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Sphingosylphosphorylcholine Induces Thrombospondin-1 Secretion in MCF10A Cells via ERK2

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Abstract

Sphingosylphosphorylcholine (SPC) is one of the bioactive phospholipids that has many cellular functions such as cell migration, adhesion, proliferation, angiogenesis, and Ca^{2+} signaling. Recent studies have reported that SPC induces invasion of breast cancer cells via matrix metalloproteinase-3 (MMP-3) secretion leading to WNT activation. Thrombospondin-1 (TSP-1) is a matricellular and calcium-binding protein that binds to a wide variety of integrin and non-integrin cell surface receptors. It regulates cell proliferation, migration, and apoptosis in inflammation, angiogenesis and neoplasia. TSP-1 promotes aggressive phenotype via epithelial mesenchymal transition (EMT). The relationship between SPC and TSP-1 is unclear. We found SPC induced EMT leading to mesenchymal morphology, decrease of E-cadherin expression and increases of N-cadherin and vimentin. SPC induced secretion of thrombospondin-1 (TSP-1) during SPC-induced EMT of various breast cancer cells. Gene silencing of TSP-1 suppressed SPC-induced EMT as well as migration and invasion of MCF10A cells. An extracellular signal-regulated kinase inhibitor, PD98059, significantly suppressed the secretion of TSP-1, expressions of N-cadherin and vimentin, and decrease of E-cadherin in MCF10A cells. ERK2 siRNA suppressed TSP-1 secretion and EMT. From online PROGgene V2, relapse free survival is low in patients having high TSP-1 expressed breast cancer. Taken together, we found that SPC induced EMT and TSP-1 secretion via ERK2 signaling pathway. These results suggests that SPC-induced TSP-1 might be a new target for suppression of metastasis of breast cancer cells.

Key Words: Sphingosylphosphorylcholine, Thrombospondin-1, Epithelial mesenchymal transition, ERK2

INTRODUCTION

Cancer metastasis encompasses several fundamental biological processes: cancer initiation, epithelial-mesenchymal transition (EMT), breach of the basement membrane barrier, neighbor invasion, intravasation, mesenchymal-epithelial transition (MET), extravasation, colonization and subsequent adaptation to foreign tissue microenvironments (Eccles and Welch, 2007; Valastyan and Weinberg, 2011; Wang *et al*., 2013; Kim *et al*., 2015). Cancer metastasis accounts for approximately 90% of all cancer-related deaths. Effective approaches to inhibit cancer metastasis have not yet been developed (Cao *et al*., 2013).

EMT is the first stage among several steps of metastasis. EMT is a cellular process during which epithelial cells show loss of cell-cell and cell-matrix junction, adopt a mesenchymal cell shape, and acquire a de-differentiated, migratory and invasive behavior (Tiwari *et al*., 2012; Nieto *et al*., 2016). Over the past few decades, activation of an EMT program has been proposed as the critical mechanism for the acquisition of malignant phenotypes by epithelial cancer cells. But, it is still unclear what specific signals induce EMT in carcinoma cells (Kalluri and Weinberg, 2009; Nieto *et al*., 2016).

Sphingosylphosphorylcholine (SPC) is one of the bioactive sphingolipids that have many cellular functions (Nixon *et al*., 2008). Increased levels of SPC are found in atopic dermatitis, Niemann-Pick disease (NPD), and malignant ascites among patients with tumors (Xiao *et al*., 2001; Kurokawa *et al*., 2009). SPC induces invasion via secretion of matrix metalloproteinase-3 (MMP-3) and perinuclear reorganization of keratin 8 (K8) filaments that contribute to the viscoelasticity of metastatic cancer cells (Beil *et al*., 2003; Kim *et al*., 2015, 2016).

Thrombospondin-1 (TSP-1) is a matricellular and calcium-binding glycoprotein which was first discovered in activated

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platelets (Lawler *et al*., 1978). Thrombospondin structural homology repeats (TSRs) are involved in neurite outgrowth, TGF-β-activation, inhibition of proliferation, inhibition of angiogenesis by CD36 activation, and induction of apoptosis (Adams and Tucker, 2000; Jimenez *et al*., 2000). TSP-1 supports metastasis of breast cancer cells (Yee *et al*., 2009). Endothelial cell derived TSP-1 induces sustained breast cancer cell quiescence (Ghajar *et al*., 2013).

However, nothing is known about the relationship between SPC and TSP-1 in cancer cells, especially in breast cancer and related cells.

In this study, we found that SPC induces EMT and secretion of TSP-1 in breast cancer and related cells. We also found that ERK2 is involved in SPC-induced TSP-1 secretion.

MATERIALS AND METHODS

Materials and plasmids

D-erythro- SPC and L-threo-SPC were obtained from Matreya (Pleasant Gap, PA, USA). Mouse monoclonal antibody specific for thrombospondin-1 was purchased from Abcam (Cambridge, UK). Mouse monoclonal antibodies against β-actin and vimentin were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against N-cadherin and E-cadherin, monoclonal antibody specific for ERK1+ERK2 (phosphor T202+Y204+T185+Y187), and goat polyclonal antibody against STAT3 were purchased from Abcam (Cambridge, UK). Peroxidase-labeled secondary antibodies were acquired from Santa Cruz Biotechnology. Alexa Fluor 488 goat anti-rabbit and 594 goat anti-mouse antibodies were obtained from Molecular Probes, Inc (Eugene, OR, USA). Dulbecco's Modified Eagle's Medium (DMEM) and DMEM-F12, RPMI 1640 and defined fetal bovine serum (FBS) were obtained from Welgene, Inc (Daegu, Korea). Lipofectamine 2000 Reagent was purchased from Invitrogen (Carlsbad, CA, USA), and SB203580, SP600125, and PD98059 were purchased from Calbiochem (La Jolla, CA, USA).

Cell culture, transfection and siRNA

The immortalized human mammary epithelial cell line MCF10A was maintained in DMEM/F-12 medium supplemented with 5% heat-inactivated horse serum (Welgene, Inc.), 10 μg/ml insulin, 20 ng/ml EGF, 0.5 μg/ml hydrocortisone, streptomycin (100 μg/ml), and penicillin (100 U/ml). Serum free medium for MCF10A was DMEM/F12, supplemented with 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, streptomycin (100 μg/ml), and penicillin (100 U/ml). MCF7 and MDA-MB-231 and HS578T, SKBR3 cells were obtained from ATCC (Rockville, MD, USA). The cells were cultured in DMEM without phenol red, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), streptomycin (100 μg/ml), and penicillin (100 U/ml) at 37 °C in a 5% CO2 atmosphere. T47D, ZR-75-1, MDA-MB-453 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin streptomycin solution (PS) (Welgene, Inc.). All cells were grown at 37°C in a humidified, 5% CO_2 atmosphere. The cells were washed three times in Dulbecco's phosphate buffered saline (DPBS) and incubated in serum-free medium for 18 h before the respective experiments.

For transient knockdown of TSP-1, non-targeting siRNA and TSP-1 siRNAs were purchased from ST Pharm. Co (Siheung, Korea). A small interfering RNA (siRNA) duplex targeting human TSP-1, 5'-GGA GUU CAG UAC AGA AAU ATT-3' and 5'-GUA CAG AAA UAA CGA GGA ATT-3' (ST Pharm. Co.) was introduced into the cells using Lipofectamine™ 2000 reagent (Invitrogen), according to the manufacturer's instructions. The cells were then cultured with or without SPC (5 μM).

Western blot

Western blot analysis was performed, as described previously (Jeong *et al*., 2016). MCF10A cells were harvested and lysed in 50 mM Tris-HCl (pH7.5), 150mM NaCl, 1% triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, sterile solution and protease inhibitors, phosphatase inhibitor (Gendepot, Barker, TX, USA) (Beil *et al*., 2003; Park *et al*., 2010). The protein concentrations in the supernatants were determined using Coomassie Plus (Pierce Biotechnology Inc., Rockford, IL, USA), as recommended by the manufacturer. The protein lysates were loaded onto a precast 8 or 10% polyacrylamide gradient gel. The proteins were separated by SDS PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Invitrogen) with glycine transfer buffer [192 mM glycine, 25 Mm Tris-HCl (pH 8.8), 10% MeOH (v/v)]. After blocking the nonspecific site with 3% non-fat dry milk, the membrane was incubated with a specific primary antibody in 3% bovine serum albumin (BSA) at 4°C overnight. The membranes were washed with TBS+0.1% Tween 20 and further incubated for 60 min with a peroxidase-conjugated secondary antibody (1:5000, Santa Cruz) at room temperature. Immunoactive proteins were detected using the PowerOpti-ECL Western blotting detection reagent (Animal Genetics Inc., Suwon, Korea).

Confocal microscopy

Confocal microscopy was performed as described previously (Zheng *et al*., 2016). MCF10A cells were grown on coverslips and fixed via SPC treatment 48 h later with methanol at -20°C. Fixed cells were permeabilized with a 10 min wash in 0.1% Triton X-100 at room temperature followed by several washes in PBS with 3% bovine serum albumin (PBS/BSA). Anti-N-cadherin or anti-E-cadherin or anti-vimentin (1:250) was incubated with coverslips overnight at 4°C. Excess antibody was removed with four washes in PBS/BSA. Species-specific second antibodies conjugated to goat anti-rabbit IgG antibody (Alexa Fluor 488, 1:500, Molecular Probes, Inc.) or goat anti-mouse IgG antibody (Alexa Fluor 594, 1:500, Molecular Probes, Inc.) were then reacted with the coverslips for 1 h at room temperature followed with four washes in PBS/BSA. The final samples were mounted onto slides and visualized using a Nikon confocal microscope.

Cell migration assay

Migration assays were performed using a multi-well chamber (Neuroprobe Inc., Gaithersburg, MD, USA) coated with 10 μg/ml fibronectin as a chamoattractant (Park *et al*., 2013; Lim *et al*., 2015). Briefly, MCF10A cells were suspended in DMEM at 1×10^6 cells/ml, and a 25 μl aliquot of this suspension was placed into the upper well of the chamber. Next, the aliquot was separated from the 3% serum-containing lower well by an 8 μm polyhydrocarbon filter. After incubation for 4 h at 37°C, non-migrated cells on the upper surface of the membrane were scraped off, and the migrated cells on the lower surface were stained by Diff-Quick, which were subsequently counted under less than five randomly chosen high power fields

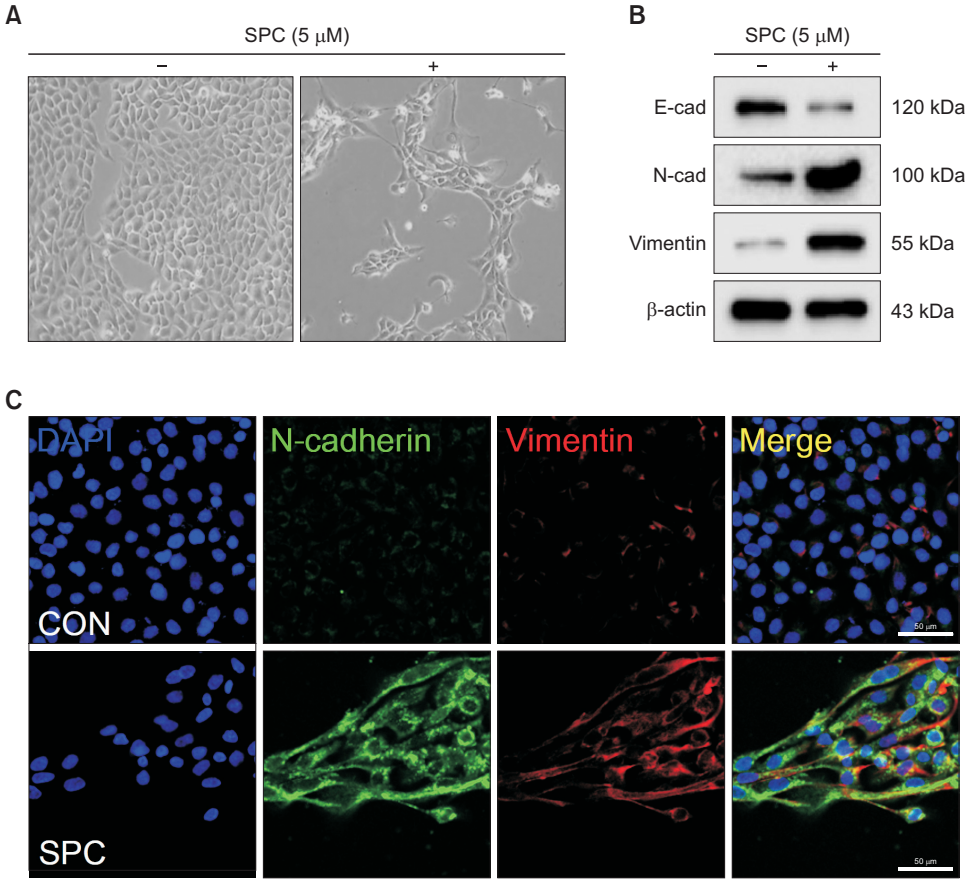

Fig. 1. SPC induces epithelial mesenchymal transition in MCF10A cells. (A) Change of morphology in MCF10A cells treated with the indicated D-erythro-SPC (5 μM) for 48 h under serum free media condition. (B) Expression level of EMT marker proteins under the same conditions. (C) Confocal microscopic examination of N-cadherin and vimentin in MCF10A cells were treated with SPC (5 μM) for 48 h. Immunofluorescence staining was performed using N-cadherin (green), and vimentin (red).

(400×). All experiments were repeated at least three times with three replicates each.

Cell invasion assay

Cell invasion assay was performed as described previously (De *et al*., 2015). Invasion assays were performed using a 24-well Transwell unit with polycarbonate filters having a diameter of 6.5 mm and a pore size of 8.0 mm (Corning, NY, USA). Briefly, a fixed number of cells (5×10^4 cells/chamber) were used for each invasion assay. The lower and upper parts of the Transwell were coated with 20 ml of a 1:2 mixture of Matrigel: DMEM. Cells were plated on the Matrigel-coated Transwell. The medium in the lower chambers contained 0.1 mg/ml of bovine serum albumin. After incubation for 24 h at 37°C, cells invading the lower surface of the membrane were fixed with methanol, and stained with hematoxylin and eosin (H&E stain), which were subsequently counted under five randomly selected fields (400×). All experiments were repeated at least three times with three replicates each.

Acetone precipitation

Cell culture supernatants were collected and centrifuged at 1,000 rpm, 5 min. The cell free supernatant was incubated with 80% cold acetone at -20°C for 60 min. Subsequently, the samples were centrifuged at 15,000 rpm at 4°C for 30 minutes. The supernatant was carefully aspirated and the pellets were allowed to air dry at 23°C.

Prognostic evaluation of TSP-1

Prognostic evaluation of TSP-1 was done according to instruction provided by Goswami and Nakshatri (2014).

Statistical analysis

The data are expressed as the mean ± SEM of at least three independent experiments performed in triplicate. $p<0.05$ was considered statistically significant.

RESULTS

SPC induces EMT in MCF10A cells

SPC was found in patients with malignant tumors (Seufferlein and Rozengurt, 1995; Calcerrada *et al*., 1999; Boguslawski *et al*., 2000; Meyer zu Heringdorf *et al*., 2002; Xu, 2002). SPC induces invasion of MCF10A cells via secretion of MMP-3 and phosphorylation and reorganization of keratin 8. Sustained keratin phosphorylation leads to keratin loss (Beil *et al*., 2003; Kim *et al*., 2015, 2016). These results suggested

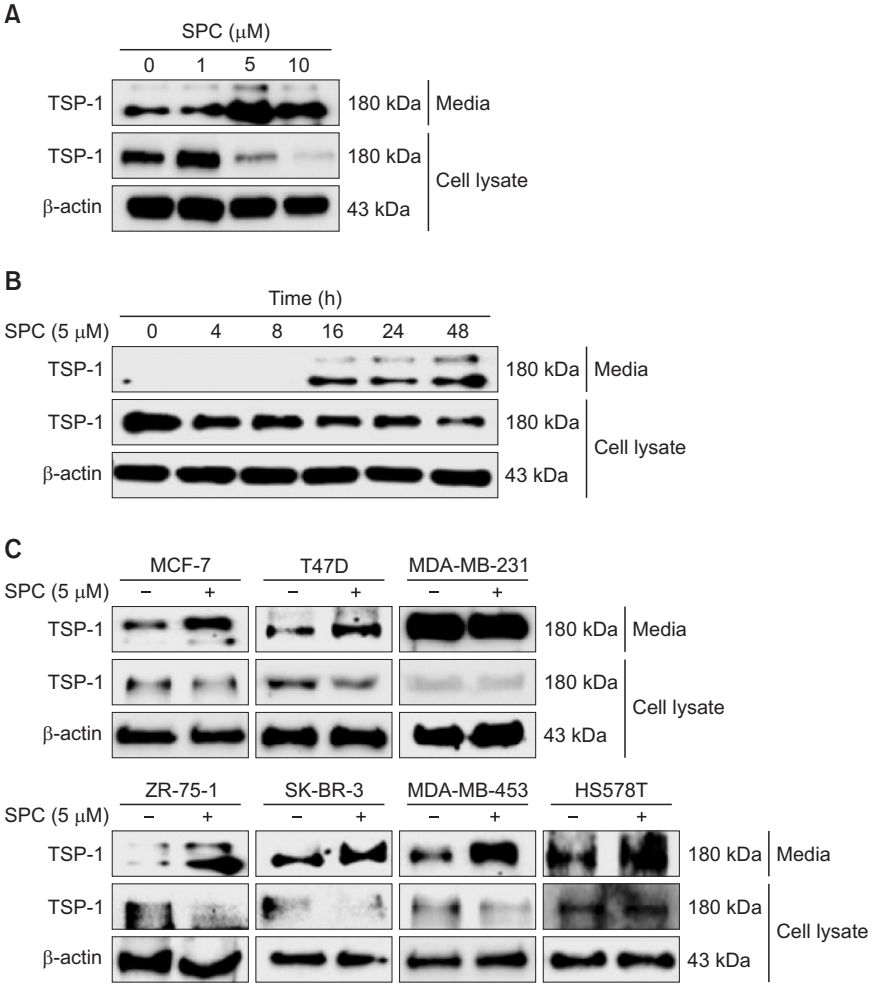

Fig. 2. Effects of SPC on thrombospondin secretion in MCF10A cells. (A) Dose dependent secretion level of TSP-1 in MCF10A cells stimulated with the indicated concentrations of D-erythro-SPC for 48 h. (B) Time dependent secretion of TSP-1. MCF10A cells were treated with 5 μM SPC for the times indicated. (C) Secretion level of TSP-1 in various cancer cell lines. In (A) and (B), SPC treatment was conducted using horse serum free or 2% horse serum media condition.

that SPC might be involved in EMT. Therefore, we examined whether SPC induced epithelial-mesenchymal transition in breast related MCF10A cells. SPC induced a mesenchymal morphology-long spindle shaped-change in MCF10A, an epithelial breast immortalized cell line (Fig. 1A). Loss of E-cadherin expression was observed in SPC-treated MCF10A cells compared with non-treated cells (Fig. 1B). Mesenchymal markers including N-cadherin, and vimentin were increased in SPC treated MCF10A cells (Fig. 1B). These observations were also confirmed by confocal microscopy (Fig. 1C). SPC induced migration and invasion of MCF10A cells (Supplementary Fig. 1, 2).

SPC induces TSP-1 secretion in MCF10A cells

Several studies have reported that TSP-1 promoted cell migration and invasion of different cells (Sid *et al*., 2008; Firlej *et al*., 2011). The type 1 repeats of TSP-1 activate latent TGF-β1 which is a typical EMT inducer (Schultz-Cherry *et al*., 1994; Park *et al*., 2013). Therefore, we investigated whether SPC induced TSP-1 in the course of EMT in breast cancer and related cell lines. SPC dose-dependently induced secretion of TSP-1 in MCF10A cells (Fig. 2A). The TSP-1 secretion was first detectable after 16 h and reached a maximum after 48 h incubation in the presence of SPC (Fig. 2B). SPC-induced secretion of TSP-1 was also confirmed in other breast cancer cell lines such as MCF-7, T47D, ZR-75-1, SK-BR-3, MDA-MB-453, and HS578T (Fig. 2C).

Effects of TSP-1 knock-down on SPC-induced EMT in MCF10A cells

We examined the involvement of TSP-1 in SPC-induced EMT using a TSP-1 siRNA. Knock-down of TSP-1 inhibited SPC-induced EMT in breast cancer cells (Fig. 3A). Gene silencing of TSP-1 in MCF10A cells restored the E-cadherin expression decreased by SPC and reversed the SPC-induced vimentin and N-cadherin expressions (Fig. 3A). Gene silencing of TSP-1 was confirmed by Western blot (Fig. 3A). These findings were also confirmed by confocal microscopy (Fig. 3B). TSP-1 si-RNA significantly blocked SPC-induced migration and invasion (Fig. 3C). These observations suggested

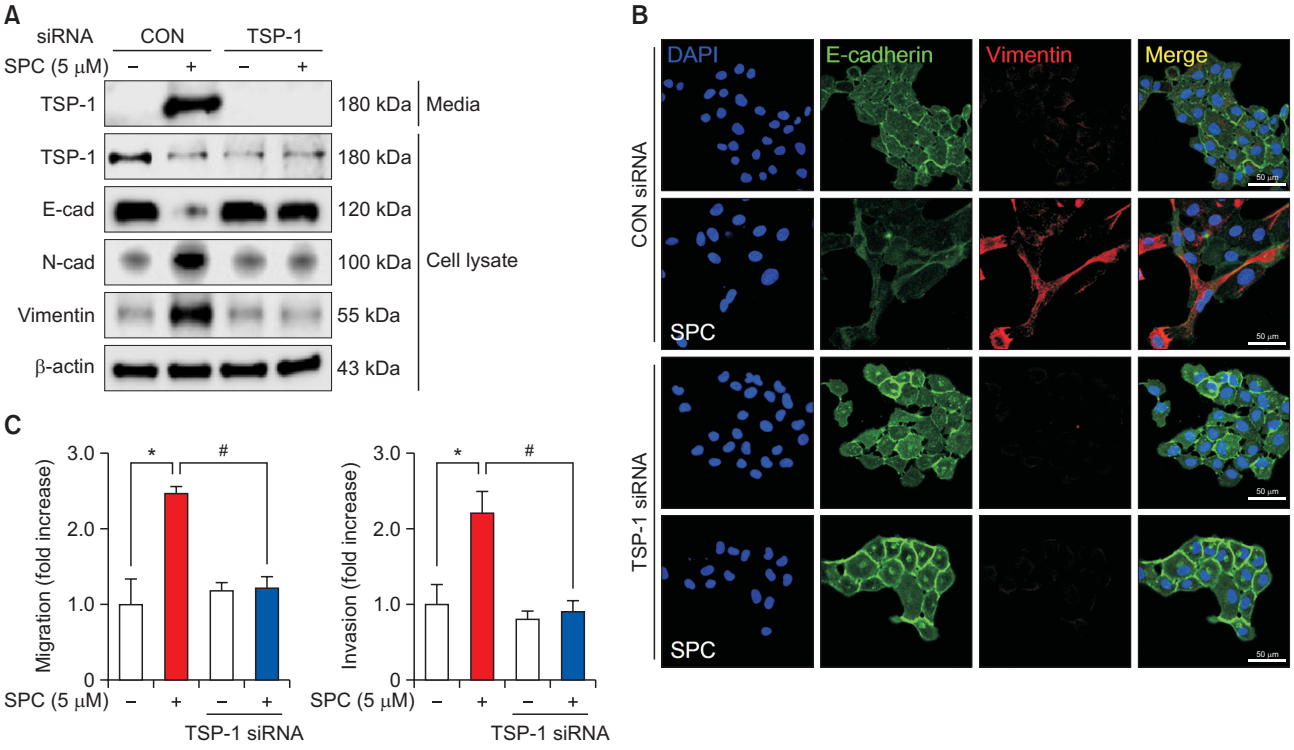

Fig. 3. Knock-down of TSP-1 reduced SPC-induced EMT. (A, B) Effect of Thrombospondin-1 gene silencing on SPC-induced EMT in MCF10A cells. MCF10A cells were treated with or without 5 μM SPC for 48 h after transfection with Thrombospondin-1 siRNA (10 nM) and control siRNA (10 nM). (C) Effect of TSP-1 gene silencing on SPC-induced migration and invasion of MCF10A cells. For gene silencing of TSP-1, the MCF10A cells were transfected with amount of TSP-1 siRNA (10 nM) and treated with SPC (5 μM). *p<0.05, $^{\#}p$<0.05 compared to control or SPC (5 μM). All experiments were repeated at least three times with three replicates (n=3) each.

that TSP-1 is involved in SPC-induced EMT of MCF10A cells.

Effects of MAP kinase inhibitors on SPC-induced TSP-1 expression and secretion in MCF10A cells

SPC-induced TSP-1 secretion is involved in SPC-induced EMT but the mechanism of TSP-1 secretion remains unclear. Therefore, we investigated the signaling pathway involved in SPC-induced TSP-1 secretion using MAP kinase inhibitors including PD98059 (MEK inhibitor), SB203580 (p38 kinase inhibitor), and SP600125 (JNK inhibitor). PD98059 suppressed TSP-1 secretion, N-cadherin and vimentin expressions, and restored E-cadherin expression (Fig. 4A, 4B). However, SP600125 (JNK inhibitor) and SB203580 (p38 kinase inhibitor) did not have any of the effects that PD98059 had (Fig. 4A). SPC induced ERK1/2 activation and PD98059 suppressed it (Fig. 4B). PD98059 also suppressed the SPC-induced migration of MCF10A cells (Supplementary Fig. 3).

Involvement of ERK2 in SPC-induced thrombospondin-1 secretion in MCF10A cells

Inhibition of SPC-induced TSP-1 secretion and EMT by PD98059 suggested that the ERK pathway is involved in SPC-induced EMT and TSP-1 secretion. Therefore, we examined the involvement of ERK2 in these processes using siRNA against ERK2 since ERK2 but not ERK1 is involved in EMT process (Shin *et al*., 2010). Both of two ERK2 siRNA suppressed the SPC-induced TSP-1 secretion and N-cadherin and vimentin expressions, and restored the E-cadherin expression (Fig. 5A). These observations were confirmed by confocal microscopy (Fig. 5B). In contrast, ERK2 overexpression increased the SPC-induced TSP-1 secretion and EMT in MCF10A cells (Fig. 5C). ERK2 siRNA suppressed the SPC-induced migration and invasion of MCF10A cells (Fig. 5D).

Effect of TSP-1 expression on relapse-free survival and metastasis-free survival

We examined the effect of TSP-1 expression on relapse-free survival and metastasis-free survival in patients with breast cancer using the online KMPLOT site, PROGgene V2. The breast cancer patients were divided into two subgroups, TSP-1^{high} and TSP-1^{low} on the basis of their median expression level. The survival curves displayed a significant difference in the probability of relapse-free survival (p-value=0.04). The survival curves in the other microarray set (GSE48408) showed a significant difference in the probability of metastasis- free survival (p-value=0.014) (Wang *et al*., 2005; Sørensen *et al*., 2013). A high TSP-1 expression was associated with poor relapse-free and metastasis-free survivals in patients with breast cancer (Fig. 6A, 6B). Using the Cox regression analysis, the estimated hazard ratios for TSP-1^{high} compared with TSP-1^{low} in relapse-free survival were 1.27 in one microarray (GSE2034) and 1.36 in metastasis-free survival in the other microarray set (GSE48408).

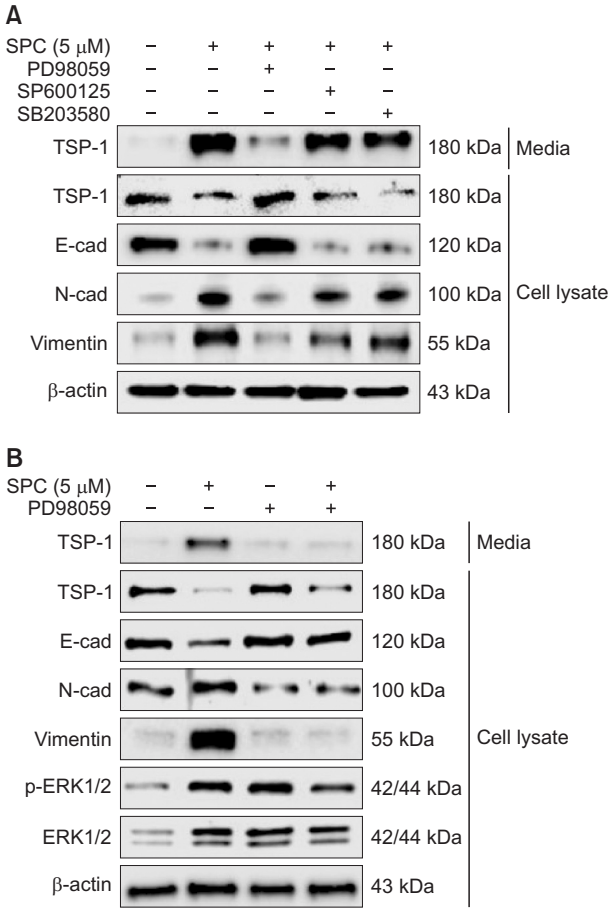

Fig. 4. Effects of MAP kinase inhibitors on SPC-induced TSP-1 secretion and EMT in MCF10A cells. (A) Effect of several kinase inhibitors on SPC-induced increase of TSP-1 secretion in MCF10A cells. MCF10A cells were treated with SPC (5 μM) for 48 h in the presence of the PD98059 (MEK inhibitor, 10 μM) SP600125 (JNK inhibitor, 5 μM), SB203580 (p38 kinase inhibitor, 10 μM). (B) Effect of MEK inhibitors on SPC-induced increase of TSP-1 secretion in MCF10A cells. MCF10A cells were treated with or without 5 μM SPC for 48 h in the presence of the PD98059.

DISCUSSION

Breast cancer accounts for the second highest percentage of deaths according to the American Cancer Society and it is mostly metastasized to brain, bone, and lungs, liver (Foulkes *et al*., 2010). Especially, triple-negative breast cancer (TNBC), which is a subtype of breast cancer that does not express the estrogen and progesterone receptors (ER/PR) and HER2, is correlated with a poor disease prognosis, high risk of recurrence and poor disease-free survival (Foulkes *et al*., 2010; Fosu-Mensah *et al*., 2015).

SPC induces EMT which plays an important role in cancer metastasis because it can promote metastasis to other organs (Nieto *et al*., 2016) (Fig. 1). Several inducers including TGF-β1 induce EMT in several cancer types. SPC might be added to these lists. SPC-induced EMT might be linked with TGF-β1 signaling since SPC induces differentiation of mesenchymal transition by TGF-β1 dependent mechanism (Jeon *et al*., 2006).

SPC dose- and time-dependently induced TSP-1 secretion during the SPC-induced EMT and TSP-1 is involved in SPC-induced EMT (Fig. 2, 3). The reports which show that TSP-1 promotes cell migration and invasion suggested that SPC-induced TSP-1 expression might play a key role in EMT since migration and invasion are the key indicators of EMT (Sid *et al*., 2008; Firlej *et al*., 2011). Furthermore, SPC-induced TSP-1 secretion suggested that TGF-β1, a typical induce of EMT, might be involved in SPC-induced EMT since TSP-1 activates latent TGF-β1 (Schultz-Cherry *et al*., 1994; Murphy-Ullrich and Poczatek, 2000).

PD98059 suppressed SPC-induced TSP-1 secretion and EMT (Fig. 4A). ERK is a downstream kinase that is phosphorylated by MEK. PD98059 inhibited SPC-induced phosphorylation of ERK (Fig. 4B). ERK2 but not ERK1 is involved in EMT (Shin and Blenis, 2010; Shin *et al*., 2010). Therefore, we examined the effects of ERK2 in SPC-induced EMT (Fig. 5). Secretion of TSP-1 was significantly suppressed by ERK2 siRNA (Fig. 5A, 5B). Overexpression of ERK2 enhanced the secretion of TSP-1 (Fig. 5C). It is still unclear whether SPC induce expression and secretion of TSP-1 or SPC induce only secretion of TSP-1 but not expression of TSP-1. However, it is clear that ERK2 seemed to be involved in the SPC-induced secretion of TSP-1 (Fig. 2, 5). Actually, ERK1/2 phosphorylates the proteins involved in the secretion process (Farhan and Rabouille, 2011).

How does SPC induce secretion of TSP-1β In fact, SPC

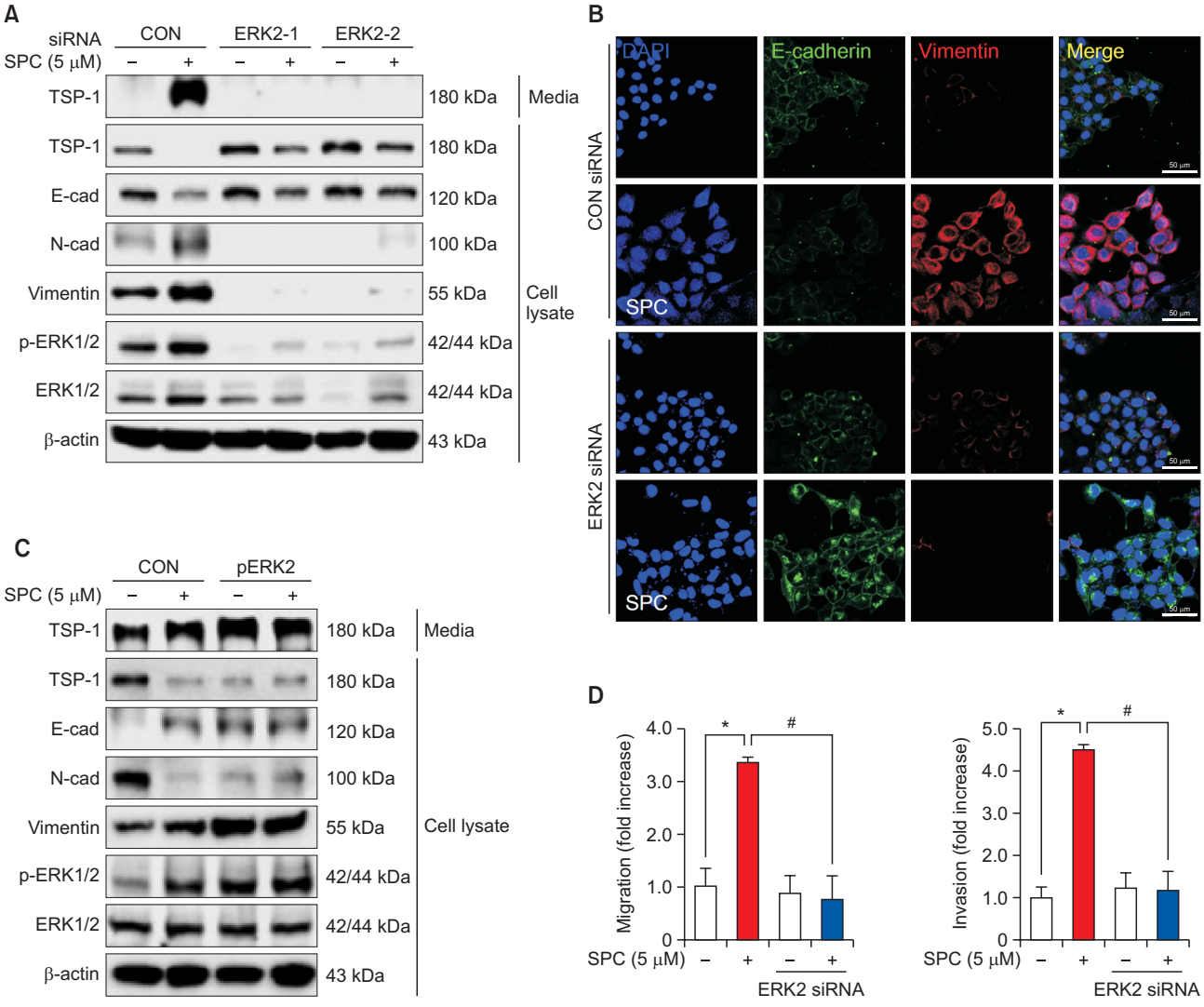

Fig. 5. Effects of ERK2 siRNA on SPC-induced TSP-1 secretion in MCF10A cells. (A) Effect of ERK2 gene silencing on TSP-1 secretion and SPC-induced EMT in MCF10A cells. MCF10A cells were treated with or without 5 μM SPC for 48 h after transfection with ERK2 siRNA (5 nM) and control siRNA (5 nM). (B) Confocal microscopic examination of E-cadherin and vimentin as EMT markers in Control and ERK2 gene silenced MCF10A cells. Each MCF10A cells were treated with or without 5 μM SPC for 48 h. Immunofluorescence staining was performed using E-cadherin (green), and vimentin (red). (C) Effects of ERK2 overexpression on SPC-induced TSP-1 secretion, N-cadherin, vimentin, and E-cadherin expressions. MCF10A cells were treated with or without SPC (5 μM) for 48 h after transfection with a plasmid containing ERK2 and control.(D) Effects of ERK2 siRNA on SPC-induced migration and invasion. *p<0.05, $^{\#}p$<0.05 compared to control or SPC (5 μM). All experiments were repeated at least three times with three replicates (n=3) each.

was reported to be a ligand for G protein coupled receptors (GPCR) including GPR3, GPR4, GPR12 and OGR1 which are important categories for drug development (Xu *et al*., 2000; Zhu *et al*., 2001; Ignatov *et al*., 2003; Im, 2003; Schlyer and Horuk, 2006; Yang *et al*., 2012). However, the reports about GPR4 and OGR1 were retracted (Retraction, 2005, 2006). Recently, we reported that GPR12 might mediate SPC-induced keratin phosphorylation and reorganization (Park *et al*., 2016). However, the involvement of GPCR in SPC-induced TSP-1 secretion can be checked using pertussis toxin since SPC is believed to modulate cellular response via pertussis toxin-sensitive GPCR (Lee *et al*., 2011).

High expression of TSP-1 was related to poor prognosis of breast cancer patients in terms of relapse free survival and metastasis free survival (Fig. 6). These observations were consistent with the reports that TSP-1 promotes an aggressive phenotype through epithelial-to-mesenchymal transition in human melanoma and TSP-1 is a bad prognositic marker for TNBC (Jayachandran *et al*., 2014; Campone *et al*., 2015). TSP-1 is highly expressed in desmoplastic components of invasive ductal carcinoma of the breast and associated with lymph node metastasis (Horiguchi *et al*., 2013).

Taking together the above results, the increase in TSP-1 secretion is involved in SPC-induced EMT. ERK activation by SPC controls TSP-1 secretion. Therefore, inhibition of SPC-induced TSP-1 expression might be a new way of controlling the SPC-induced EMT.

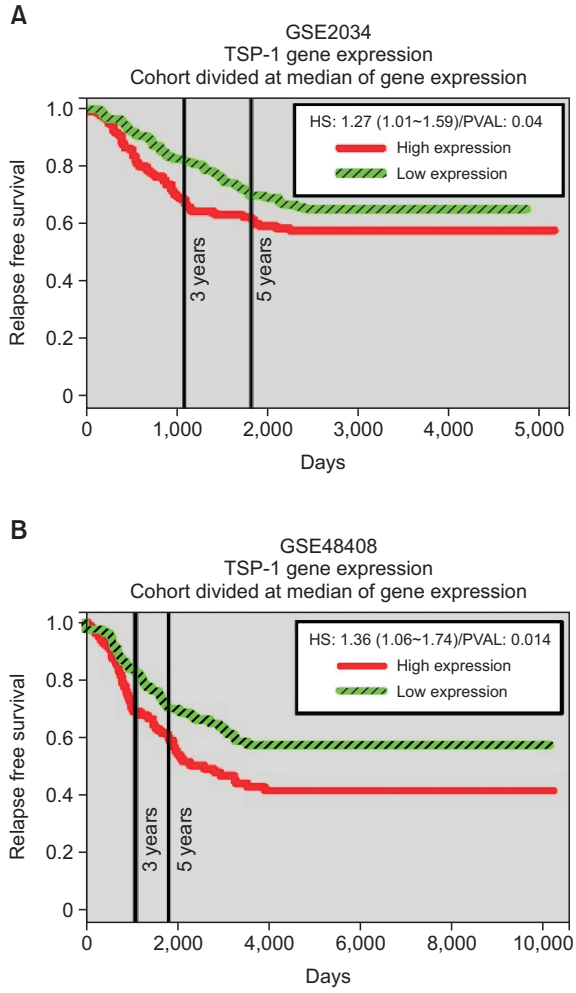

Fig. 6. Effects of TSP-1 on survival of breast cancer patients. (A) Effects of TSP-1 on relapse free survival of breast cancer patients. (B) Effects of TSP-1 on metastasis free survival of breast cancer patients. The prognostic value of TSP-1 was estimated in a public microarray datatbases of breast cancers from 285 (A) or 163 (B) patients, respectively (Wang *et al*., 2005; Sørensen *et al*., 2013; Goswami and Nakshatri, 2014). Kaplan-Meier curves showed the relapse-free survival or metastasis-free survival of breast cancer patients having above or below median expression of TSP-1. HR=Hazard ratio based on relative risk of event occurrence between the group of patients with TSP-1 expression above median value in the selected population compared to the group with TSP-1 expression below the median value.

ACKNOWLEDGMENTS

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REFERENCES

Adams, J. C. and Tucker, R. P. (2000) The thrombospondin type 1 repeat (TSR) superfamily: diverse proteins with related roles in neuronal development. *Dev. Dyn.* **218**, 280-299.

Beil, M., Micoulet, A., von Wichert, G., Paschke, S., Walther, P., Omary, M. B., Van Veldhoven, P. P., Gern, U., Wolff-Hieber, E., Eggermann, J.,Waltenberger, J., Adler, G., Spatz, J. and Seufferlein, T. (2003) Sphingosylphosphorylcholine regulates keratin network architecture and visco-elastic properties of human cancer cells. *Nat. Cell Biol.* **5**, 803-811.

Boguslawski, G., Lyons, D., Harvey, K. A., Kovala, A. T. and English, D. (2000) Sphingosylphosphorylcholine induces endothelial cell migration and morphogenesis. *Biochem. Biophys. Res. Commun.* **272**, 603-609.

Calcerrada, M. C., Miguel, B. G., Catalan, R. E. and Martinez, A. M. (1999) Sphingosylphosphorylcholine increases calcium concentration in isolated brain nuclei. *Neurosci. Res*. **33**, 229-232.

Campone, M., Valo, I., Jézéquel, P., Moreau, M., Boissard, A., Campion, L., Loussouarn, D., Verriele, V., Coqueret, O. and Guette, C. (2015) Prediction of recurrence and survival for triple-negative breast cancer (TNBC) by a protein signature in tissue samples. *Mol. Cell Proteomics* **14**, 2936-2946.

Cao, Z., Shang, B., Zhang, G., Miele, L., Sarkar, F. H., Wang, Z. and Zhou, Q. (2013) Tumor cell-mediated neovascularization and lymphangiogenesis contrive tumor progression and cancer metastasis. *Biochim. Biophys. Acta* **1836**, 273-286.

De, U., Kundu, S., Patra, N., Ahn, M. Y., Ahn, J. H., Son, J. Y., Yoon, J. H., Moon, H. R., Lee, B. M. and Kim, H. S. (2015) A new histone deacetylase inhibitor, MHY219, inhibits the migration of human prostate cancer cells via HDAC1. *Biomol. Ther. (Seoul)* **23**, 434-441.

Eccles, S. A. and Welch, D. R. (2007) Metastasis: recent discoveries and novel treatment strategies. *Lancet* **369**, 1742-1757.

Farhan, H. and Rabouille, C. (2011) Signalling to and from the secretory pathway. *J. Cell Sci.* **124**, 171-180.

Firlej, V., Mathieu, J. R., Gilbert, C., Lemonnier, L., Nakhle, J., Gallou-Kabani, C., Guarmit, B., Morin, A., Prevarskaya, N., Delongchamps, N. B. and Cabon, F. (2011) Thrombospondin-1 triggers cell migration and development of advanced prostate tumors. *Cancer Res.* **71**, 7649-7658.

Fosu-Mensah, N., Peris, M.S., Weeks, H.P., Cai, J. and Westwell, A.D. (2015) Advances in small-molecule drug discovery for triple-negative breast cancer. *Future Med. Chem.* **7**, 2019-2039.

Foulkes, W. D., Smith, W. D. and Reis-Filho, J. S. (2010) Triple-negative breast cancer. *N. Engl. J. Med*. **363**, 1938-1948.

Ghajar, C. M., Peinado, H., Mori, H., Matei, I. R., Evason, K. J., Brazier, H., Almeida, D., Koller, A., Hajjar, K. A., Stainier, D. Y., Chen, E. I., Lyden, D. and Bissell, M. J. (2013) The perivascular niche regulates breast tumour dormancy. *Nat. Cell Biol.* **15**, 807-817.

Goswami, C. P. and Nakshatri, H. (2014) PROGgeneV2: enhancements on the existing database. *BMC Cancer* **14**, 970.

Horiguchi, H., Yamagata, S., Rong Qian, Z., Kagawa, S. and Sakashita, N. (2013) Thrombospondin-1 is highly expressed in desmoplastic components of invasive ductal carcinoma of the breast and associated with lymph node metastasis. *J. Med. Invest.* **60**, 91-96.

Ignatov, A., Lintzel, J., Hermans-Borgmeyer, I., Kreienkamp, H. J., Joost P., Thomsen, S., Methner, A. and Schaller, H. C. (2003) Role of the G-protein-coupled receptor GPR12 as highaffinityreceptor for sphingosylphosphorylcholine and its expression and functionin brain development. *J. Neurosci.* **23**, 907-914.

Im, D. S. (2003) Linking Chinese medicine and G-protein-coupled receptors. *Trends Pharmacol. Sci.* **24**, 2-4.

Jayachandran, A., Anaka, M., Prithviraj, P., Hudson, C., McKeown, S. J., Lo, P. H., Vella, L. J., Goding, C. R., Cebon, J. and Behren, A. (2014) Thrombospondin 1 promotes an aggressive phenotype through epithelial-to-mesenchymal transition in human melanoma. *Oncotarget* **5**, 5782-5797.

Jeon, E. S., Moon, H. J., Lee, M. J., Song, H. Y., Kim, Y. M., Bae, Y. C., Jung, J. S. and Kim, J. H. (2006) Sphingosylphosphorylcholine induces differentiation of human mesenchymal stem cells into smooth-muscle-like cells through a TGF-beta-dependent mechanism. *J. Cell Sci.* **119**, 4994-5005.

Jeong, Y. H., Park, J. S., Kim, D. H. and Kim, H.-S. (2016) Lonchocarpine increases Nrf2/ARE-mediated antioxidant enzyme expression by modulating AMPK and MAPK signaling in brain astrocytes. *Biomol. Ther. (Seoul)* **24**, 581-588.

Jimenez, B., Volpert, O. V., Crawford, S. E., Febbraio, M., Silverstein, R. L. and Bouck, N. (2000) Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nat. Med.* **6**, 41-48.

Kalluri, R. and Weinberg, R. A. (2009) The basics of epithelial-mesenchymal transition. *J. Clin. Invest.* **119**, 1420-1428.

Kim, E. J., Kim, H. J., Park, M. K., Kang, G. J., Byun, H. J., Lee, H. and Lee, C. H. (2015) Cardamonin suppresses TGF-β1-induced epithelial mesenchymal transition via restoring protein phosphatase 2A expression. *Biomol. Ther. (Seoul)* **23**, 141-148.

Kim, H. J., Choi, W. J. and Lee, C. H. (2015) Phosphorylation and reorganization of keratin networks: Implications for carcinogenesis and epithelial mesenchymal transition. *Biomol. Ther. (Seoul)* **23**, 301-312.

Kim, H. J., Kang, G. J., Kim, E. J., Park, M. K., Byun, H. J., Nam, S., Lee, H. and Lee, C. H. (2016) Novel effects of sphingosylphosphorylcholine on invasion of breast cancer: Involvement of matrix metalloproteinase-3 secretion leading to WNT activation. *Biochim. Biophys. Acta* **1862**, 1533-1543.

Kurokawa, T., Yumiya, Y., Fujisawa, H., Shirao, S., Kashiwagi, S., Sato, M., Kishi, H., Miwa, S., Mogami, K., Kato, S., Akimura, T., Soma, M., Ogasawara, K., Ogawa, A., Kobayashi, S. and Suzuki, M. (2009) Elevated concentrations of sphingosylphosphorylcholine in cerebrospinal fluid after subarachnoid hemorrhage: a possible role as a spasmogen. *J. Clin. Neurosci.* **16**, 1064-1068.

Lawler, J. W., Slayter, H. S. and Coligan, J. E. (1978) Isolation and characterization of a high molecular weight glycoprotein from human blood platelets. *J. Biol. Chem.* **253**, 8609-8616.

Lee, H. Y., Lee, S. Y., Kim, S. D., Shim, J. W., Kim, H. J., Jung, Y. S., Kwon, J. Y., Baek, S. H., Chung, J. and Bae, Y. S. (2011) Sphingosylphosphorylcholine stimulates CCL2 production from human umbilical vein endothelial cells. *J. Immunol.* **186**, 4347-4353.

Lim, S. C., Lee, K. M. and Kang, T. J. (2015) Chitin from cuttlebone activates inflammatory cells to enhance the cell migration. *Biomol. Ther. (Seoul)* **23**, 333-338.

Meyer zu Heringdorf, D., Himmel, H. M. and Jakobs, K. H. (2002) Sphingosylphosphorylcholine-biological functions and mechanisms of action. *Biochim. Biophys. Acta* **1582**, 178-189.

Murphy-Ullrich, J. E. and Poczatek, M. (2000) Activation of latent TGF-beta by thrombospondin-1: mechanisms and physiology. *Cytokine Growth Factor Rev.* **11**, 59-69.

Nieto, M. A., Huang, R. Y., Jackson, R. A. and Thiery, J. P. (2016) EMT: 2016. *Cell* **166**, 21-45.

Nixon, G. F., Mathieson, F. A. and Hunter, I. (2008) The multi-functional role of sphingosylphosphorylcholine. *Prog. Lipid Res.* **47**, 62-75.

Park, K. S., Kim, H. K, Lee, J. H., Choi, Y. B., Park, S. Y., Yang, S. H., Kim, S. Y. and Hong, K. M. (2010) Transglutaminase 2 as a cisplatin resistance marker in non-small cell lung cancer. *J. Cancer Res. Clin. Oncol.* **136**, 493-502.

Park, M. K., You, H. J., Lee, H. J., Kang, J. H., Oh, S. H., Kim, S. Y. and Lee, C. H. (2013) Transglutaminase-2 induces N-cadherin expression in TGF-beta1-induced epithelial mesenchymal transition via c-Jun-N-terminal kinase activation by protein phosphatase 2A down-regulation. *Eur. J. Cancer* **49**, 1692-1705.

Park, M. K., Park, S., Kim, H. J., Kim, E. J., Kim, S. Y., Kang, G. J., Byun, H. J., Kim, S. H., Lee, H. and Lee, C. H. (2016) Novel effects of FTY720 on perinuclear reorganization of keratinnetwork induced by sphingosylphosphorylcholine: Involvement of protein phosphatase 2A and G-protein-coupled receptor-12. *Eur. J. Pharmacol.* **775**, 86-95.

Retraction (2005) Sphingosylphosphorylcholine and lysophosphatidylcholine are ligands for the G protein-coupled receptor GPR4. *J. Biol. Chem.* **280**, 43280.

Retraction (2006) Sphingosylphosphorylcholine is a ligand for ovarian cancer G-protein-coupled receptor 1. *Nat. Cell Biol.* **8**, 299.

Schlyer, S. and Horuk, R. (2006) I want a new drug: G-protein-coupled receptors in drug development. *Drug Discov. Today* **11**, 481-493.

Schultz-Cherry, S., Lawler, J. and Murphy-Ullrich, J. E. (1994) The type 1 repeats of thrombospondin 1 activate latent transforming growth factor-β. *J. Biol. Chem.* **269**, 26783-26788.

Seufferlein, T. and Rozengurt, E. (1995) Sphingosylphosphorylcholine rapidly induces tyrosine phosphorylation of p125FAK and paxillin, rearrangement of the actin cytoskeleton and focal contact assembly. Requirement of p21rho in the signaling pathway. *J. Biol. Chem.* **270**, 24343-24351.

Shin, S. and Blenis, J. (2010) ERK2/Fra1/ZEB pathway induces epithelial-to-mesenchymal transition. *Cell Cycle* **9**, 2483-2484.

Shin, S., Dimitri, C. A., Yoon, S. O., Dowdle, W. and Blenis, J. (2010) ERK2 but not ERK1 induces epithelial-to-mesenchymal transformation via DEF motif-dependent signaling events. *Mol. Cell* **38**, 114-127.

Sid, B., Langlois, B., Sartelet, H., Bellon, G., Dedieu, S. and Martiny, L. (2008) Thrombospondin-1 enhances human thyroid carcinoma cell invasion through urokinase activity. *Int. J. Biochem. Cell Biol.* **40**, 1890-1900.

Sørensen, K. P., Thomassen, M., Tan, Q., Bak, M., Cold, S., Burton, M., Larsen, M. J. and Kruse, T. A. (2013) Long non-coding RNA HOTAIR is an independent prognostic marker of metastasis in estrogen receptor-positive primary breast cancer. *Breast Cancer Res. Treat.* **142**, 529-536.

Tiwari, N., Gheldof, A., Tatari, M. and Christofori, G. (2012) EMT as the ultimate survival mechanism of cancer cells. *Semin. Cancer Biol.* **22**, 194-207.

Valastyan, S. and Weinberg, R. A. (2011) Tumor metastasis: molecular insights and evolving paradigms. *Cell* **147**, 275-292.

Wang, X., Zhu, Y., Ma, Y., Wang, J., Zhang, F., Xia, Q. and Fu, D. (2013) The role of cancer stem cells in cancer metastasis: new perspective and progress. *Cancer Epidemiol.* **37**, 60-63.

Wang, Y., Klijn, J. G., Zhang, Y., Sieuwerts, A. M, Look, M. P., Yang, F., Talantov, D., Timmermans, M., Meijer-van Gelder, M. E., Yu, J., Jatkoe, T., Berns, E. M., Atkins, D. and Foekens, J. A. (2005) Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet* **365**, 671-679.

Xiao, Y. J., Schwartz, B., Washington, M., Kennedy, A., Webster, K., Belinson, J. and Xu, Y. (2001) Electrospray ionization mass spectrometry analysis of lysophospholipids in human ascitic fluids: comparison of the lysophospholipid contents in malignant vs nonmalignant ascitic fluids. *Anal. Biochem.* **290**, 302-313.

Xu, Y. (2002) Sphingosylphosphorylcholine and lysophosphatidylcholine: G protein-coupled receptors and receptor-mediated signal transduction. *Biochim. Biophys. Acta* **1582**, 81-88.

Xu, Y., Zhu, K., Hong, G., Wu, W., Baudhuin, L. M., Xiao, Y. and Damron, D. S., (2000) Sphingosylphosphorylcholine is a ligand for ovarian cancer G-protein-coupled receptor 1. *Nat. Cell Biol.* **2**, 261-267.

Yang, C. R., Wei, Y., Qi, S. T., Chen L., Zhang, Q. H., Ma J. Y., Luo Y. B., Wang Y. P., Hou, Y., Schatten, H., Liu, Z. H. and Sun, Q. Y. (2012) The G protein coupled receptor 3 is involved in cAMP and cGMP signaling and maintenance of meiotic arrest in porcine oocytes, *PLoS ONE* **7**, e38807.

Yee, K. O., Connolly, C. M., Duquette, M., Kazerounian, S., Washington, R. and Lawler, J. (2009) The effect of thrombospondin-1 on breast cancer metastasis. *Breast Cancer Res. Treat.* **114**, 85-96.

Zheng, M., Zhang, X., Min, C., Choi, B. G., Oh, I. J. and Kim, K. M. (2016) Functional regulation of dopamine D_3 receptor through interaction with PICK1. *Biomol. Ther. (Seoul)* **24**, 475-481.

Zhu, K., Baudhuin, L. M., Hong, G., Williams, F. S., Cristina, K. L., Kabarowski, J. H., Witte, O. N. and Xu, Y. (2001) Sphingosylphosphorylcholine and lysophosphatidylcholine are ligands for the G protein-coupled receptor GPR4. *J. Biol. Chem.* **276**, 41325-41335.