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S1P differentially regulates migration of human ovarian cancer and human ovarian surface epithelial cells

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Abstract

Epithelial ovarian cancer (EOC) arises from the epithelial layer covering the surface of ovaries and intra-peritoneal metastasis is commonly observed at diagnosis. Sphingosine-1-phosphate (S1P), a bioactive lipid signaling molecule, is potentially involved in EOC tumorigenesis. We have found that S1P is elevated in human EOC ascites. We show that physiologically relevant concentrations of S1P stimulate migration and invasion of EOC cells, but inhibit migration of human ovarian surface epithelial (HOSE) cells. In addition, S1P inhibits lysophosphatidic acid (LPA)-induced cell migration in HOSE, but not in EOC cells. We have provided the first line of evidence that the expression levels of S1P receptor subtypes are not the only determinants for how cells respond to S1P. Even though $S1P_1$ is expressed and functional in HOSE cells, the inhibitory effect mediated by $S1P_2$ is dominant in those cells. The cellular pre-existing stress fibers are also important determinants for the migratory response to S1P. Differential S1P-induced morphology changes are noted in EOC and HOSE cells. Pre-existing stress fibers in HOSE cells are further enhanced by S1P treatment, resulting in the negative migratory response to S1P. By contrast, EOC cells lost stress fibers and S1P treatment induces filopodium-like structures at cell edges, which correlates with increased cell motility. In addition, inhibition of the protein kinase C pathway is likely to be involved in the inhibitory effect of S1P on LPA-induced cell migration in HOSE cells. These findings are important for the development of new therapeutics targeting S1P and LPA in EOC.

Keywords

Sphingosine-1-phosphate (S1P); Epithelial Ovarian Cancer (EOC); Human Ovarian Surface Epithelial (HOSE); Migration; Stress Fibers

Introduction

Epithelial ovarian cancer (EOC) is the most deadly gynecologic disease, mainly due to the lack of highly sensitive and specific methods for early detection and effective treatments for late-stage diseases (1). Approximately 70 percent of patients with the initial diagnosis of EOC are

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at Stage III or IV (2), presenting peritoneal metastases and beyond. Metastasis, rather than the primary tumor, is the major cause of morbidity and mortality in patients with ovarian and other solid cancers. The metastatic process and genes controlling this process in EOC are poorly understood.

We have shown that the levels of lysophosphatidic acid (LPA), a bioactive lipid molecule, are elevated in malignant ovarian ascites from EOC patients (3,4). LPA stimulates both migration and invasion of EOC cells *in vitro* and tumor metastasis *in vivo* (5-13).

Sphingosine-1-phosphate (S1P), another bioactive lipid factor, has recently been shown to be involved in ovarian tumorigenesis *in vivo* and it stimulates migration and invasion of EOC cells *in vitro*. We have shown that both LPA and S1P stimulate expression and secretion of the proangiogenic factor interleukin-8 (IL-8) in EOC cell lines (14). Recently, several groups have shown S1P induces cell migration or invasion in EOC cell lines Dov13 and OVCAR3 (15-17). However, some of these studies have used concentrations of S1P that are much higher than what has been detected in pathophysiological conditions (15-17). More importantly, Visentin *et al.* have shown that an anti-S1P monoclonal antibody (mAb) substantially reduced or eliminated SKOV3 (an EOC cell line) intraperitoneal tumor progression in nude mice (18). The anti-S1P mAb significantly reduced levels of certain proangiogenic cytokines (IL-6, IL-8 and VEGF) *in vivo* and *in vitro*. However, the effect of this anti-S1P mAb on EOC cell migration has not been evaluated (18). Since migration is an important step in tumor metastasis, it is critical to further investigate the role of S1P in migration of EOC cells and the mechanisms involved. The effect of S1P on migration of human ovarian surface epithelial (HOSE) cells, the source of EOC, has yet to be reported.

S1P affects cell migration through its receptors. To date, five receptors for S1P have been identified, which consist of EDG1 ($S1P_1$), EDG5 ($S1P_2$), EDG3 ($S1P_3$), EDG6 ($S1P_4$) and EDG8 ($S1P_5$) (19,20). Among them, $S1P_1$, $S1P_2$ and $S1P_3$ are widely expressed in various tissues, whereas the expression of $S1P_4$ is mainly confined to lymphoid tissues and platelets, and that of $S1P_5$ to central nervous system. S1P can exert either stimulatory or inhibitory effects on cell migration. In many of the different cell types (mostly non-malignant cells) studied, $S1P_1$ and/or $S1P_3$ mediate S1P-induced stimulation of cell migration, whereas $S1P_2$ mediates S1P-induced inhibition of cell migration. The migratory response of a particular cell type to S1P has been well correlated with their receptor subtype expression patterns (21-24). For example, S1P enhances migration of endothelial cells, which predominantly express $S1P_1$ and $S1P_3$ (25), while it inhibits migration of C2C12 myoblasts (26) and B16 melanoma cells (24), which have high levels of $S1P_2$. Thus, the expression levels of S1P receptor subtypes appear to be a major factor in determining how cells respond to S1P during migration.

Oncogenic transformation is always accompanied by pronounced morphological changes resulting from alterations in the organization of the actin cytoskeleton and adhesive interactions (27). The small GTPase RhoA acts upstream of ROCK. RhoA induces the assembly of actin stress fibers and associated focal adhesions in non-malignant cells (28). However, in transformed cells, RhoA activity does not necessarily correlate with stress fiber levels. Loss of stress fibers and lack of focal adhesions contribute directly to the enhanced motility of transformed cells. Constitutively active Raf/MEK/ERK signaling pathway has been shown to play an important role in the uncoupling of RhoA from stress fiber formation (29,30). Constitutive activation of the ERK signaling pathway in EOC cells has previously been reported (31).

In the present study, we tested S1P's effect on migration of EOC and HOSE cells and determined the mechanisms involved. We report that S1P stimulates migration of human EOC cells but inhibits migration of HOSE cells. In addition, the expression levels of S1P receptor

subtypes are not the only determinant for how cells respond to S1P in migration. In particular, the difference in pre-existing stress fibers in EOC and HOSE cells plays an important role in affecting the cellular migratory response to S1P.

Materials and Methods

Reagents

Oleoyl-LPA (18:1-LPA), S1P and $S1P_{1/3}$ antagonist VPC23019 were from Avanti Polar Lipids (Birmingham, AL). $S1P_2$ antagonist JTE013 was from Tocris Bioscience (Ellisville, MI). $S1P_1$ agonist SEW-2871 was from Biomol (Plymouth Meeting, PA). MEK inhibitor U0126, phorbol-12-myristate-13-acetate (PMA) and PKC inhibitor Gö6976 were from Calbiochem (San Diego, CA). Small interfering RNA (siRNA) specific for S1P receptor $S1P_2$ and control siRNA were from Dharmacon (Lafayette, CO). Actin cytoskeleton staining kit was from Chemicon (Temecula, CA).

Ovarian surface epithelial cell cultures

All studies with human tissues were approved by the local IRB. Slices from normal human ovaries were obtained during surgery from nonmalignant gynecological disorders. Pieces of an ovary were cultured as explants in medium 199:MCDB105 medium (1:1 Sigma, St. Louis, MO) containing 20% FBS (Hyclone, Logan, UT). Once cells grew out from the specimen, the tissue was transferred to another culture dish. After cells reached approximately 50% confluence, they were passaged and grown in a medium supplemented with 10% FBS. Upon passage, samples of each culture were plated in multi-well slides, grown for 4 days, and prepared for immunocytochemistry for cytokeratin (Anti-cytokeratin Antibody AE_1/AE_3, Millipore, Billerica, MA) and vimentin (Anti-vimentin VIM 3B4, Millipore, Billerica, MA). Passaged cells were 95% positive for both of these markers. Three batches of primary HOSE cell cultures (n602, n605 and n615) from different subjects were used in this study.

The immortalized HOSE cell lines used in this study were T103*p53i*, T137*p53i*, T151*p53i*, and HOSE 642. T103*p53i*, T137*p53i*, and T151*p53i* were established by ectopic expression of hTERT and p53 shRNA (32). HOSE 642 was established by expression of human papilloma viral oncogenes E6/E7 (33). The immortalized cell lines were cultured under the same conditions as described above for primary HOSE cells.

Cell lines and transfection

Ovarian cancer cell lines HEY and OCC1 were cultured in RPMI 1640 supplemented with 10% FBS (Hyclone). HEY and T103*p53i* cells were transfected by nucleofection using the Nucleofector machine according to the manufacturer's instructions (Amaxa, Cologne, Germany). Dominant negative and constitutively active MEK were provided by Dr. D. Templeton (Case Western Reserve University, Cleveland, OH). The small interfering RNA (siRNA) oligonucleotide duplexes targeting $S1P_2$ was from Dharmacon ON-TARGETplus SMARTpool with the following sequences: 5′-UUGCCAAGGUCAAGCUGUAUU-3′, 5′-CCAACAAGGUCCAGGAACAUU-3′, 5′-GACAAGAGCUGCCGCAUGCUU-3′, 5′-GUGACCAUCUUCUCCAUCAUU-3′. To establish HEY cells that stably expressing $S1P_2$, cells were transfected with pCR3.1/$S1P_2$ (a kind gift from Dr. Kevin Lynch, University of Virginia Health Science Center) and selected for resistance to G418 (400 μg/mL) 48 hrs after transfection. For transient transfection, experiments were conducted 48-72 hrs after transfection.

Detection of S1P receptors by reverse transcription-PCR analysis

The expression levels of $S1P_{1-3}$ were detected by reverse transcription-PCR. In brief, total RNA was extracted from cells and reverse transcribed. Derived cDNAs were used as template in PCR. The primer sequences for $S1P_{1-3}$ were as follows: $S1P_1$, 5′-TGCTCTCCATCGTCATTCTG and 5′-CCAGGAAGTACTCCGCTCTG; $S1P_2$, 5′-TGCCAAGGTCAAGCTGTATG and 5′-CAGAAGGAGGATGCTGAAGG; $S1P_3$, 5′-GGTGACCATCGTGATCCTCT and 5′-TTCGGAGAGTGGCTGCTATT. GAPDH was amplified in a separate tube as a housekeeping gene with primers 5′-GAAGGTGAAGGTCGGAGT and 5′-GAAGATGGTGATGGGATTTC. PCR reactions were conducted for 30 cycles.

Migration and invasion assay

Migration assays were performed as previously described (6). In brief, the lower phase of the chamber was coated with laminin (10 μg/mL). Cells (5×10^4 per well in 300 μL serum-free medium) were added to the top chamber of the transwell and S1P or LPA was added to the bottom chamber. For antagonist treatment, 1 μM VPC23019 or 1 μM JTE013 were added to both chambers. Invasion was assayed by measuring cells that invaded through the Matrigel Invasion Chambers (Becton Dickinson, Bedford, MA) as previously reported (7). Cells (5×10^4 cells per well in 300 μL serum-free medium) were allowed to invade for 16 hrs.

Immunofluorescence microscopy

Cells cultured in 8-chamber glass slides were treated as indicated, washed once with PBS, fixed in 4% paraformaldehyde in PBS for 20 min, and then permeabilized with 0.1% Triton X-100 in PBS for 15 min. The cells were then incubated with TRITC-phalloidin for 45 min to visualize filamentous actin (F-actin). Stained cells were mounted and images were captured using a Zeiss LSM 510 confocal microscope at 63× magnification (Carl Zeiss, Thornwood, NY).

Statistical analysis

Data are presented as the mean ± SD and statistical differences between two groups were analyzed using Student's *t* test. $P < 0.05$ was considered significant.

Results

Levels of S1P were elevated in ascites samples from patients with ovarian cancer

We have developed mass spectrometry (MS)-based methods to analyze lysophospholipids (LPLs) in human body fluids (4,34). Using improved extraction and MS detection methods (Supplemental Materials and Methods), we recently analyzed several pairs of ascites samples from ovarian cancer patients versus ascites samples from patients with benign diseases and showed that both LPA (in a concentration range of 5-40 μM in EOC samples) and S1P (in a concentration range of 60-100 nM in EOC samples) were 5-10 times up-regulated in ovarian cancer patients ascites when compared to patients with benign liver diseases (Supplemental Fig. S1). In blood samples, S1P concentrations were measured in the 75-1100 nM range. In ovarian cancer ascites, the concentration range of oleoyl-LPA (18:1-LPA) was 1-6 μM (4, 34). To be more pathophysiologically relevant, we have conducted our experiments based on the concentrations of lipids found in the blood and/or ascites fluid of the EOC patients. Therefore, S1P (0.01-1 μM) and 18:1-LPA (1-5 μM) were used in most of our studies.

S1P induced more potent migration of ovarian cancer cells than LPA

Cell migration is one of the most important steps of tumor metastasis. Recently, S1P (0.01 to 1 μM) has been shown to induce migration of OVCAR3 cells where fibronectin was used as the extracellular matrix (17). Here, we compared S1P's migration-inducing activity to another

well characterized and EOC ascites-elevated lipid signaling factor, LPA. S1P dose-dependently increased migration of HEY cells when laminin was coated on the lower phase of the migration chambers (Fig. 1A). Higher concentrations of S1P (> 1 μM) were not tested, since they were not usually detected in human blood or ascites as mentioned above (Supplemental Fig. S1). The dose-dependent migratory effect induced by LPA has been previously reported (6). S1P (0.1 μM) was significantly more effective than LPA (5 μM) in stimulating migration in both HEY and OCC1 EOC cell lines (Fig. 1B and Supplemental Fig. S2). S1P also stimulated stronger *invasion* of HEY cells when compared to LPA (Fig. 1C). Since LPA and S1P co-exist in human ovarian cancer ascites, we tested the effect when both LPA (5 μM) and S1P (0.1 μM) were present. We found that there was no additive effect (Fig. 1B and Supplemental Fig. S2), suggesting that the signaling pathways utilized by LPA and S1P may overlap in EOC cells.

To determine whether S1P induced cell migration is chemokinetic or chemotactic, S1P was added either to the top, bottom, or both chambers in the migration assays. S1P in the bottom chamber stimulated maximal cell migration, with an approximately 10-fold more cell migration than when S1P was added to the top chamber only or to both chambers. Thus, S1P-stimulated HEY cell migration was mainly chemotactic (Fig. 1D). A similar chemotactic effect of S1P was also detected in OCC1 cells (data not shown).

S1P inhibited migration of HOSE cells

We conducted similar experiments in HOSE cells to determine whether S1P had the same effect on the non-malignant cells that are precursors of ovarian cancer cells (35). At a very low concentration, S1P (0.01 μM) stimulated migration of an immortalized HOSE cell line T103*p53i* (Fig. 2A). However, different from its effect on EOC cells, increased concentrations of S1P (0.1-0.5 μM) exhibited an inhibitory effect on HOSE cell migration (Fig. 2A).

A dramatic inhibitory effect of S1P was observed in the presence of LPA. In contrast to S1P and similar to its effect in EOC cells, LPA alone stimulated migration in HOSE cells (Figs. 2B-D and Supplemental Table S1). When S1P was added together with LPA to the bottom chamber of the transwell, it strongly inhibited LPA-induced cell migration in all HOSE cells tested including three batches of primary HOSE cell cultures and four immortalized HOSE cell lines (Figs. 2B-D and Supplemental Table S1). This is in sharp contrast to the strong stimulatory effect of S1P in EOC cells. No inhibitory effect of S1P was observed in any EOC cell lines tested even when a high concentration of S1P (1 μM) was used, either in the presence or absence of LPA (Fig. 1). Taken together, our data suggest that EOC cells have altered responses to S1P in cell migration. These characteristics may be related to their adaptation to an increased motility favoring tumor development.

The S1P's inhibitory effect on LPA-induced cell migration has been previously reported. In NIH3T3 cells, S1P (0.1 μM) inhibited LPA (0.25 μM)-induced cell migration only when it was added to the bottom chamber of the transwell and it promoted the retraction of pseudopodia (36). To test whether S1P exerted its inhibitory effect via a similar mechanism in HOSE cells, S1P was added to either the top or the bottom chamber while LPA was used as a chemoattractant in the bottom chamber. S1P effectively inhibited LPA-induced cell migration regardless of whether it was added to the bottom or the top chamber (Fig. 2D), suggesting a different mechanism(s) may be involved in the inhibitory effect of S1P in HOSE cells.

$S1P_{1/3}$ mediated the stimulatory effect of S1P in EOC cells and $S1P_2$ was involved in the inhibitory effect

Our findings described above are critically important for designing new therapeutics targeting S1P and LPA in ovarian cancer. To determine the mechanisms by which S1P exerts its

differential effect, we first focused on S1P receptors. $S1P_{1/3}$ and $S1P_2$ have been shown to be involved in the stimulatory and inhibitory effects of S1P, respectively, in migration of several other cell types, and relative expression levels of the two types of receptors correlated with the particular S1P activity seen (37-39). To determine whether EOC cell lines and HOSE cells differentially express these receptors, which may be a cause of their differential response to S1P in cell migration, we first examined the expression pattern of these receptors in EOC and HOSE cells. Although RT-PCR is not quantitative, our data indicate that all cell lines/types tested express $S1P_{1-3}$ (Fig. 3A and data not shown). In particular, the "stimulatory" $S1P_1$ and $S1P_3$ were expressed in all primary HOSE cells and immortalized HOSE cell lines tested, whereas the inhibitory effect of S1P was dominant. This result is consistent with the previous report that both primary HOSE and immortalized HOSE cells express all three S1P receptors ($S1P_{1-3}$) (40). These results suggest that expression levels of $S1P_{1-3}$ in different cell lines or types cannot be the only determinant for the response to S1P in cell migration.

To determine which S1P receptors are functionally involved in the effect of S1P on EOC cell migration, we tested the effects of VPC23019 (an $S1P_{1/3}$ antagonist) and JTE013 (an $S1P_2$ antagonist) in HEY cells. While both of these inhibitors reduced the basal level of cell migration, VPC23019 (1 μM) effectively blocked (~78%) S1P-induced cell migration, implying that one or both of these receptors mediate the stimulatory effect of S1P on EOC cell migration (Fig. 3B). Similar results were obtained in OCC1 cells (Data not shown).

In contrast, JTE013 did not block the stimulatory effect of S1P (Fig. 3B). The negative role of $S1P_2$ in cell migration was more clearly demonstrated by overexpressing $S1P_2$ in HEY cells, which resulted in a robust inhibition of cell migration (Fig. 3B). Taken together, our data suggest that in EOC cells, as seen in other cell types, $S1P_{1/3}$ mediate the stimulatory effect and $S1P_2$ mediates the inhibitory effect. The stimulatory effect dominates over the inhibitory effect when S1P was used as a chemoattractant in migration of EOC cells. This balance can be altered if the expression levels of these receptors are artificially changed or the activity and/or binding to the ligand are pharmacologically modulated.

$S1P_1$ and its signaling pathways were intact in HOSE cells and $S1P_2$ mediated inhibitory effect of S1P was dominant in HOSE cells

While HOSE cells express "stimulatory" $S1P_1$ and $S1P_3$ (Fig. 3A), how S1P induces its inhibitory effect in these cells has yet to be determined. One possibility is that the protein expression and/or the membrane location of these receptors do not correlate with their RNA levels. We have tested many commercially available S1P receptor antibodies and have not found them to be reliable in detecting endogenous S1P receptors (data not shown). A better way to address this issue is to determine whether S1P receptors are functional in HOSE cells. To this end, we first tested the effect of a specific $S1P_1$ agonist, SEW-2871, on HOSE cell migration. When SEW-2871 was added to the bottom chamber as a chemoattractant, it promoted potent migration of T103*p53i* cells (Fig. 3C, a). This strongly suggests that not only is the $S1P_1$ receptor functional, but also that the signaling pathways activated via $S1P_1$ leading to cell migration are intact and functional in these cells. These data also imply that while SEW-2871 only selectively activates $S1P_1$, S1P, the natural ligand, activates multiple receptors and the effect mediated by $S1P_2$ may be dominant in HOSE cells. To further investigate the role of $S1P_{1/3}$ in HOSE cells, we treated the cells with VPC23019 and found that cell migration in response to S1P was further decreased, supporting the positive role of $S1P_{1/3}$ in cell migration in HOSE cells (Fig. 3C, b).

To determine the functional involvement of $S1P_2$ in S1P-inhibited HOSE cell migration, JTE013 was used to block $S1P_2$ in T103*p53i* cells. This treatment dramatically reversed the inhibitory effect of S1P, suggesting that $S1P_2$ is responsible for the inhibitory effect of S1P in HOSE cells (Fig. 3C, b). An siRNA against $S1P_2$ abrogated the endogenous expression of the

receptor at the mRNA level and reversed the inhibitory effect of S1P in HOSE cell migration (Fig. 3D).

EOC and HOSE cells showed different cell morphology

Collectively, our data suggest that HOSE and EOC cells are similar to many other cell types that have been previously reported in having $S1P_{1/3}$ as the "stimulatory" receptors and $S1P_2$ as the "inhibitory" receptor. However, while LPA indistinguishably stimulated cell migration in both EOC and HOSE cells, S1P exhibited dual effects. Not only did S1P display a biphasic dose response in HOSE cells, but it also had opposing effects in HOSE and EOC cells. Since these cells express functional $S1P_1$, $S1P_2$, and $S1P_3$, our data indicate that expression of functional S1P receptors is not the only determinant of cellular response to S1P in cell migration.

In an effort to seek out additional determinants, we have noticed different cell morphologies among HOSE and EOC cells. Cytoskeleton reorganization occurs during cellular transformation, causing poor adhesion, increased motility, invasiveness, and contact-independent growth (27). To visualize the morphology of EOC and HOSE cells and the potential effect of S1P on cell morphology, F-actin was stained by TRITC-conjugated phalloidin before and after S1P (0.5 μM) treatment. In two EOC cell lines, HEY and OCC1, which respond to S1P with increased cell migration, no obvious stress fibers were observed before or after S1P treatment (Figs. 4A, a, a′, b and b′). S1P treatment induced filopodium-like structures at cell edges (Figs. 4A a′ and b′). These projections were short (~2-10 μm), which are usually found at the leading edge of migratory epithelial cells where they are functionally involved in cell migration (41). It is possible that the formation of filopodium-like structures is correlated with the stimulatory effect of S1P in these two cells.

By contrast, both primary HOSE cells (n615 p3) and immortalized HOSE cells (T103*p53i*) showed strong stress fibers which were further enhanced by S1P treatment (Figs. 4B, a, a′, b and b′). This is also correlated with the inhibitory effect of S1P in these cells, since strong stress fibers result in high levels of cell adhesion and decreased cell motility (42). LPA (1 μM) treated T103*p53i* cells showed loose stress fibers and membrane ruffles at cell edges (Fig. 4B, a″).

Inhibition of MEK/ERK pathway restored stress fibers in HEY cells and blocked S1P-induced cell migration

It is imperative to test whether the pre-existing stress fibers are functionally related to the cellular response to S1P. Since disruption of the MEK/ERK pathway has been shown to restore stress fibers in transformed fibroblasts (30,43) and EOC cells were reported to express constitutively active ERK signaling pathway (31), HEY cells were treated with the MEK/ERK pathway inhibitor U0126 (10 μM) for 24 hrs, which restored stress fibers (Fig. 5A). We then tested the migration response of U0126-treated cells to S1P. As expected, cell migration in the presence of S1P was dramatically inhibited after U0126 treatment (Fig. 5B). Similar results were obtained with dominant negative MEK (MEK 2A) (Figs. 5A and 5B), which restored stress fibers and inhibited HEY cell migration in response to S1P. These results have established a functional correlation between cell morphology (stress fibers in particular) and the direction of response to S1P in terms of cell migration.

To test whether releasing stress fibers in HOSE cells would change the cellular response to S1P, an immortalized HOSE cell line (T103*p53i*) was transfected with constitutively active MEK (MEK 2E). Although MEK 2E partially reduced the amount of stress fibers in T103*p53i* cells (Fig. 5C), the inhibitory effect of S1P on T103*p53i* cell migration was not reversed (Fig. 5D). It is possible that further reduction of stress fibers in HOSE cells is required for S1P to convert the stimulatory effect or additional factor(s) are involved in EOC cells for

their positive response to S1P in cell migration. The first possibility was supported by the fact that when T103*p53i* cells were treated with phorbol-12-myristate-13-acetate (PMA), a factor known to be able to dismantle stress fibers (44), stress fibers were lost completely (Fig. 5C). Under these conditions, S1P stimulated cell migration (Fig. 6). These data support the notion that the pre-existing stress fibers are important determinants for how cells respond to S1P in cell migration.

PKC involvement in the inhibitory effect of S1P in HOSE cells

The most dramatic effect of S1P was that it completely blocked LPA-induced cell migration in HOSE cells. PMA not only changed the inhibitory effect of S1P when it was applied alone, but also almost completely reversed the inhibitory effect of S1P on LPA-induced cell migration (Fig. 6). Since it is well known that PMA pre-treatment (0.1 μM for 1 hr) can activate classical and novel protein kinase C (PKC) isoforms, we further investigated the potential involvement of PKC in HOSE cell migration. PMA did not significantly change cell migration induced by LPA. The two PKC inhibitors, bisindoylmaleimide (Bis, a broad spectrum inhibitor of PKC) and Gö6976 (selectively inhibits the classical PKC) completely inhibited LPA-stimulated cell migration, suggesting that activation of PKC is required for LPA to induce cell migration in HOSE cells (Fig. 6 and data not shown). In contrast, PMA pretreatment converted S1P's inhibitory effect to stimulatory when it was applied alone or together with LPA. The two PKC inhibitors, Bis and Gö6976, did not change S1P's inhibitory effect when they were used alone or with LPA, but they did block the effect of PMA, suggesting that PMA's targets are PKCs (Fig. 6 and data not shown). Similar results were obtained with primary HOSE cells (data not shown). These results suggest that the inhibitory effect of S1P involves inhibition of the PKC pathway.

Discussion

Although S1P-induced cell migration and invasion in EOC cell lines have been reported previously (15-17), here we report three lines of novel and interactive observations: 1) S1P levels are elevated in ascites from patients with ovarian cancer when compared to ascites from patients with benign diseases; 2) pathophysiological concentrations of S1P, but not LPA, induced differential effects in HOSE vs. ovarian cancer cell; and 3) S1P inhibits LPA's migratory activity in HOSE, but not in EOC cells. Along with the published data using OVCAR3 and Dov13 cell lines, the positive response to pathophysiological concentrations of S1P in cell migration appears to be a relatively general phenomenon for EOC cells. In contract, S1P's (0.1-0.5 μM) inhibitory effect on cell migration was consistently observed in all HOSE cells tested, including a total of three batches of primary HOSE cells derived from different human subjects and four immortalized HOSE cell lines. These novel observations are potentially pathophysiologically relevant in terms of our understanding of the molecular events and mechanisms of cellular transformation and tumor development, as well as designing new strategies to antagonize S1P's promoting effect on tumorigenesis and/or metastasis of EOC. Therefore, in this work, we have focused to answer the *one* most critical question: why HOSE and EOC cells respond differently to S1P.

We first examined whether lack of expression of “stimulatory” S1P receptors in HOSE cells is one of the major factors for this difference and whether the same S1P receptor may have different functions in different cell types. We presented the first line of evidence that expression levels of functional S1P receptor subtypes are not the *only* determinant for how cells respond to S1P in cell migration. Consistent with previous reports indicating that $S1P_{1/3}$ and $S1P_2$ are involved in the stimulatory and inhibitory effects of S1P on migration, respectively (37-39), we have shown that the same receptors also play similar roles in EOC and HOSE cells. However, our data strongly suggest that the expression of these receptor subtypes in cells

cannot be the sole factor for determining how these cells respond to S1P. Our observation that a very low concentration of S1P (0.01 μM) actually stimulates migration of HOSE cells suggests that S1P may have different dose-responsive curves for each of its receptors in HOSE cells. Low concentrations of S1P may primarily activate $S1P_1$ and/or $S1P_3$, resulting in a dominant stimulatory effect. Higher concentrations of S1P (0.1-0.5 μM) may stimulate $S1P_2$, leading to an inhibitory effect in HOSE cells, which masks the stimulatory effect mediated by $S1P_1$ and/or $S1P_3$. This notion is supported by our results showing that PMA alone did not stimulate cell migration, however, when combined with 0.5 μM S1P, which was inhibitory when administrated alone, a strong stimulation of cell migration was observed (Fig. 6). These data suggest that PMA has released the negative effect of S1P that occurs through $S1P_2$, resulting in the positive effect of S1P through $S1P_{1/3}$ becoming dominant.

Secondly, we have found that stress fibers strongly influence the directions of S1P-modulated cell migration. Pre-existing stress fibers are likely to play an important role in the differential migratory effects of S1P on EOC and HOSE cells. All HOSE cells tested displayed strong stress fibers, which were further enhanced by S1P treatment (Fig. 4). Cells with strong stress fibers are generally less motile (44). On the contrary, EOC cells lost stress fibers and S1P stimulated filopodium-like structures, which have been shown to be related to enhanced cell motility (41). The functional relationship between the stress fibers and the effects of S1P on cell migration was further supported by restoration of stress fibers in EOC cells through blocking the MEK/ERK signaling pathway and loose of stress fibers by PMA treatment in HOSE cells, accompanied by corresponding cell migratory behavior changes in response to S1P. Changing of cell morphology (including stress fibers), cell adhesion, and cell motility is a well known and critical event of cellular transformation and tumor metastasis (27). It is also interesting to note that the loss of stress fibers is not a prerequisite for LPA-induced cell migration. Similar to EOC cells, LPA stimulated cell migration in HOSE cells, but it only weakly reduced stress fibers in these cells.

Finally, we have found that S1P inhibited LPA-induced cell migration only in HOSE cells. PMA can reverse this effect, suggesting an involvement of PKC, which is in agreement with our PKC inhibitor results. PKC isoforms have been shown to play central roles in a vast number of cellular processes, including cell morphology and migration (45). Since the classical PKC pathway is likely to be required for LPA-induced cell migration (our inhibitor assays in Fig. 6), S1P may block LPA's effect by inhibiting the classical PKCs, a possibility that requires further investigation. However, PMA is known to be able to bind and activate other proteins, such as chimaerins, protein kinase D, RasGRPs, Munc13s and DAG kinase γ, and its effect on stress fibers can be mediated through PKC-dependent or -independent mechanisms (46). The precise mechanisms by which S1P inhibits LPA's effects only in HOSE cells remains to be further investigated. For example, RhoA has been shown to be a critical regulator of stress fiber formation (42), which may be differentially modulated by S1P in the context of cell transformation. In addition, the MEK/ERK pathway is one of the candidates that could uncouple RhoA from stress fiber formation (47) and ERK is constitutively activated in many EOC cells but not in HOSE cells (31).

Taken together, our data suggest that multiple factors may contribute to S1P's differential effects on cell migration in EOC and HOSE cells, which may include 1) different strength and character of pre-existing stress fibers in nonmalignant HOSE and malignant EOC cells; 2) different activation of PKC, RhoA, and/or other regulator(s) of stress fibers by S1P in the two types of cells; and 3) different coupling between the regulator and the stress fibers in those cells. These hypotheses warrant further studies. The present study has provided important information which may be the foundation for further development of novel therapeutic approaches targeting S1P and LPA in EOC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations List

S1P, sphingosine-1-phosphate; EOC, epithelial ovarian cancer; HOSE, human ovarian surface epithelial; LPA, lysophosphatidic acid; PKC, protein kinase C; IL-8, interleukin-8; PMA, phorbol-12-myristate-13-acetate; siRNA, small interfering RNA; MS, mass spectrometry; LPL, lysophospholipid.

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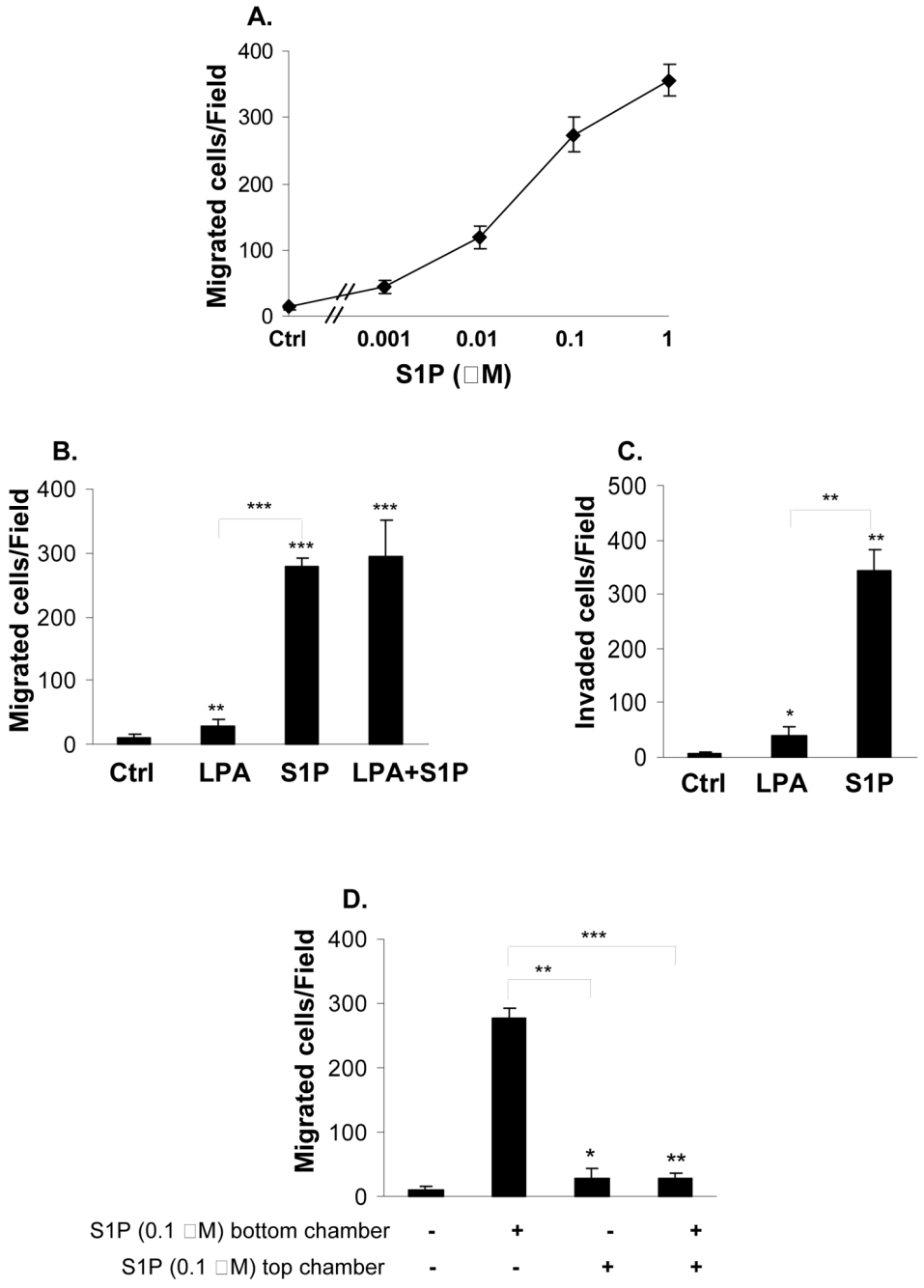

Figure 1.

S1P- and LPA-induced cell migration and invasion in HEY EOC cells. A. Dose curve of S1P-induced migration. B. Cell migration in response to LPA (5 μM) and/or S1P (0.1 μM). C. Cell invasion in response to LPA (5 μM) or S1P (0.1 μM). D. S1P was added to bottom, top or both chambers to modulate cell migration. Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Statistical analyses were conducted by comparing each case with the control (Ctrl) unless specified.

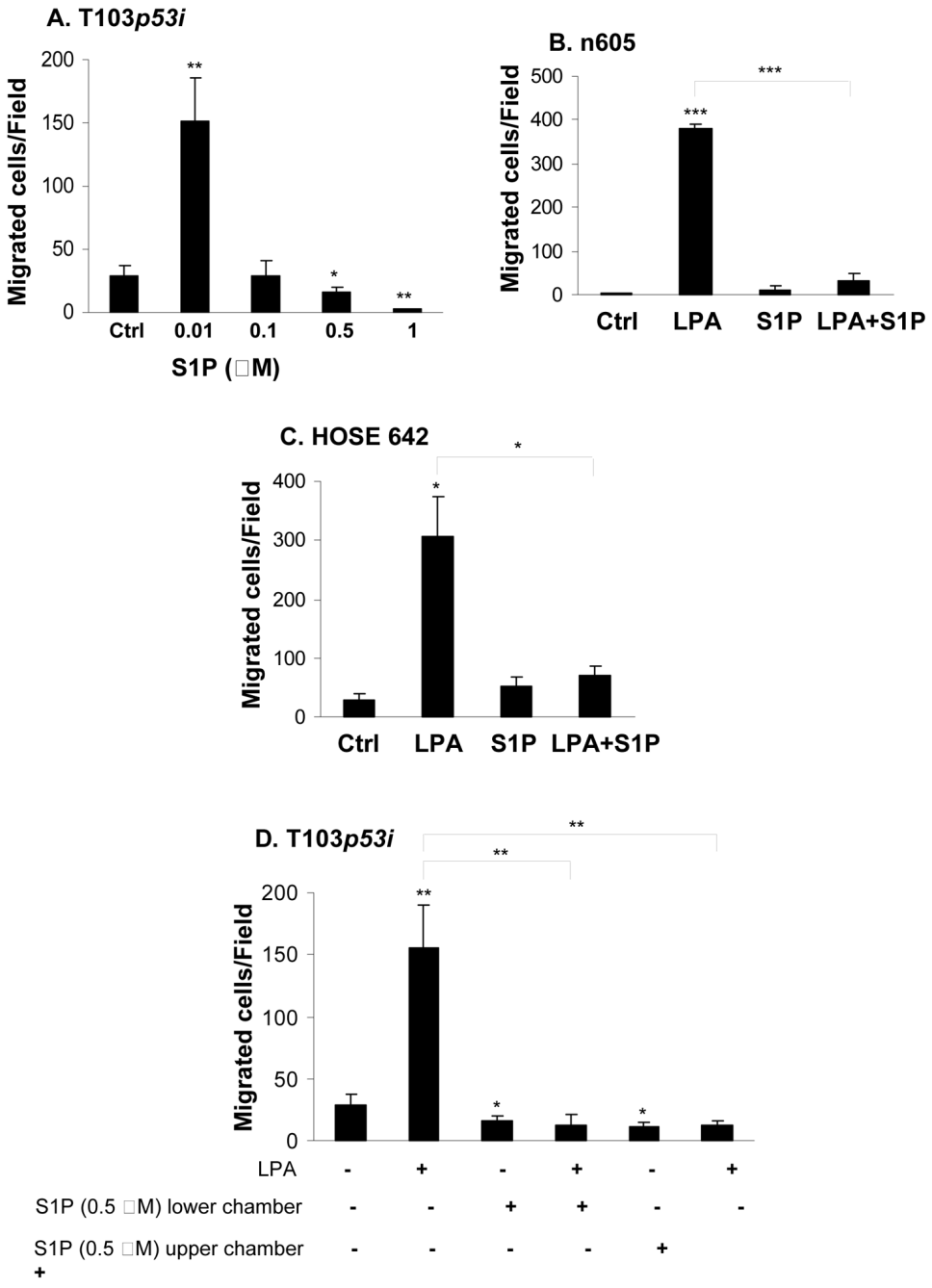

Figure 2.

The effect of S1P in cell migration in HOSE cells. A. Dose curve of S1P-induced migration in T103*p53i* cells. B and C. Primary HOSE n605 and immortalized HOSE 642 cell migration in response to LPA (1 μM) and/or S1P (0.5 μM). D. T103*p53i* cell migration in the presence of LPA (1 μM) and/or S1P (0.5 μM) with S1P was added to the bottom or the top chamber. Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Statistical analyses were conducted by comparing each case with the control (Ctrl) unless specified.

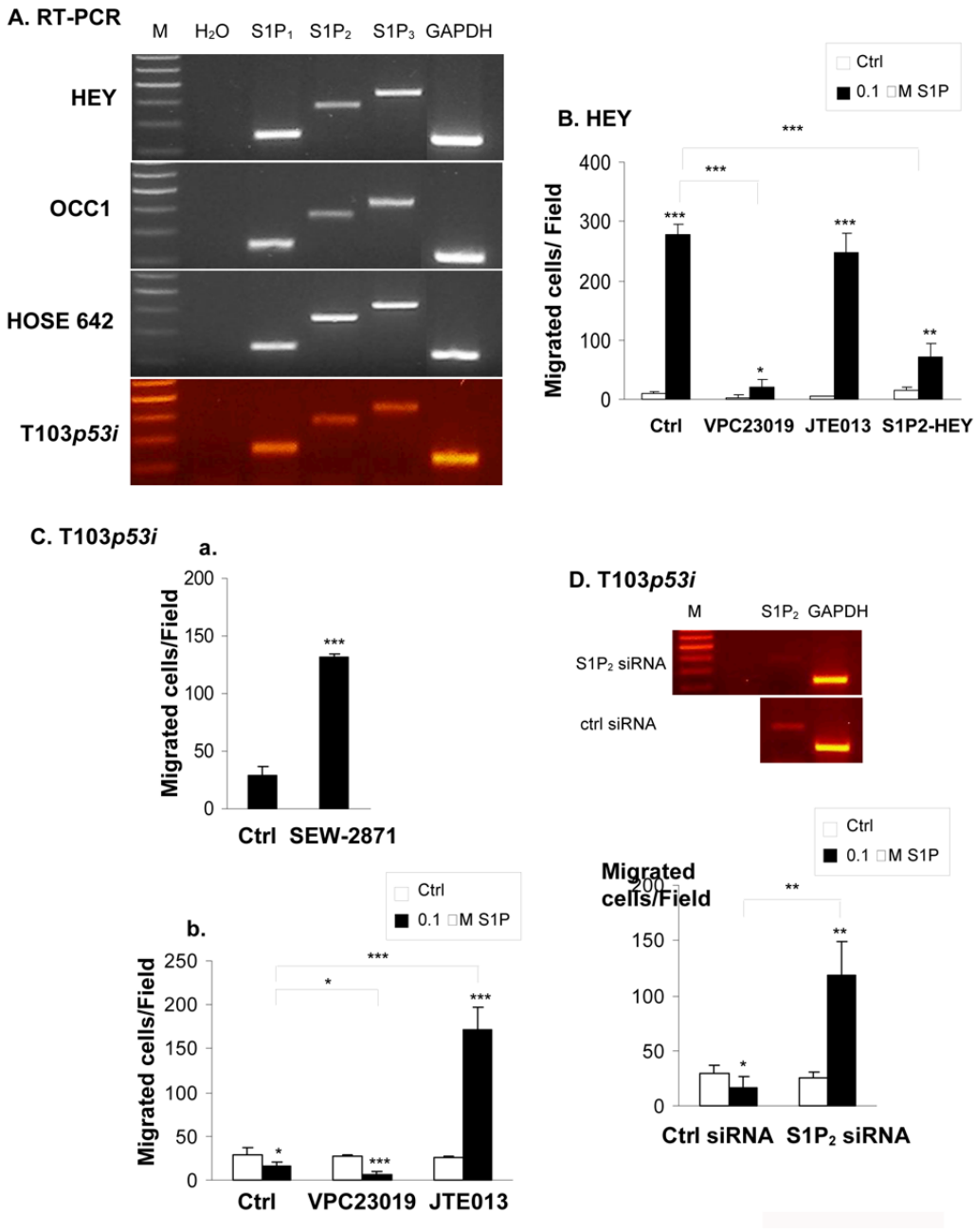

Figure 3.

The role of S1P receptors in migration of EOC and HOSE cells. A. $S1P_{1\text{-}3}$ expression in EOC and HOSE cells detected by RT-PCR. B. Effect of S1P with or without the $S1P_2$ antagonist JTE013 (1 μM), the $S1P_{1/3}$ antagonist VPC23019 (1 μM) on HEY cell migration and $S1P_2$ stably expressing HEY cells' migration in response to S1P. C. Migration response of T103*p53i* cells to SEW-2871 (a) and to S1P (0.1 μM) with or without the $S1P_2$ antagonist JTE013 (1 μM), the $S1P_{1/3}$ antagonist VPC23019 (1 μM) (b). D. Selective depletion of $S1P_2$ expression by target siRNA but not control siRNA was identified by RT-PCR. Migration response to S1P was test 72 hrs after siRNA transfection. Student's *t* test, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Statistical analyses were conducted by comparing each case with the control (Ctrl) unless specified.

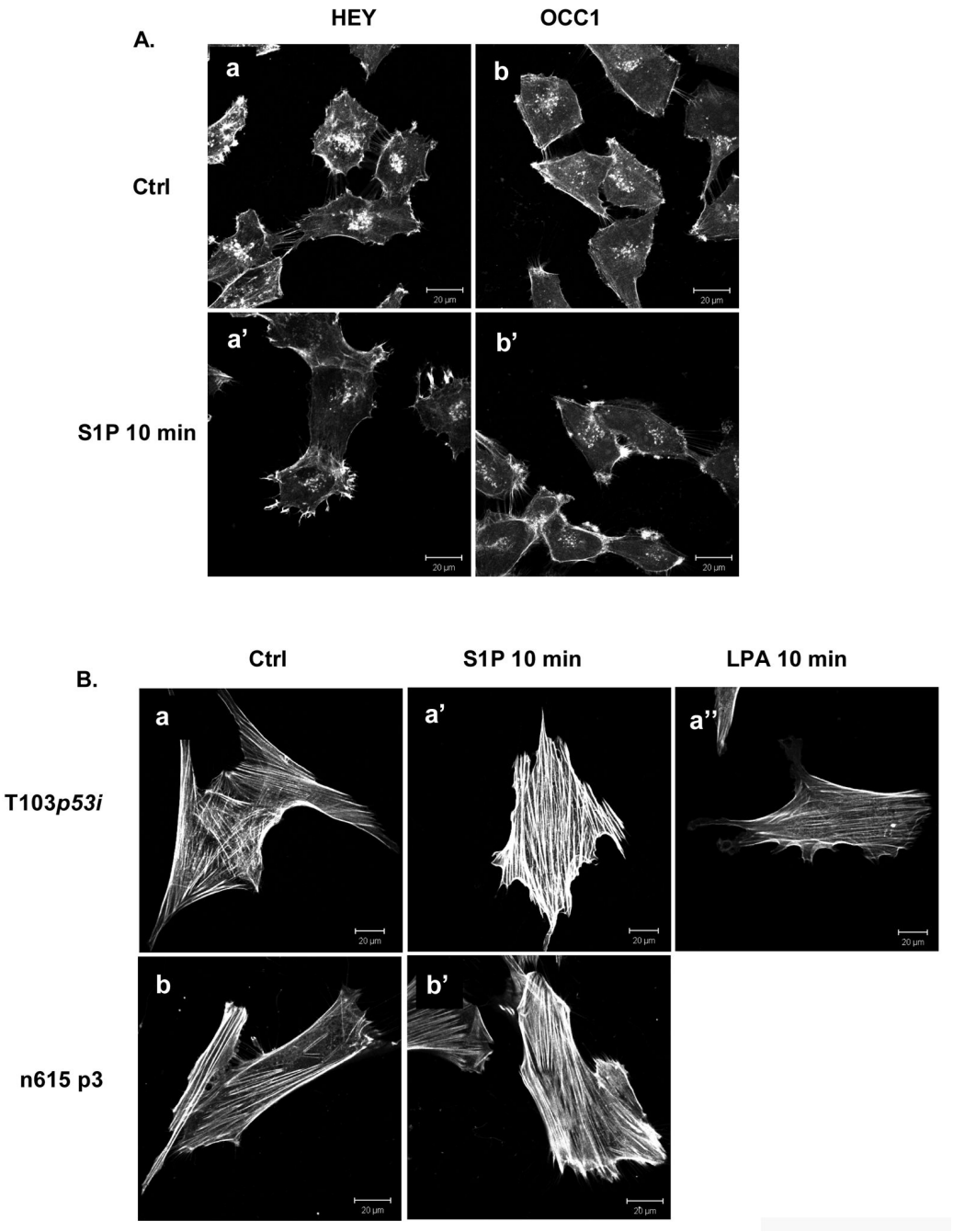

Figure 4.

Cell morphology of EOC and HOSE cells and response to S1P treatment. Two EOC cells (HEY and OCC1) (A) and two HOSE cells (T103*p53i* and n615 p3) (B) were plated onto glass slides, treated with or without S1P (0.5 μM) or LPA (5 μM) for 10 min as indicated, and then F-actin was stained with TRITC-phalloidin. The pictures are representative images of two independent experiments.

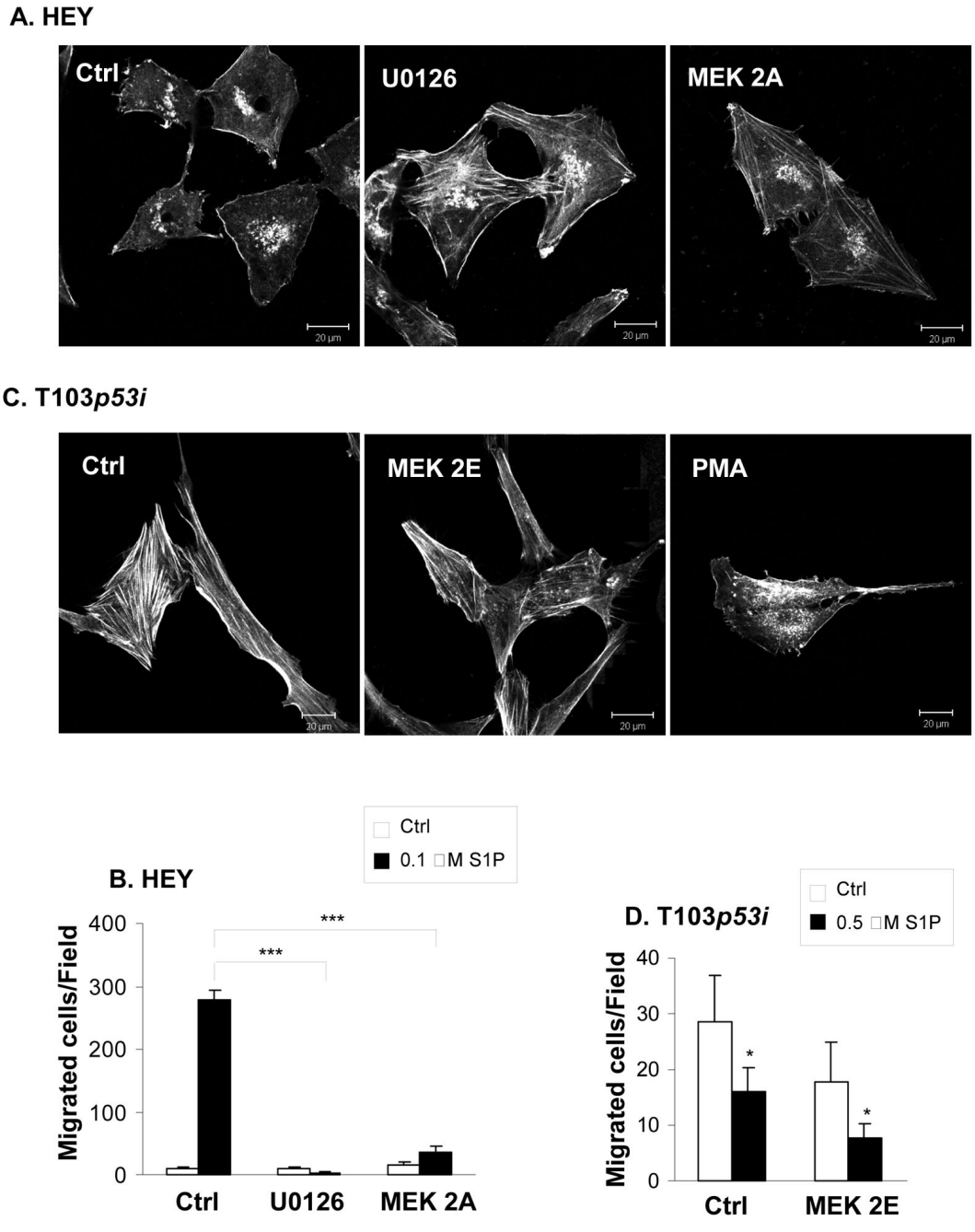

Figure 5.

MEK-regulated stress fibers were correlated with cell migration in response to S1P. A. HEY cells were treated with U0126 (10 μM) for 24 hrs or transfected with MEK 2A and then stained F-actin with TRITC-phalloidin. B. 24 hrs after treatment with U0126 (10 μM or 48 hrs after MEK 2A transfection, cell migration in response to S1P (0.1 μM) was tested. C. T103*p53i* cells were transfected with MEK 2E and then stained F-actin with TRITC-phalloidin. D. Forty-eight hrs after transfection, cell migration in response to S1P (0.5 μM) was tested. Student's *t* test, *, $P < 0.05$; ***, $P < 0.001$.

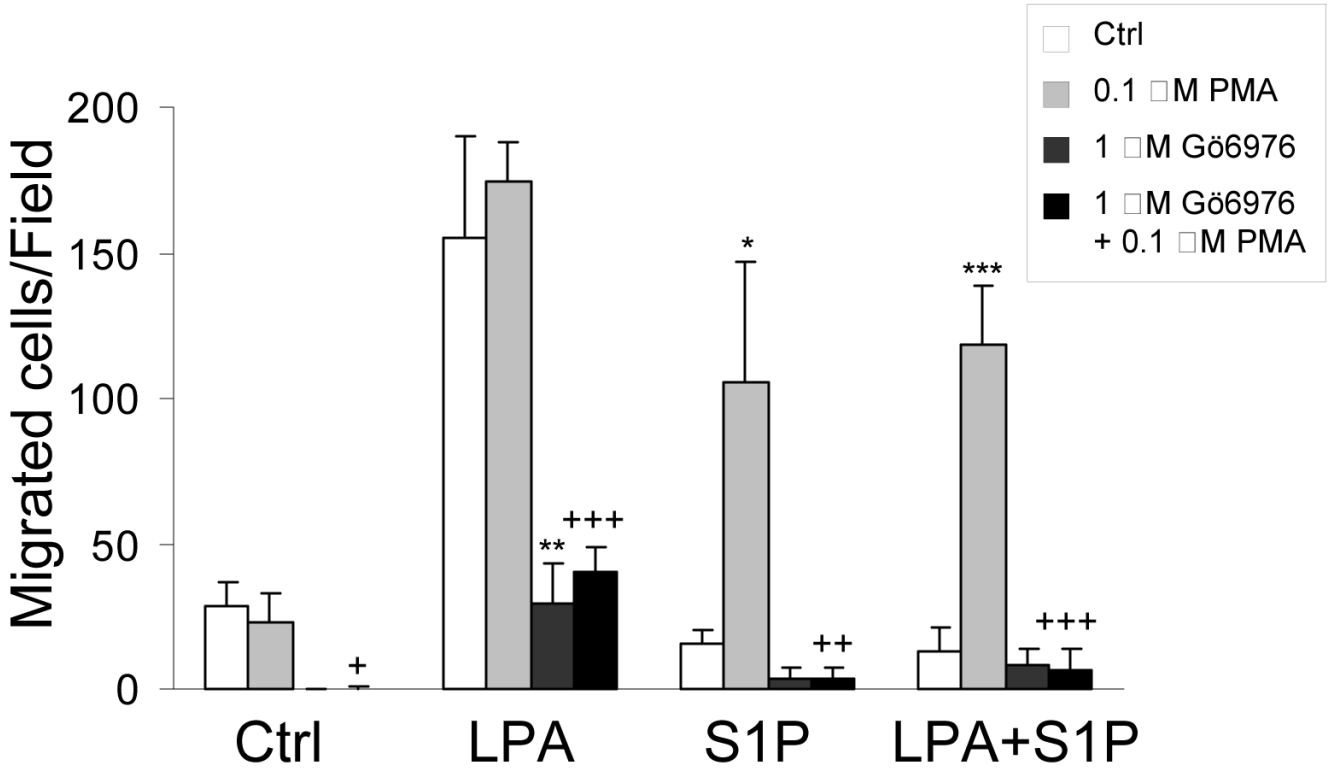

Figure 6.

Activation of the PKC pathway by PMA pretreatment reversed the inhibitory effect of S1P. T103*p53i* cells were untreated or pretreated with PMA (0.1 μM) for 1 hr, Gö6976 (1 μM) for 30 min, or Gö6976 (1 μM) for 30 min followed by PMA (0.1 μM) for 1 hr. Cell migration in response to LPA or/and S1P was then tested. Student's *t* test, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; compared with the correspondent control (Ctrl, the first bar in the same group). Student's *t* test, +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$; compared with the correspondent PMA results (0.1 μM PMA, the second bar in the same group).