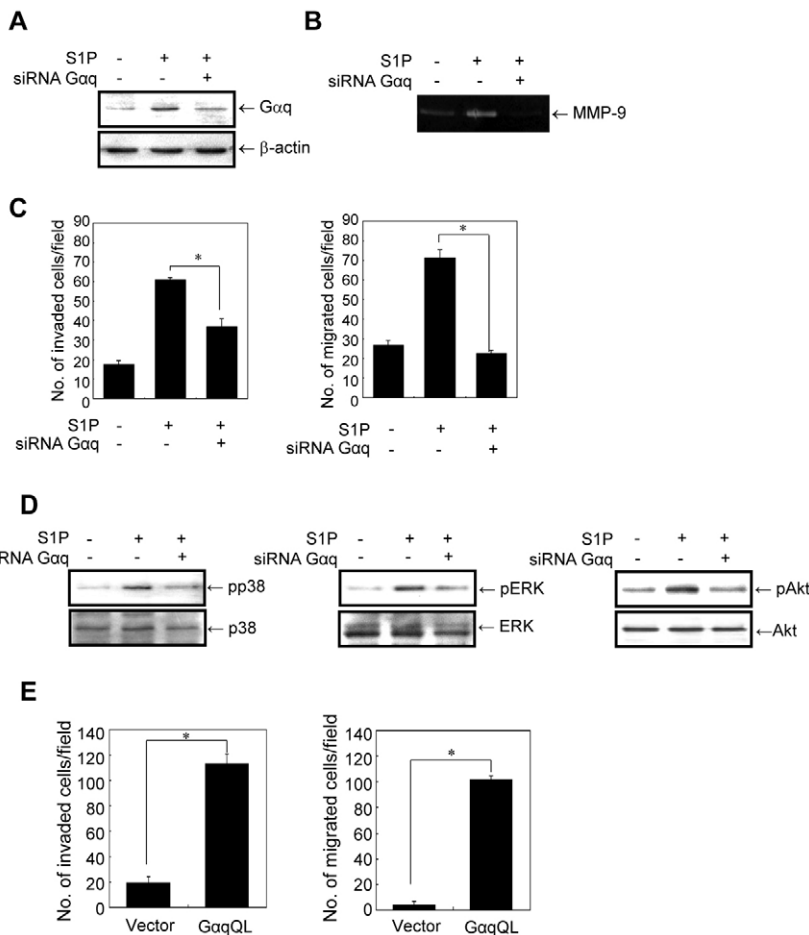

Fig. 4. $G_{\alpha q}$ is crucial for S1P-induced invasive and migratory phenotypes of MCF10A cells. (**A**) Cells were transfected with control siRNA or siRNAs targeting $G_{\alpha q}$ (50 pmol). Knockdown of $G_{\alpha q}$ was confirmed by immunoblot analysis. (**B**) The conditioned media were analyzed by gelatin zymogram assay. (**C**) The transfected cells were subjected to in vitro invasion and transwell migration assays (*$P<0.01$). (**D**) The levels of activated p38 MAPK, ERKs and Akt were determined by immunoblot analyses using phospho-specific antibodies (pp38, pERKs and pAkt, respectively). (**E**) Cells were transfected with vector or $G_{\alpha q}$QL. The transfected cells were subjected to in vitro invasion and transwell migration assays. *$P<0.01$.

1994), a constitutively active mutant of $G_{\alpha q}$. As shown in Fig. 4E, invasive and migratory abilities were significantly induced by activation of $G_{\alpha q}$, demonstrating that the activation of $G_{\alpha q}$ confers invasive/migratory phenotypes on MCF10A cells.

S1P₃ receptor is involved in S1P-induced cell responses of MCF10A cells

Among the S1P receptors, $S1P_2$ and $S1P_3$ have been shown to couple to $G_{\alpha q}$ (Takabe et al., 2008). Because $S1P_2$ negatively regulates S1P-mediated responses of vascular endothelial cells, whereas $S1P_3$ induces migration, morphogenesis and cytoskeletal reorganization (Yatomi, 2006; Taha et al., 2004), we focused on the $S1P_3$ receptor. To address the role of $S1P_3$ in S1P-induced cell responses, we used an siRNA targeting the $S1P_3$ gene transcript. The siRNA transfection effectively decreased the level of the $S1P_3$ mRNA, as confirmed by RT-PCR (Fig. 5A).

The S1P-induced upregulation of MMP-9 was markedly inhibited by an siRNA targeting $S1P_3$ and by treatment with CAY10444, an antagonist of $S1P_3$ (Fig. 5B, left and right, respectively). The activation of MEK1/2, ERK1/2, p38 and Akt were reduced by knockdown of $S1P_3$ (Fig. 5C). These data demonstrate that $S1P_3$ is essential for the S1P-induced MMP-9 upregulation and activation of signaling pathways. Our results suggest that the coupling of $S1P_3$ to $G_{\alpha q}$ may mediate these cellular responses triggered by S1P in MCF10A cells.
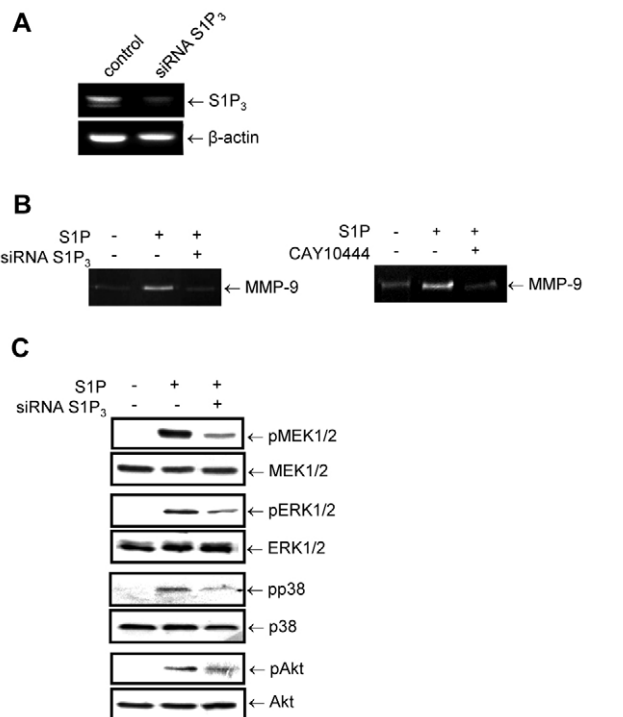
Fig. 5. S1P-induced MMP-9 upregulation is mediated through the S1P₃ receptor. (**A**) Cells were transfected with control siRNA or siRNAs targeting $S1P_3$ (50 pmol). Knockdown of $S1P_3$ was confirmed by RT-PCR. (**B**) Left: the siRNA-transfected cells were subjected to gelatin zymogram assay. Right: gelatin zymogram assay was performed on cells treated with 10 μM S1P for 48 hours in the absence or presence of 100 μM CAY10444. (**C**) The levels of activated MEK1/2, ERKs, p38 MAPK and Akt were determined by immunoblot analyses using phospho-specific antibodies (pMEK1/2, pERKs, pp38 and pAkt, respectively).

Ca²⁺ signaling is required for S1P-induced MMP-9 upregulation and invasion

Exogenous S1P increases intracellular Ca^{2+} through activation of PLC in many cell types (Okajima et al., 1996; Sato et al., 1999). We next examined whether the PLC-Ca^{2+} pathway participated in S1P-induced MMP-9 upregulation of MCF10A cells. The intracellular Ca^{2+} level was increased by S1P treatment, as measured using a Ca^{2+} indicator, Fura-2/AM (Fig. 6A). We then determined the effect of S1P on the expression of PLC-β_4, which was shown to be activated by $G_{\alpha q}$ (Peng et al., 1997). As shown in Fig. 6B, S1P induced the expression of PLC-β_4 in a time-dependent manner. This induction was inhibited by knockdown of $G_{\alpha q}$ (Fig. 6C). To examine whether Rac1 is crucial to the S1P-induced PLC-β_4 expression, the level of PLC-β_4 was detected in cells transfected with an siRNA targeting Rac1. Knockdown of Rac1 was confirmed
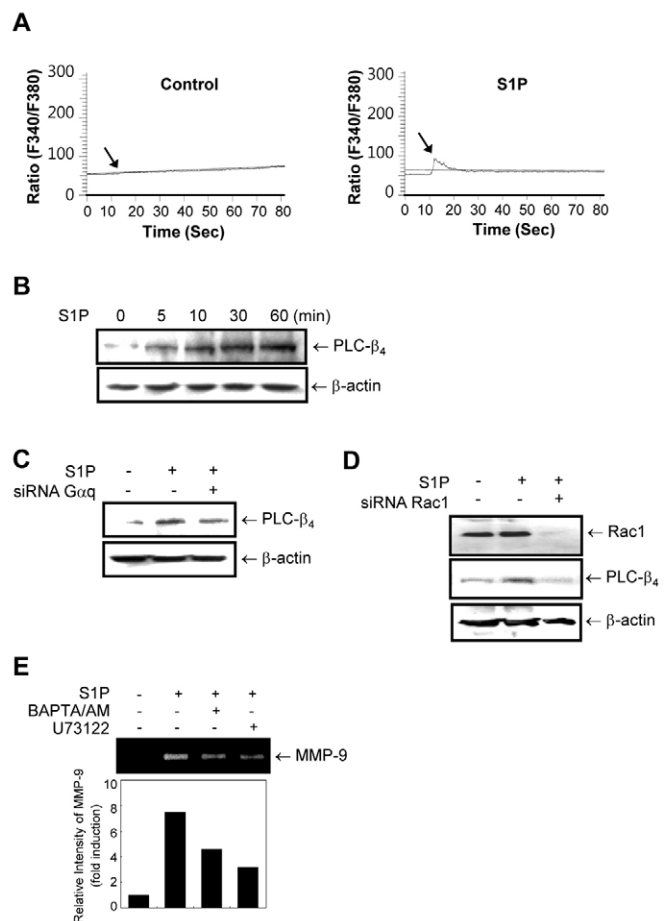
Fig. 6. Ca²⁺ signaling is required for S1P-induced MMP-9 upregulation. (**A**) Cells were loaded with the Ca^{2+} indicator Fura-2/AM, and then intracellular calcium level was measured using a fluorescence spectrophotometer. The arrow indicates the time of addition of 40 μM S1P. Min, minutes; sec, seconds. (**B**) Cells were treated with 10 μM S1P for the indicated time. PLC-β_4 expression was detected by immunoblot analysis. (**C**) Cells were transfected with control siRNA or siRNAs targeting $G_{\alpha q}$ (50 pmol). Expression of PLC-β_4 in siRNA-transfected cells was subjected to immunoblot analysis. (**D**) Cells were transfected with control siRNA or siRNA targeting Rac1. Knockdown of Rac1 by siRNA was confirmed by immunoblot analysis. Expression of PLC-β_4 in siRNA-transfected cells was examined by immunoblot analysis. (**E**) A gelatin zymogram assay was performed on cells treated with 50 μM BAPTA-AM or 5 μM U73122 for 48 hours.

by immunoblot analysis. As shown in Fig. 6D, S1P-induced PLC-β_4 expression was almost completely blocked by knockdown of Rac1. These data demonstrate that Rac1 mediates S1P-induced PLC-β_4 expression in MCF10A cells. Consistent with our results, regulation of PLC-β isoforms by Rac has been reported previously (Snyder et al., 2006; Hicks et al., 2008).

We next investigated the role of intracellular Ca^{2+} and PLC-β_4 in S1P-induced MMP-9 upregulation and cell invasion. Pre-treatment of MCF10A cells with an intracellular Ca^{2+} chelator BAPTA-AM for 2 hours before stimulation with S1P for 48 hours significantly inhibited MMP-9 upregulation (Fig. 6D). U73122, an inhibitor of PLC, also significantly decreased S1P-induced MMP-9 upregulation. These results show the involvement of intracellular Ca^{2+} and the $G_{\alpha q}$–PLC pathway in S1P-induced MMM-9 upregulation of MCF10A cells.

FTY720 reverses S1P-induced MMP-9 upregulation and invasion

FTY720, an immune modulator, has been shown to function as an antagonist of all S1P receptors, except S1P$_2$ (Paugh et al., 2003; Brinkmann et al., 2002). When phosphorylated, FTY720 acts as an agonist (Brinkmann et al., 2002). We examined the effects of FTY720 and its phosphorylated form on S1P-induced cellular responses. FTY720 potently inhibited S1P-induced MMP-9 upregulation, whereas the MMP-9 level was increased by phosphorylated FTY720 (Fig. 7A). The results imply that FTY720 is not phosphorylated in MCF10A cells, and thus acts as an antagonist. Invasion and migration induced by S1P were significantly inhibited by FTY720 (Fig. 7B). We then examined the effect of FTY720 on the activation of signaling molecules induced by S1P. The levels of phosphorylated forms of MEK1/2, ERK1/2, p38 and Akt were markedly decreased by FTY720 treatment (Fig. 7C). The results demonstrate that FTY720 can reverse the activation of signaling pathways leading to MMP-9 upregulation and invasive phenotypes triggered by S1P in MCF10A cells. These data also confirm that the S1P-triggered cellular responses in MCF10A cells were not through the S1P$_2$ receptor.

Discussion

The inflammatory microenvironment plays a crucial role in the regulation of invasion, migration and metastasis of breast cancer. Inflammatory breast cancer is highly metastatic and exhibits an invasive phenotype both in vivo and in vitro (Charafe-Jauffret et al., 2010; Wu et al., 2009). It has been reported that S1P, a cell membrane-derived phospholipid, plays an important role in the processes of inflammatory responses and cell migration in many cell types (reviewed by Nixon, 2009; Spiegel and Milstien, 2003). Despite the collective body of evidence of S1P-mediated inflammatory responses, the detailed mechanism underlying the role of S1P in cell invasion has not been revealed. This study aimed to elucidate the cellular mechanisms of S1P receptor activation on the changes in invasiveness of breast epithelial cells.

On the basis of our previous studies (Shin et al., 2005; Song et al., 2006) and the observations obtained from this study, we propose a working model of signaling networks that lead to MMP-9 gene expression responsible for the S1P$_3$–$G_{\alpha q}$ coupling-mediated invasion/migration of MCF10A cells (Fig. 8). It should be noted, however, that MMP-9 may not be the sole contributor to the S1P-mediated invasive phenotypic change of the cells, because tumor cell invasion requires diverse signaling components, resulting in multiple activities other than MMP-9-mediated protease activity. Recent studies have shown that S1P enhances cell invasion through interaction with several other proteins including MMP-2, uPA and MT1-MMP (Devine et al., 2008; Young et al., 2009). Because our study showed that S1P had no effect on MMP-2 expression in MCF10A cells (Fig. 2A,B), possible contributors other than MMP-9 would be uPA and MT1-MMP.

MMP-9 expression was also detected at the primary tumor site and the stromal compartment in 68% of cases of breast tumors (Jones et al., 1999). In addition, a high level of MMP-9 expression was detected at sites of inflammation, thus promoting migration of inflammatory cells across the basement membrane (Gong et al., 2008). Thus, it is highly plausible that expression of the MMP-9 gene plays a crucial role in the enhancement of tissue invasion of breast cancer cells with inflammation, and thereby causes a more aggressive cancer phenotype. Given that the induction of mammary epithelial cell invasion can also occur as a normal physiological process (Traurig, 1967), it needs to be further elucidated whether the invasive breast cancer cells produce more S1P than non-invasive ones to associate S1P with cancer progression.

The effect of S1P-stimulated MMP-9 expression and increase in the invasive/migratory phenotypes of MCF10A cells was significantly impaired by inhibition of the $G_{\alpha q}$ pathway (Fig. 4), indicating that $G_{\alpha q}$ is involved in the regulation of MMP-9
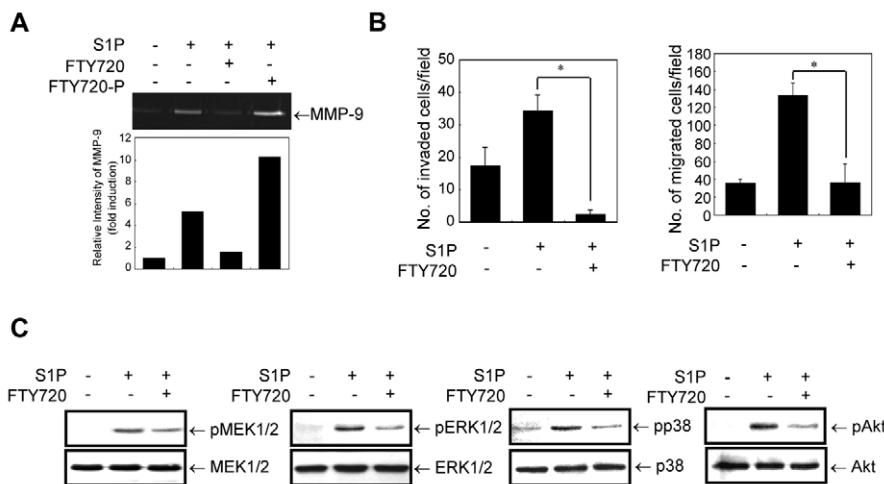
Fig. 7. FTY720 reverses S1P-induced MMP-9 upregulation and the invasive phenotype in vitro. (A) A gelatin zymogram assay was performed on cells treated with 10 μM S1P for 48 hours in the absence or presence of FTY720 and phosphorylated FTY720 (FTY720-P). (B) In vitro invasion and Transwell migration assays were conducted on cells treated with 10 μM S1P for 17 hours in the absence or presence of FTY720. Values are means + s.e.m. of triplicate samples (*P<0.01). (C) The levels of activated MEK1/2, ERKs, p38 MAPK and Akt were determined by immunoblot analyses using phospho-specific antibodies (pMEK1/2, pERKs, pp38 and pAkt, respectively).
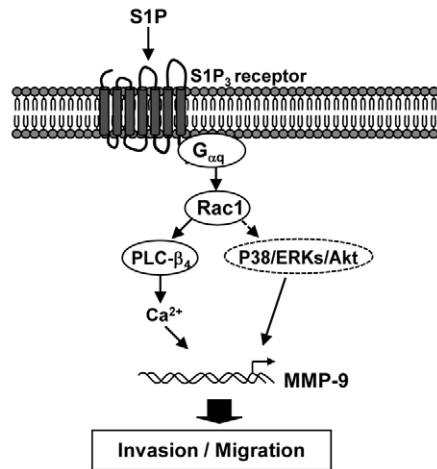
Fig. 8. A proposed model of the mechanisms involved in S1P-induced malignant phenotypic conversion of MCF10A human breast epithelial cells.

expression and breast cell invasion induced by S1P. Interestingly, we have previously shown that $G_{\alpha 12}$ and $G_{\alpha 13}$ upregulate MMP-2, rather than MMP-9, which plays a crucial role in the conversion of MCF10A cells to a malignant phenotype (Kim et al., 2010). These results suggest that the $G_{\alpha 12/13}$ and $G_{\alpha q}$ differentially regulate MMPs in the processes of breast cell invasion.

Rac, a member of the Rho small G protein family, has been shown to induce cell migration and invasion in various types of cancer such as breast, renal and liver carcinomas (Keely et al., 1997). The activity of Rac is modulated by a variety of GPCR-mediated inflammatory signaling pathways, such as the activation of S1P and lysophosphatidic acid receptors (Taha et al., 2004; Moolenaar et al., 2004). It has been suggested that S1P-induced activation of Rac may be mediated by $G_{\alpha i}$ in CHO cells (Okamoto et al., 2000). In the present study, Rac1 was preferentially activated when MCF10A cells were stimulated by S1P, whereas Rho was not affected by S1P (Fig. 3A).

We also showed that S1P stimulation induced the expression of MMP-9 and invasion/migration by the concerted action of multilayer intracellular signaling molecules, including MEK, ERK1/2, MKK3/6, p38 and Akt, which was mediated by $G_{\alpha q}$. Consistent with our results, activation of p38 by $G_{\alpha q}$ has been reported in HaCaT human keratinocytes (Seo and Juhnn, 2010).

S1P receptor subtypes differentially regulate cell migratory responses upon exposure to chemoattractants. $S1P_1$ and $S1P_3$ act as attractant receptors mediating migration, whereas $S1P_2$ acts as a repellant receptor inhibiting chemotaxis upon S1P treatment (Takashima et al., 2008). It has been reported that vascular $S1P_1$ and $S1P_3$ are involved in vascular smooth muscle cell proliferation and migration, demonstrating the role of these receptor subtypes in regulating angiogenesis (Waeber et al., 2004). It has been shown that the $S1P_3$ promotes cell migration through $G_{\alpha i}$-mediated Rac stimulation, whereas $G_{\alpha 12/13}$-mediated RhoA stimulation is not required for cell migration in CHO cells (Takuwa, 2002). Among S1P receptors, $S1P_3$ has been shown to be involved in the activation of the $G_{\alpha q}$-coupled signaling cascade by S1P (Windh et al., 1999; Jongsma et al., 2009). Knockdown of the $S1P_3$ receptor or $G_{\alpha q}$ significantly abolished S1P-induced MMP-9 expression, indicating that S1P-activated $S1P_3$ mediates invasion and migration of

MCF10A cells via the expression of MMP-9, which is mediated by $G_{\alpha q}$ (Figs 4 and 5). Our results demonstrate that in addition to the previously suggested signaling pathway, the $S1P_3$–$G_{\alpha i}$–PI3K pathway, as shown in CHO cells (Takuwa, 2002) and human neutrophils (Akasaki et al., 1999), the $S1P_3$–$G_{\alpha q}$–PI3K signaling pathway may also play a role through activation of Rac in promoting the migration of MCF10A cells. Given that targeting S1P receptors may be complicated by the presence of multiple isoforms with opposing actions on tumor cells, and thus selectivity of receptor antagonism is a key element in successful S1P receptor-based therapeutic interventions (Sabbadini, 2006), our results implicate the receptor $S1P_3$ as a potentially valuable therapeutic target for designing new drugs for regulating invasiveness of breast cells in the inflammatory microenvironment.

$G_{\alpha q}$ has been shown to regulate the activity of PLC-β. $G_{\alpha q}$ activates PLC-β, and thus induces protein kinase C activation and intracellular Ca^{2+} mobilization (Mizuno and Itoh, 2009). $G_{\alpha q}$ coupling mediates cell migration through stimulation of PLC (Hla et al., 2001). In addition, PLC activation is preferentially regulated by $S1P_3$ in mouse embryonic fibroblasts cells (Ishii et al., 2001; Ishii et al., 2002). S1P-induced oxygen free radical generation in migrating smooth muscle cells requires $G_{\alpha 12/13}$-mediated PLC activation (Roztocil et al., 2007). Our results clearly demonstrate that S1P-induced $S1P_3$ receptor stimulation triggers the $G_{\alpha q}$-PI3K and $G_{\alpha q}$-PLC–Ca^{2+} pathways, which are crucial for the regulation of S1P-induced MMP-9 expression. Consequently, these signaling events are necessary the invasive and migratory phenotypes of MCF10A cells (Fig. 6).

Because there is an increasing body of data implicating S1P as a potent tumorigenic agent, strategies for cancer therapy that target S1P have been pursued (reviewed by Sabbadini, 2006). FTY720, a structural analog of sphingosine, acts as an agonist for S1P receptor subtypes, such as $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$, when it is phosphorylated by sphingosine kinase (Paugh et al., 2003; Brinkmann et al., 2002). Non-phosphorylated FTY720, however, is known to act as an antagonist; non-phosphorylated FTY720 has been shown to inhibit the migration of lymphocytes (Gräler and Goetzl, 2004), to lead to a significant reduction in tumor volume in an in vivo mouse breast cancer model (Azuma et al., 2002), and to inhibit angiogenesis and tumor vascularization (LaMontagne et al., 2006). In the current study, treatment of the cells with FTY720 completely inhibited S1P-induced MMP-9 expression and the consequential enhancement of invasion and migration of MCF10A cells (Fig. 7). In contrast, phosphorylated FTY720 did not inhibit S1P-induced MMP-9 expression, but significantly enhanced its expression, indicating that FTY720 is not phosphorylated in MCF10A human breast epithelial cells. In summary, we have demonstrated the enhancement of invasive/migratory phenotypes of human breast epithelial cells by the inflammatory lipid S1P, suggesting that the control of S1P-mediated signaling pathways can potentially provide sphingolipid-based therapeutics for the treatment of inflammatory breast cancer.

Materials and Methods
Cell lines
MCF10A cells were cultured, as previously described (Moon et al., 2000). Cells were cultured in DMEM–F12 supplemented with 5% horse serum, 0.5 µg/ml hydrocortisone, 10 µg/ml insulin, 20 ng/ml EGF, 0.1 µg/ml cholera enterotoxin, 100 units/ml penicillin and streptomycin, 2 mM L-glutamine and 0.5 µg/ml amphotericin B.

FTY720 and FTY720-P treatment
FTY720 {2-amino-2-[2-(octyl-phenyl) ethyl]-1,3-propanediol hydrochloride} and FTY720-phosphate (FTY720-P) were kindly provided by Sanghee Kim (Seoul

National University, Seoul, Korea). FTY720 and FTY720-P were dissolved in dimethylsulphoxide (DMSO) to a final concentration of 5 mM. FTY720 and FTY720-P were added 2 hours before S1P treatment.

Immunohistochemistry
C57BL/6 mice were anesthetized with ketamine and infused with S1P (0.1 mg/kg) for 30 minutes via the femoral vein. Control mice were injected with lipopolysaccharide (1 mg/kg). Animals were killed 3 hours after treatment and liver sections were prepared. Briefly, the tissues were dehydrated in graded alcohol and embedded in paraffin. Sections of 6-μm thickness prepared on glass slides were deparaffinized in xylene and rehydrated via ethanol and placed in PBS. Antigen retrieval was performed by boiling the sections for 10 minutes in citrate buffer (0.1 M, pH 6.0) in a microwave oven. Endogenous peroxidase activity was blocked with 3% H_2O_2 in PBS. After dewaxing the sections, endogenous peroxidase activity was blocked with 3% H_2O_2 in phosphate-buffered saline. Non-specific adsorption was minimized by pre-incubating the sections in 10% normal donkey serum for 40 minutes. The sections were then incubated overnight at 4°C with a 1:200 dilution of anti-MMP-9 antibody (Neuromics, Edina, MN, USA), then with a biotin-conjugated donkey anti-goat serum (1:200; Jackson ImmunoResearch, West Grove, PA, USA) and an extravidin peroxidase complex (Sigma, St Louis, MO, USA). The peroxidase label was detected using diaminobenzidine hydrochloride (DAB; Sigma). Images were captured using a Leica DMR microscope.

Transfection
Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Immunoblot analysis
Immunoblot analysis was performed, as previously described (Kim et al., 2003). Anti-p38 MAPK, anti-phospho-p38 MAPK, anti-MEK1/2, anti-phospho-MEK1/2, anti-ERK1/2, anti-phospho-ERK1/2, anti-JNK, phospho-JNK, anti-MKK3, anti-phospho-MKK3/6, anti-Akt, anti-phospho-Akt and anti-β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-MMP-2 antibody was purchased from R&D Systems (Minneapolis, MN, USA). Anti-MMP-9, anti-$G_{\alpha q}$, anti-PLC-β_4 and anti-Jun antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

RT-PCR
RNA was extracted from cell using TRIzol and reverse transcribed with RT-Superscript-III reverse transcriptase (Invitrogen). RT-PCR was performed using primers for S1P$_3$, MMP-2, MMP-9 and β-actin. The thermocycler conditions have been previously described (Kim et al., 2010). Equal volumes of each PCR product were analyzed by 1% agarose gel electrophoresis; S1P$_3$ (209 bp), MMP-2 (119 bp), MMP-9 (136 bp) and β-actin (175 bp) bands were detected.

Electrophoretic mobility shift assay (EMSA)
A Panomics EMSA kit (Redwood City, CA, USA) was used to identify AP-1 transcription factor. Nuclear extracts were prepared as previously described (Song et al., 2006). A total of 10 μg of the extracts was incubated with a biotin-labeled AP-1 probe (5′-CGCTTGATGACTCAGCCGGAA-3′) or NFκB probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′), and was then subjected to electrophoresis on a 6% non-denaturing polyacrylamide gel. The gel content was then transferred to a Biodyne nylon membrane (Pall, Tokyo, Japan) and developed using a chemiluminescence system, according to the manufacturer's instructions (Panomics EMSA Gel-Shift Kit), and exposed to film. Immunoinhibition analysis was performed with anti-Jun, anti-JunD, anti-Fra-1, anti-fos, and anti-p53 antibodies (Santa Cruz Biotechnology, Inc.).

Chromatin immunoprecipitation (ChIP) assay
This assay was conducted as previously described (Song et al., 2006). PCR was performed with primers for the AP-1-binding sites (–533 bp and –79 bp) in the MMP-9 promoter (–533 bp: forward, 5′-AAGACATTTGCCCGAGGTCC-3′; reverse, 5′-AAAGTGATGGAAGACTCCCTGAGAC-3′; –79 bp: forward, 5′-CCCTCCCTTTCATACAGTTCCC-3; reverse, 5′-TGTGTGTGTGTGTGTGTGT-GGC-3′).

Detection of Rho-GTP and Rac1-GTP
To detect Rho GTPase activity, cells were washed with ice-cold PBS and lysed in Mg^{2+} lysis/wash buffer (MLB) buffer. Cell lysates were clarified by centrifugation and incubated with GST-RBD bound to glutathione-Sepharose beads (Upstate, Charlottesville, VA, USA). The beads were washed four times with MLB buffer and bound Rho proteins were detected by immunoblotting with an antibody against Rho (Upstate). The level of Rac1–GTP was measured by affinity precipitation using the PAK-1 p21-binding domain Rac assay reagent (Upstate) following the manufacturer's instructions, as previously described (Shin et al., 2005).

Gelatin zymogram assay
Cells were cultured in serum-free DMEM–F12 medium for 48 hours. Gelatinolytic activity of the conditioned medium was determined by the gelatin zymogram assay, as previously described (Moon et al., 2000).

MMP-9 activity assay
The level of active MMP-9 in the conditioned medium was assessed using a Fluorokine E enzyme activity assay kit (R&D Systems) according to the manufacturer's instructions. Briefly, samples were incubated in 96-well plates coated with MMP-9 monoclonal antibody that captures all three MMP-9 forms (92, 82 and 65 kDa). To measure the activity of MMP-9, a standard curve was obtained using an MMP-9 standard and the chemical activator, p-aminophenylmercuric acetate (APMA). The endogenous level of active MMP-9 in the cell supernatant was detected without APMA treatment. The wells were washed, and the fluorogenic substrate, linked to a quencher molecule, was added and incubated. The monoclonal antibody-bound active MMP-9 cleaves the substrate between the fluorophore and quencher and the fluorescence was read at a wavelength of 340 nm using a SmartSpec spectrophotometer (Bio-Rad, Hercules, CA, USA).

In vitro and in vivo invasion assays
An in vitro invasion assay was performed using a 24-well Transwell unit with polycarbonate filters (Corning Costar, Cambridge, MA, USA). The lower side of the filter was coated with type I collagen and the upper side was coated with Matrigel (Collaborative Research, Lexington, KY, USA). The lower compartment was filled with serum-free medium containing 0.1% BSA. S1P and FTY720 were added to the Transwell insert in the lower well. Cells were placed in the upper part of the Transwell plate, incubated for 17 hours, fixed with methanol and stained with Hematoxylin for 10 minutes followed by a brief staining with Eosin. The invasive phenotypes were determined by counting the cells that migrated to the lower side of the filter, using microscopy at 400× magnification. Thirteen fields were counted for each filter, and each sample was assayed in triplicate.

For the in vivo invasion assay, MCF10A cells were seeded on the upper layer of the CAM of a 9-day-old chick embryo. After incubation for 48 hours, the lower layer of the CAM was stored frozen at –80°C. Genomic DNA of the frozen tissue was later isolated, as described previously (Nyberg et al., 2003). PCR was performed on the isolated DNA using human Alu primers (forward, 5′-ACGCCTGTA ATCCCAGCACTT-3′; reverse, 5′-TCGCCCAGGCTGGAGTGCA-3′). The PCR conditions were as follows: 95°C for 10 minutes, 95°C for 30 seconds, 58°C for 45 seconds, 73°C for 45 seconds and 72°C for 10 minutes. The PCR product (220 bp) was electrophoresed on a 2% agarose gel.

In vitro migration assay using Transwell units
An in vitro migration assay was performed using a 24-well Transwell unit with polycarbonate filters, as previously described (Kim et al., 2003; Shin et al., 2005). Experimental procedures were the same as the in vitro invasion assay described above, except that the filter was not coated with Matrigel for the migration assay.

Luciferase reporter assay
Luciferase and β-galactosidase activities were assayed using a luciferase assay kit (Promega, Madison, WI, USA) and a Galacto-Light Kit (Tropix Inc., Bedford, MA, USA), respectively, and measured with a luminometer (Tuner Designs, Sunnyvale, CA, USA) as previously described (Song et al., 2006). For MMP-2 and MMP-9 promoter assays, full-length human MMP-2 (Bian and Sun, 1997) and MMP-9 promoter–luciferase constructs (Ma et al., 2001) were kindly provided by Etty N. Benveniste (Department of Cell Biology, University of Alabama, Birmingham, AL, USA).

Knockdown of $G_{\alpha q}$ and S1P$_3$ by small-interfering (si) RNA molecules
Knockdown of S1P$_3$ receptor and $G_{\alpha q}$ were performed with siRNA molecules targeting S1P$_3$ or $G_{\alpha q}$ (Santa Cruz Biotechnology, Inc.). Cells were plated in 6-well plates at 1.5×10^5 cells/well, grown for 24 hours, then transfected with 50 pmol siRNA for 6 hours using Lipofectamine 2000 reagent and OPTI-MEMI (Invitrogen). Control cells were treated with negative control siRNA (Santa Cruz Biotechnology, Inc.).

Determination of intracellular Ca^{2+} levels
Ca^{2+} levels were measured, as described previously (Wu et al., 2009). Cells were washed with PBS, then incubated in DMEM–F-12 containing 5 μM Fura-2/AM for 30 minutes in the dark at 37°C. Fura-2/AM-loaded cells were washed with HEPES-buffered Krebs–Ringer bicarbonate (HKRB) buffer to remove unbound Fura-2/AM. The fluorescence of Ca^{2+}-bound and unbound Fura-2 was measured using a F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) and an excitation wavelengths between 340 and 380 nm.

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