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Sphingosine-1 phosphate promotes intestinal epithelial cell proliferation via S1PR2

Tanzhou Chen[1], Zhiming Huang[1], Runping Liu[2], Jing Yang[2], Phillip B. Hylemon[2,3], Huiping Zhou[1,2,3]

[1]Department of Gastroenterology and Hepatology, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325000, China, [2]Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, VA 23298, USA, [3]McGuire VA Medical Center, Richmond, VA 23235, USA

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1. ABSTRACT

Sphingosine-1 phosphate (S1P) is a potent bioactive lipid mediator that acts both as an intracellular signaling molecule and a natural ligand of five different G protein-coupled receptors (GPCRs), S1PR1-5. The level of S1P in intestinal tissue is abundant. Previous studies have reported that S1P protects intestinal epithelial cell from apoptosis by activating the ERK and Akt signaling pathways. However, the effect of S1P on intestinal epithelial cell proliferation under physiological conditions and the underlying signaling mechanisms remain to be elucidated. Here, we show that, except for S1PR4, all S1PRs are expressed in normal intestinal epithelial cells with S1PR2 being the most abundant. S1P dose-dependently stimulated cell migration and proliferation, which were inhibited by JTE-013, a selective chemical antagonist of S1PR2, and by a S1PR2 shRNA. S1P significantly upregulated the expression of c-Myc, cyclin D1, E-cadherin and zona occluden-1 (ZO-1), which was completely inhibited by downregulation of S1PR2 expression with a shRNA. In total, the results suggest that S1P-mediated activation of the S1PR2 plays an important role in regulating intestinal epithelial cell proliferation and migration.

2. INTRODUCTION

The gastrointestinal tract is the largest epithelial barrier that protects the mammalian hosts from the external environment (1). Intestinal epithelial cell proliferation and migration effectively sustains a physical and biochemical barrier between the host and its environment (2). Maintenance of an intestinal mucosal barrier is critical for gastrointestinal (GI) functions, including digestion of food, absorption of nutrients, expulsion of waste, and mechanical exclusion of bacteria. The proliferation and migration of intestinal epithelial cells are tightly regulated by various signaling molecules and contribute critically to the maintenance of the mucosal barrier (3-5). However, the exact cellular/molecular mechanisms involved in regulating intestinal barrier function have not been completely elucidated.

Sphingosine-1 phosphate (S1P) is a bioactive sphingolipid involved in regulating numerous cellular functions under both physiological and pathological conditions (6). S1P is synthesized from sphingosine by sphingosine kinase (SphK). Two distinct isoforms of SphK have been identified, SphK1 and SphK2, which are formed by alternative splicing (7). Sphk2 has a nuclear

localization sequence while SphK1 is mainly located in the cytosol. It also has been reported that Sphk2 is responsible for producing S1P in the mitochondria (8). S1P can directly function as an intracellular signaling molecule or exert its effects extracellularly through activating five different G protein-coupled receptors, S1P receptors (S1PRs) (6,9). During the last two decades, a number of studies have been done to examine the role of S1P and S1PRs in health and disease. It has been reported that S1P is a key regulator of cell proliferation, migration and survival in various types of cells (9). Five S1PRs are also found differentially expressed in different tissues or organs (10). In the human intestine, all five S1PRs are detectable, but the expression level of individual S1PRs varies. A previous study reported that S1P regulates the expression of adherens junction protein E-Cadherin and enhances intestinal epithelial cell barrier function *via* activation of S1PR1 (11). It also has shown that S1P protects intestinal epithelial cells from apoptosis *via* an Akt-dependent pathway (12). However, in these studies the authors only examined the expression of S1PR1 and S1PR3, not S1PR2 in intestinal epithelial cells. The Human Protein Atlas data indicate that all S1PRs are expressed in intestinal tissue and expression of S1PR2 is extremely high in the GI tract (http://www.proteinatlas.org). During the last decade, extensive studies have been done to identify the signaling pathways involved in regulating intestinal epithelial cell proliferation and apoptosis. Activation of ERK1/2 significantly inhibited TNF-α-induced cell apoptosis in intestinal epithelial cells (13,14). Our recent studies have shown that activation of S1PR2 by S1P not only activates the ERK1/2 and Akt pathways, but also promotes cell proliferation in cholangiocytes (15). However, the role of S1PR2 in S1P-mediated regulation of cell proliferation and migration in normal intestinal epithelial cells has not been explored.

In the current study, we examined the expression of S1PRs and the role of S1PR2 in S1P-mediated cell proliferation and migration in intestinal epithelial cells, and further identified the underlying signaling mechanisms.

3. MATERIALS AND METHODS

3.1. Reagents

Cell culture DMEM medium, trypsin-EDTA, antibiotics, and insulin were purchased from Invitrogen (Carlsbad, CA). Heat-inactivated fetal bovine serum (FBS) was purchased from ATLANTA biological, Inc (Flowery Branch, GA) and dialyzed FBS was purchased from GEMINI Bio-products (West Sacramento, CA). Protease inhibitors and phosphatase inhibitors were from Thermos Fisher Scientific Inc (Rockford, IL). S1P was purchased from Enzo Life Sciences, Inc (Farmingdale, NY). JTE-013 was purchased from Cayman Chemical (Ann Arbor, Michigan). Antibody recognizing S1PR1 was obtained from Abcam (Cambridge, MA). Anti-p-AKT antibody was from Cell Signaling Technology (Danvers, MA). All other antibodies (p-ERK, total-AKT, total-ERK, S1PR2, and S1PR3) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Donkey anti-mouse IgG IRDye-800CW, Goat anti-mouse IgM IRDye 800CW, and Goat anti-rabbit IgG IREDye 800CW were from LI-COR Biotechnology (Lincoln, NE). Antibody recognizing β-actin, IPTG and all other chemicals were from Sigma (St. Louis, MO).

3.2. Cell culture and treatment

The IEC-6 cells were derived from normal rat intestinal crypt cells and were obtained from the American Type Culture Collection (Manassas, VA) (16). The small intestinal epithelium contains a large population of undifferentiated cells in the crypt region. The proliferation, migration and differentiation of these cells are tightly regulated and play critical roles in maintaining normal epithelial barrier function. IEC-6 cells have been well-characterized and widely used to study the regulation and function of intestinal epithelial cells. Cells were cultured in DMEM with 10% heat-inactivated FBS with 1% antibiotic and 1% insulin and were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. S1P and JTE-013 were directly added into the cell culture medium and incubated for indicated time periods.

3.3. Cell migration scratch assay

The IEC-6 cells were plated onto 12-well plates at a density of 5 x 10^5 cells per well overnight to form a confluent monolayer. The “Scratch” was created by scraping the cell monolayer in a straight line with a p200 pipet tip. Cells were washed gently with culture medium to remove the debris and were cultured in 1 ml of culture medium. The first image of the scratch was captured under an Olympus 1x71 phase-contrast microscope using a 10 x objective at a marked reference point. The cells were treated with different concentrations of S1P (0.01, 0.1, and 1 μM) or vehicle control (DMSO) with or without JTE-013 (10 μM) for 24 or 48 h. At the end of treatment, the plates were placed under the microscope and the second image of the same scratch area was captured after matching the reference point. The images acquired for each treatment group were further analyzed using IPLab 4.0 (15).

3.4. Cell proliferation assay

The IEC-6 cells were plated onto 48-well plates and cultured in serum-free medium for overnight. After treatment with different concentrations of S1P (0.01, 01, and 1 μM) with or without pretreatment with JTE-013 for different time periods, the number of cells was counted using the Cellometer Vision CBA Analysis System (Nexcelom Bioscience, Lawrence, MA).

3.5. Western blot analysis

Total cell lysates were prepared using ice-cold modified radioimmunoprecipitation assay buffer (RIPA)

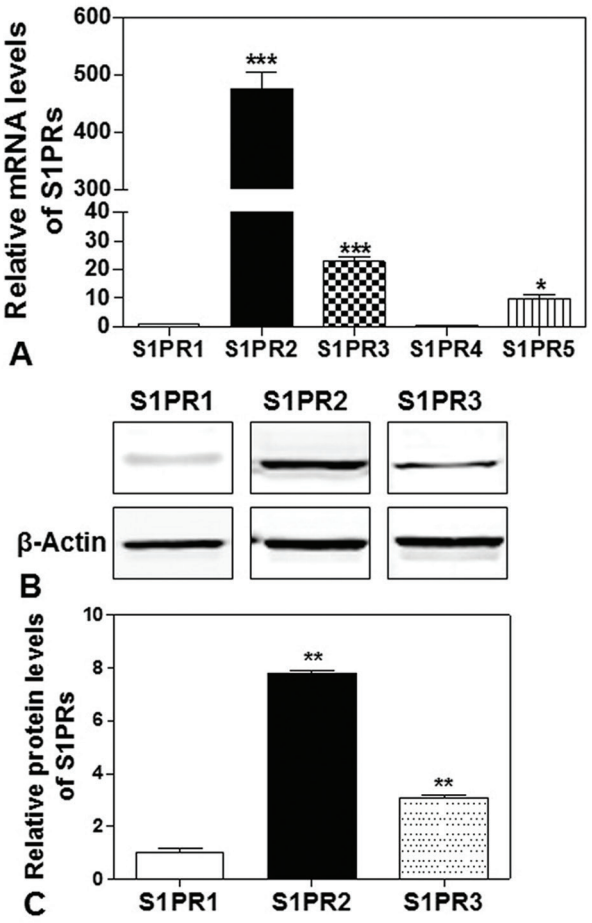

Figure 1. Differential expression of S1PRs in IEC-6 cells. (A) Total cellular RNA was isolated from IEC-6 cells. mRNA levels of individual S1PRs were determined by real-time RT-PCR, as described in materials and methods, and normalized using GAPDH as an internal control. Relative mRNA levels of individual S1PRs to S1PR1 (designated=1) are shown. Values represent the mean ± S.E. of three independent experiments. Statistical significance relative to the S1PR1 (designated=1): *P<0.05, ***P<0.001 (B) Total cell lysate of IEC-6 cells was prepared as described in Materials and Methods. Protein levels of S1PR1, S1PR2 and S1PR3 were determined by western blotting analysis using specific antibodies. β-actin was used as a loading control. Representative images are shown. (C). Relative densities of individual S1PRs were analyzed using Odyssey image software using β-actin as a loading control. Values represent the mean ± S.E. of three independent experiments. Statistical significance relative to the S1PR1 (designated=1): **P<0.01.

containing 50 mM Tris, 150 mM NaCl, 1.0.% Nonidet P-40, 0.5% sodium deoxycholate, 0.1.% SDS, protease inhibitors and phosphatase inhibitors, pH 8.0. and centrifuged at 4°C for 10 min. The protein concentration of the supernatant was measured and then boiled in SDS-PAGE loading buffer for 10 min. The fifty µg of protein were resolved on 10% Bis-Tris NuPage gels and transferred onto Nitrocellulose membranes. After blocking with 5% non-fat milk in TBS buffer, the blots were incubated with primary antibodies overnight at 4°C. The immunoreactive bands of the target proteins were detected using IRDye secondary antibodies using an Odyssey Fluorescence Imaging System (LI-COR Biosciences, USA). The densities of immunoblot bands were analyzed using Image Studio software (LI-COR)(15).

3.6. RNA isolation and quantitative real-time RT-PCR

Total cellular RNA was isolated using QIAzol Lysis Reagent after different treatments. Two µg of total RNA were reverse transcribed into the first-strand cDNA using a High Capacity cDNA Transcription Kit (Life Technologies, Carlsbad, CA). The mRNA levels of specific target genes were determined by real-time PCR using iQ^{TM} SYBR Green Supermix PCR reagents (BIO-RAD) and normalized using β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control (15).

3.7. RNA interference using lentiviral system

The recombinant lentiviruses of shRNA for S1PR2 were produced by transient transfection of HEK293FT cells as described previously (17). IEC-6 cells were transduced with lentiviral shRNA specifically targeting S1PR2 or control lentiviral shRNA for 48h. After removing the virus, cells were treated with S1P or vehicle control for specified time periods. The effect of downregulation of S1PR2 on cell proliferation and migration was measured as described in the previous sections.

3.8. Statistical analysis

All of the experiments were repeated at least three times and the results were expressed as mean ± SD. One-way ANOVA and the Duncan's test were used to analyze the differences between sets of data using GraphPad Prism 5.0. (GraphPad, San Diego, CA). A value of $P < 0.05$ was considered statistically significant.

4. RESULTS

4.1. S1PR2 is the predominant S1PR in the IEC-6 cells

S1P is a high-affinity natural ligand of five GPCRs, S1PR1-5, which are differentially expressed in cells in different tissues (10). S1P-mediated activation of GPCRs has been reported to regulate diverse physiological and pathological processes *via* coupling with different G proteins (18). A previous study reported that S1P regulated the expression of adherens junction protein E-Cadherin (E-cad) *via* S1PR1 (EDG1) in intestinal epithelial cells, but not other S1PRs (11). However, the expression of other S1PRs in intestinal epithelial cells has not been fully examined. In the current study, we first examined the mRNA levels of all S1PRs in IEC-6 cells. As shown in Figure 1A, all S1PRs were detectable except S1PR4. The relative expression level of S1PR2 was ~20-fold higher than any other S1PRs. We further confirmed the expression of S1PR1,

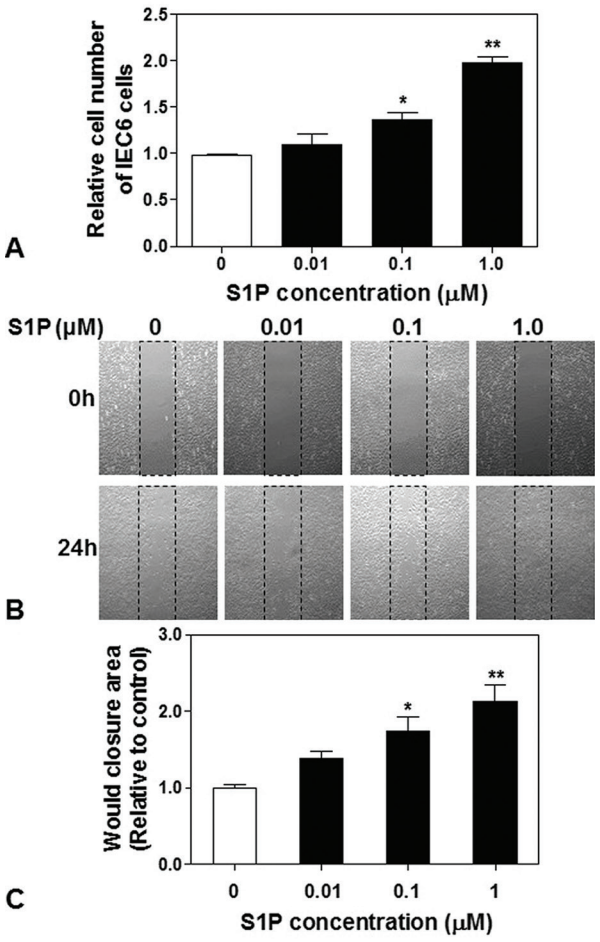

Figure 2. Effect of S1P on cell proliferation and migration in IEC-6 cells. (A) IEC-6 cells were cultured in 1% dialyzed FBS overnight and then treated with different concentrations of S1P (0, 0.01, 0.1 and 1 μM) for 48 h. At the end of the treatment period, cells were harvested and analyzed using a Cellometer Vision CBA automatic cell counter. Relative cell number, compared to vehicle control group, is shown. Values represent the mean ± S.E. of three independent experiments. Statistical significance relative to the vehicle control (designated=1): *P<0.05, **P<0.01. (B) IEC-6 cells were plated on 12-well plates until confluent. Cells were scratched to simulate a wound and images were recorded as 0 h. Cells were treated with different concentrations of S1P (0, 0.01, 0.1 and 1 mM). After 24 h, images of wound areas were recorded. Representative images are shown. (C) The area of wound was quantified using IPLab4.0. Relative wound closure was calculated. *P<0.05, **P<0.0.1, compared to control group, n=3.

S1PR2, S1PR3 and S1PR5 by DNA sequencing (Data not shown). Western blot analysis also showed that the protein level of S1PR2 was 3-8 fold higher than those of S1PR1 and S1PR3 (Figure 1B-C).

4.2. Effect of S1P on cell proliferation and migration in IEC-6 cells

S1P has been identified as a pleiotropic bioactive lipid mediator, which plays an important role in regulating numerous cellular processes including cell proliferation, survival, and migration (19). Previous studies reported that S1P enhances intestinal epithelial cell barrier function *via* regulating the expression of adherens junction protein E-cad and inhibits tumor necrosis factor-α (TNF-α)/cycloheximide (CHX)-induced apoptosis *via* activating the Akt signaling pathway (11-12). In this study, we examined whether S1P has any effect on cell proliferation and migration under normal physiological conditions. As shown in Figure 2, S1P not only dose-dependently increased cell proliferation, but also promoted cell migration of IEC-6 cells.

4.3. Role of S1PR2 in S1P-induced cell proliferation and migration in IEC-6 cells

S1P can function as a signaling molecule to directly activate multiple intracellular signaling pathways or activate GPCRs on the cell membrane to activate various signaling pathways (6,18). Although previous studies reported that S1PR1 is the major receptor involved in S1P-mediated protection of normal intestinal epithelial cells from apoptosis (12), the expression and role of other S1PRs in S1P-mediated cell proliferation and migration in IECs have not been examined. Our data indicate that S1PR2 is the predominant S1PR expressed in IEC-6 cells. In order to determine the role of S1PR2 in S1P-induced cell proliferation and migration, a specific chemical antagonist of S1PR2, JTE-013, was used. As shown in Figure 3A, S1P-mediated increase of cell proliferation was significantly inhibited by JTE-013. Similarly, S1P-induced cell migration was also blocked by JTE-013 (Figure 3B). To further delineate the specific role of S1PR2 in S1P-mediated cell proliferation and migration, a gene specific shRNA targeting S1PR2 was used as described previously (20). As shown in Figure 4, S1P-mediated cell proliferation and migration were significantly reduced in IEC-6 cells transduced with S1PR2 shRNA.

4.4. S1P can activate the ERK1/2 signaling pathway *via* S1PR2 in IEC-6 cells

Activation of ERK1/2 and Akt signaling pathways is linked to the regulation of gene expression, growth, and differentiation in many cell types including intestinal epithelial cells (21-23). In IEC-6 cells, S1P (1 μM) rapidly and significantly induced activation of the ERK1/2 signaling pathway, as shown in Figure 5A. S1P had less effect on Akt activation in IEC-6 cells (Figure 5B). To further examine the role of S1PR2 in S1P-induced ERK1/2 activation, JTE-013 was used as an antagonist. As shown in Figure 6, S1P-induced ERK1/2 activation was completely inhibited by JTE-013. Similarly, down-regulation of S1PR2 expression using shRNA also blocked S1P-induced ERK1/2 activation (data not shown). We further examined the effect of down-regulation of S1PR2 expression on S1P-induced gene expression related to cell proliferation and epithelial cell barrier function. As shown in Figures 7 and 8, knocking down S1PR2 expression with shRNA completely inhibited S1P-induced expression of c-myc, cyclin D1, E-Cad and ZO-1 in IEC-6 cells.

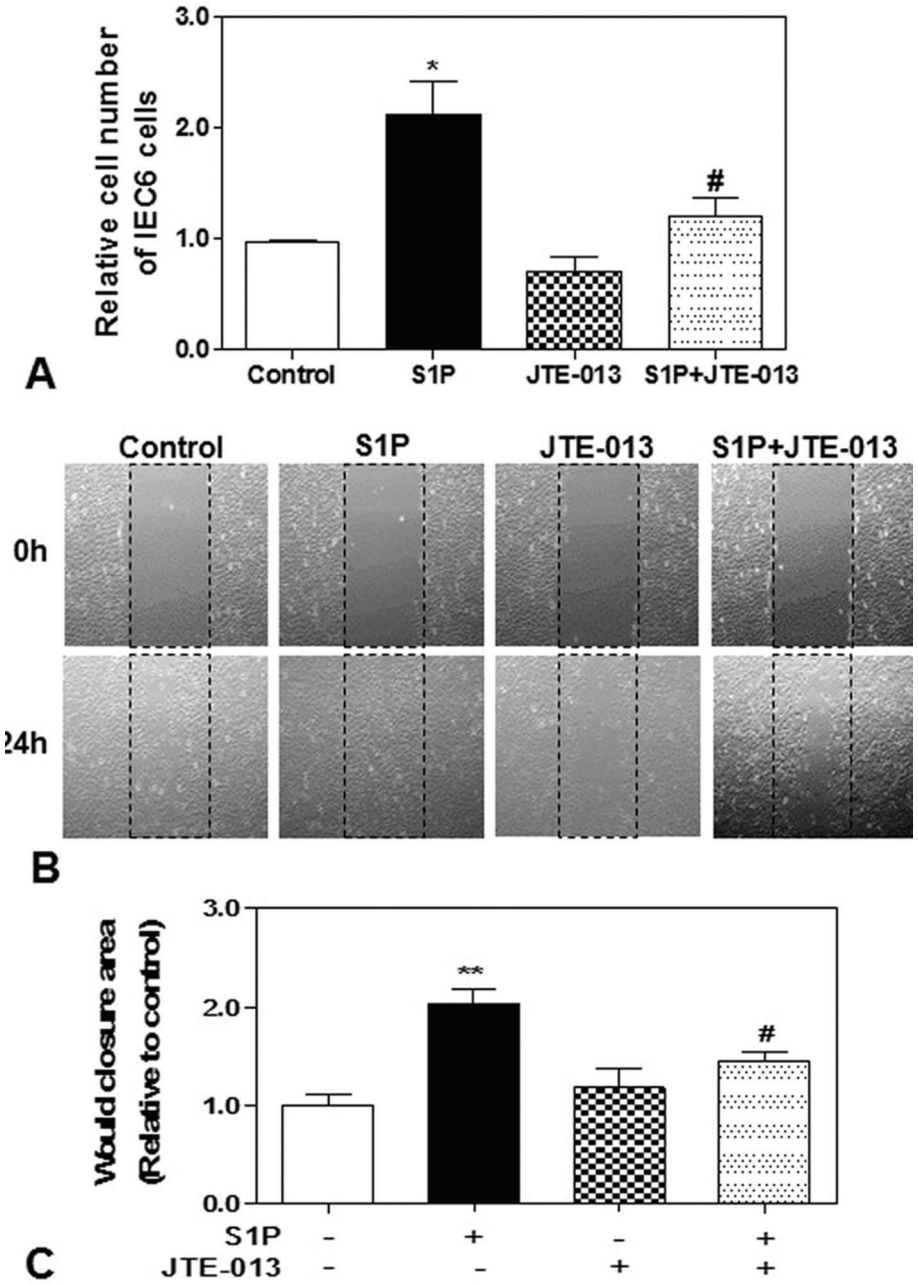

Figure 3. Effect of JTE-013 on S1P-induced cell proliferation and migration in IEC-6 cells. (A) IEC-6 cells were cultured in 1% dialyzed FBS overnight. After pretreatment with JTE-013 (10 μM) for 0.5 h, cells were treated with vehicle or S1P (1 μM) for 48 h. At the end of the treatment period, cells were harvested and analyzed using a Cellometer Vision CBA automatic cell counter. Relative cell number, compared to vehicle control group, is shown. Values represent the mean ± S.E. of three independent experiments. Statistical significance relative to the vehicle control (designated=1): *P<0.05, statistical significance relative to the S1P group, #P<0.05 (B) IEC-6 cells were plated on 12-well plates until confluent. Cells were scratched to simulate a wound and images were recorded as 0 h. Cells were pre-treated with JTE-013 (10 μM) for 0.5 h, then treated with S1P (1 μM). After 24 h, images of wound areas were recorded. Representative images are shown. (C) The area of wound was quantified using IPLab4.0. Relative wound closure was calculated. **P<0.01, compared to control group. #P<0.05, compared to S1P group, n=3.

5. DISCUSSION

The mammalian intestinal epithelium is a single cell layer comprising proliferative crypts and differentiated villi, which separates the intestinal lumen from the underlying sterile tissue (24,25). Undifferentiated intestinal epithelial cells continuously replicate in the proliferative zone within the crypts and differentiate as they migrate up the luminal surface of the intestine to replace cells lost under physiological conditions.

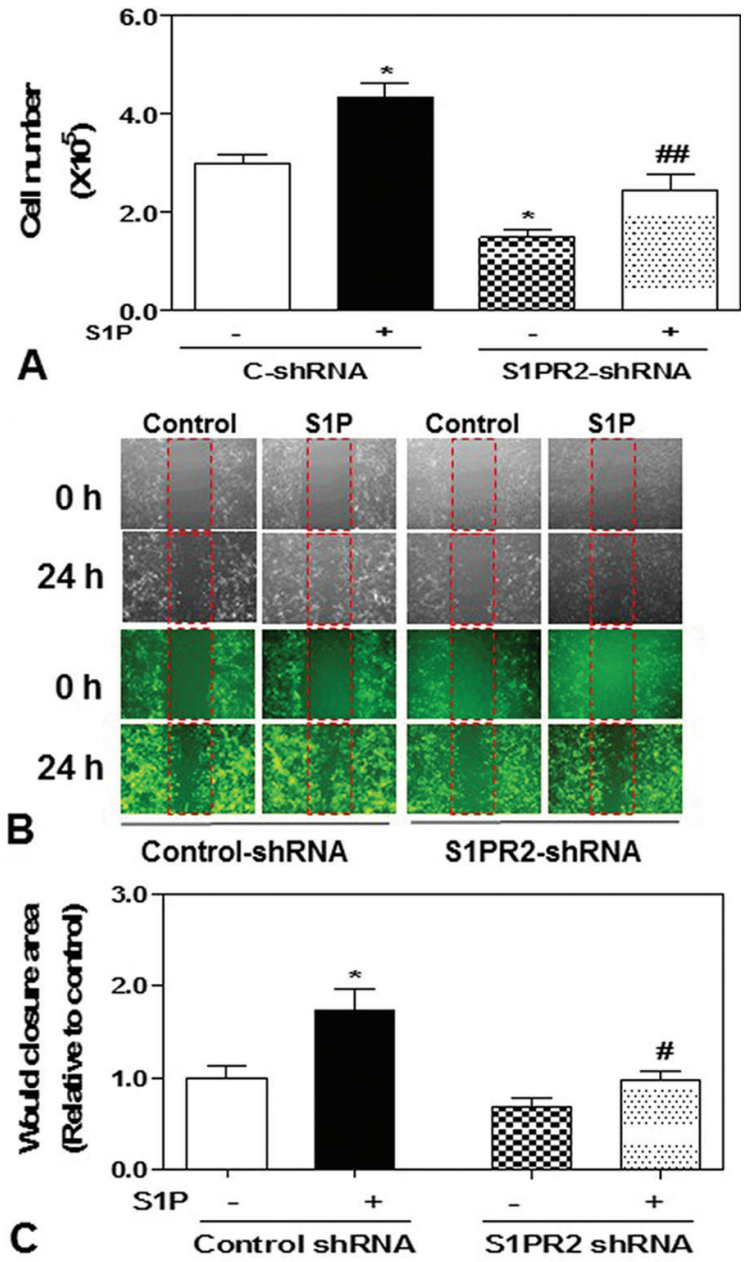

Figure 4. Effect of S1PR2 shRNA on S1P-induced cell proliferation and migration in IEC-6 cells. (A). IEC-6 cells were transfected with control or S1PR2 shRNA for 48 h. Cells were treated with S1P (1μM) for 48 h. At the end of the treatment period, cells were harvested and analyzed using a Cellometer Vision CBA automatic cell counter. Relative cell number, compared to vehicle control group, is shown. Values represent the mean ± S.E. of three independent experiments. Statistical significance relative to the vehicle control (designated=1): *P<0.05, statistical significance relative to the S1P group transfected with control shRNA, ##P<0.01 (B) IEC-6 cells were transfected with control or S1PR2 shRNA for 48 h, then plated on 12-well plates until confluent. Cells were scratched to simulate a wound and images were recorded as 0 h. Cells were treated with vehicle or S1P (1 mM). After 24 h, images of wound areas were recorded. Representative images are shown. (C) The area of the wound was quantified using IPLab4.0. Relative wound closure was calculated. *P<0.05, compared to control group. #*P*<0.05, compared to control shRNA group, n=3.

Intestinal epithelial cell proliferation and migration effectively sustain a physical and biochemical barrier between host and its environment. Disruption of intestinal epithelial barrier integrity is linked to various pathological conditions (26-30).

S1P is a highly active lipid signaling molecule with a broad spectrum of biological activities, including regulation of cytoskeletal rearrangements, angiogenesis and vascular maturation, cell proliferation and survival, calcium homeostasis, immune cell trafficking, as well as

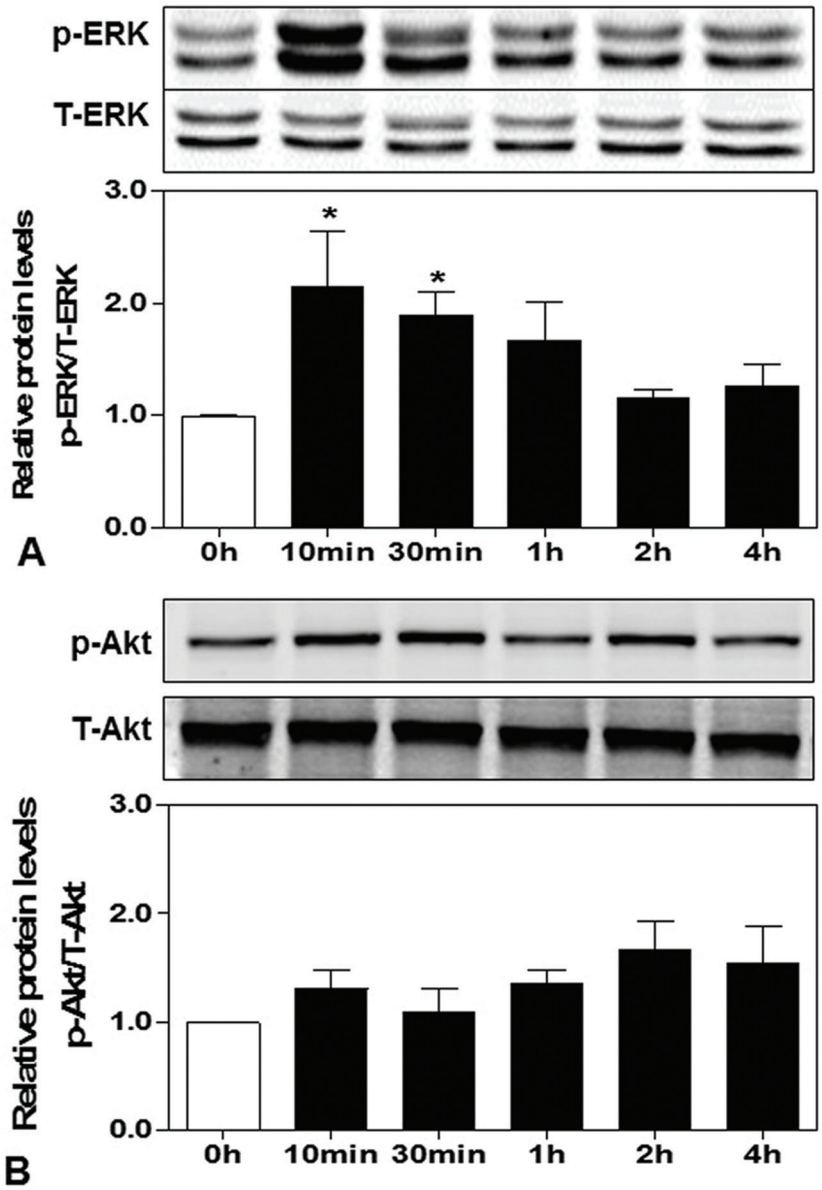

Figure 5. Effect of S1P on ERK1/2 and Akt activation in IEC-6 cells. IEC-6 cells were cultured in 1% dialyzed FBS overnight and then treated with S1P (1 mM) for 10 min, 30 min, 1h, 2h, or 4h. At the end of treatment, cells were harvested and total protein was isolated. The protein levels of phosphorylated ERK1/2 (p-ERK), total ERK1/2 (T-ERK), phosphorylated Akt1/2/3 (p-Akt), and total Akt1/2/3 (T-Akt) were detected by Western blot analysis. Representative images of the immunoblots for p-ERK, T-ERK, p-Akt, and T-Akt are shown. Relative levels of p-ERK1/2/t-ERK and p-Akt/T-Akt were calculated based on the densities of immunoblots using Odyssey image software. (A). p-ERK and T-ERK, Values represent the mean ± S.E. of three independent experiments. Statistical significance relative to the vehicle control (designated=1): *P<0.05 (B). p-Akt and T-Akt.

endothelial and epithelial barrier function (18,19,31-34). In addition to directly functioning as an intracellular signaling molecule, S1P exerts its physiological and pathological effects *via* activating five different GPCRs (S1PR1-5) on the cell membrane (10,31,33,35,36). Tissue distribution analysis of S1P indicates that its content in intestine is very high (37). Although there are a couple of studies reported that S1P protects intestinal epithelial cells from apoptosis and regulates the expression of adherens junction protein, E-cad, and enhances intestinal epithelial cell barrier function (11-12), the role of S1P-mediated activation of S1PRs in regulating intestinal epithelial cell proliferation and migration under physiological conditions has not been explored.

In the current study, we identified that S1PR2 is the predominant S1PR expressed in intestinal

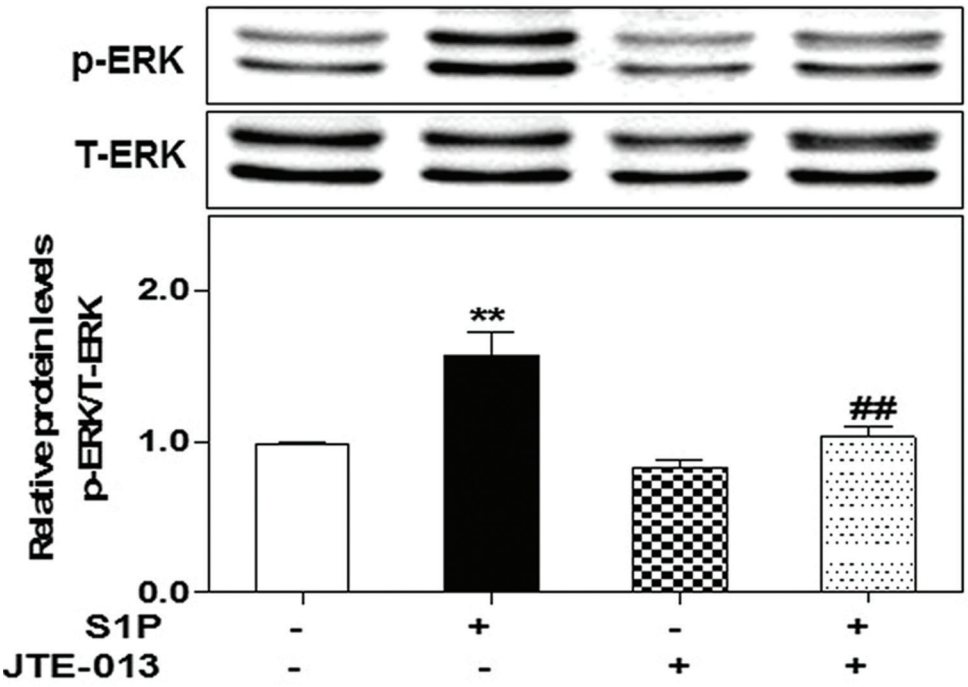

Figure 6. Effect of JTE-013 on S1P-induced ERK activation. IEC-6 cells were cultured in 1% dialyzed FBS overnight. Cells were pretreated with JTE-013 (10 μM) for 30 min, then treated with S1P (1 mM) for 30 min. At the end of treatment, cells were harvested and total protein was isolated. The protein levels of phosphorylated ERK1/2 (p-ERK) and total ERK1/2 were detected by Western blot analysis. The representative images of the immunoblots for p-ERK and T-ERK are shown. Relative levels of p-ERK1/2/t-ERK were calculated based on the densities of immunoblots using Odyssey image software. Values represent the mean ± S.E. of three independent experiments. Statistical significance relative to the vehicle control (designated=1): **P<0.01 statistical significance relative to the S1P group, ##P<0.01.

epithelial cells (Figure 1), suggesting that S1PR2 is the key S1PR involved in S1P-mediated regulation of intestinal epithelial barrier function. A previous study by Green J, *et al.* reported that only S1PR1 was expressed in IEC-6 cells, not S1PR3. But the authors did not mention whether they measured the expression of other S1PRs (11). Nevertheless, we were able to detect all S1PRs except S1PR4 by real-time RT-PCR and confirmed this by DNA sequencing. Due to the limitation of available specific antibodies for S1PR4 and S1PR5, we only examined the protein levels of S1PR1, S1PR2 and S1PR3. In contrast to the previous report, we detected S1PR1, S1PR2, and S1PR3 both at the mRNA and protein levels. Our results indicated that the expression level of S1PR2 is much higher than that of S1PR1 or S1PR3. By using a specific chemical antagonist of S1PR2 or gene-specific shRNA of S1PR2, we further demonstrated that S1P-mediated cell proliferation and migration is mainly mediated by S1PR2 (Figures 2-4). Furthermore, S1P-mediated activation of S1PR2 induced activation of the MEK/ERK1/2 signaling pathway. It has been reported that MEK/ERK activity controls JNK activation *via* regulating MAPK phosphatase (MKP-1), which prevents cell apoptosis (13). We also found that S1P-mediated cell proliferation was significantly inhibited by U0126, a chemical inhibitor of MAPK (Data not shown). A recent study also reported that S1P-induced upregulation of Downregulated-in-adenoma (DRA), the major Cl-/HCO3-exchanger involved in electroneutral NaCl absorption in the mammalian intestine, is mediated by S1PR2 (38). Consistent with our previous finding, S1P-mediated activation of ERK1/2 *via* S1PR2 represents an important signaling pathway involved in regulating intestinal epithelial cell proliferation and migration. In addition, it has also been reported that S1PR2 is positively coupled to renal mesangial cell proliferation (39). Several recent studies further demonstrated that activation of S1PR2 attenuates reactive oxygen species formation, inhibits cell death and negatively regulates macrophage activation and ameliorates atherosclerosis (40-43). Our recent studies in cholangiocarcinoma cells indicated that S1PR2 is critical to conjugated bile acid-mediated cell proliferation and invasion (15, 44). Intestinal epithelial cells are constantly exposed to high concentrations of conjugated bile acids. Our preliminary study also showed that conjugated bile acids significantly promote cell proliferation in IEC-6 cells (data not shown). Whether S1PR2 is responsible for conjugated bile acid-mediated cell proliferation in intestinal epithelia cells remains to be identified and is our current ongoing project.

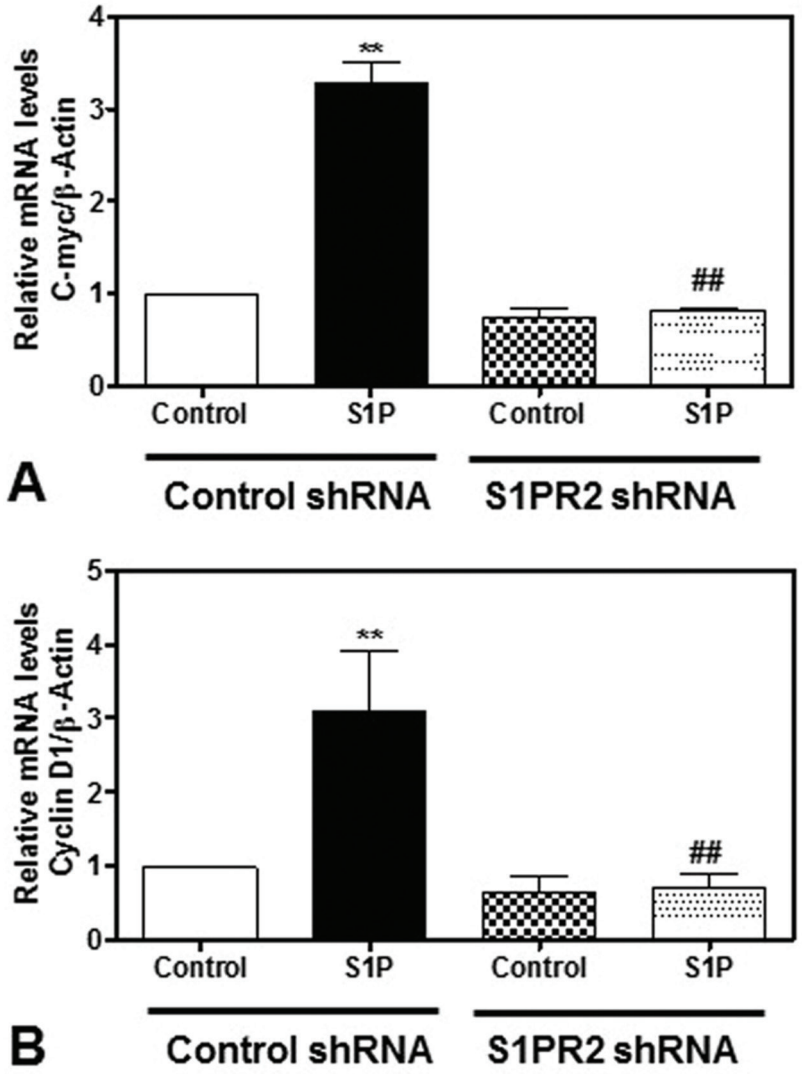

Figure 7. Effect of S1PR2 shRNA on S1P-induced expression of c-myc and cyclin D1 in IEC-6 cells. IEC-6 cells were transfected with control or S1PR2 shRNA for 48 h. Cells were treated with S1P (1 μM) for 24 h. At the end of the treatment period, cells were harvested for isolation of total RNA. The mRNA levels of c-myc and cyclin D1 were determined by real-time RT-PCR and normalized using GAPDH as an internal control. Relative mRNA levels of c-myc and cyclin D1 are shown. Values represent the mean ± S.E. of three independent experiments. Statistical significance relative to the vehicle control (designated=1): **P<0.01 Statistical significance relative to the S1P group, ##P<0.01(A). c-myc, (B) cyclin D1.

In summary, the intestinal barrier function is key for the maintenance of intestinal homeostasis. Intestinal epithelial cells not only effectively sustain a physical and biochemical barrier between host and its environment, but are also actively involved in regulating the communication between host and its environment. Our current study indicates that S1P-mediated activation of S1PR2 plays a critical role in maintaining intestinal barrier function under physiological conditions. Whether S1P/S1PR-mediated signaling pathways contribute to the pathogenesis of gastrointestinal diseases associated with barrier dysfunction remains to be identified. Understanding these signaling pathways will provide important information for the development of novel therapeutics for gastrointestinal diseases such as intestinal mucosal hypoplasia and short bowel syndrome.

6. ACKNOWLEDGEMENT

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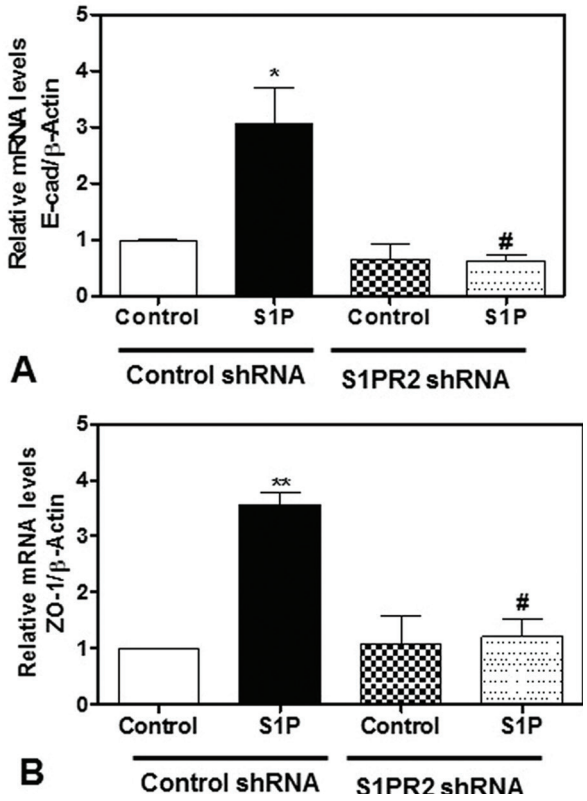

Figure 8. Effect of S1PR2 shRNA on S1P-induced expression of E-cad and ZO-1 in IEC-6 cells. IEC-6 cells were transfected with control or S1PR2 shRNA for 48 h. Cells were treated with S1P (1 μM) for 24 h. At the end of the treatment period, cells were harvested for isolation of total RNA. The mRNA levels of E-cad and ZO-1 were determined by real-time RT-PCR and normalized using GAPDH as an internal control. Relative mRNA levels of E-cad and ZO-1 are shown. Values represent the mean ± S.E. of three independent experiments. Statistical significance relative to the vehicle control (designated=1): **P<0.01 statistical significance relative to the S1P group, ##P<0.01 (A). c-myc, (B) cyclin D1.

7. REFERENCES

1. L. W. Peterson and D. Artis: Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat Rev Immunol*, 14(3), 141-53 (2014) DOI: 10.1038/nri3608
2. L. Xiao, J. N. Rao, T. Zou, L. Liu, B. S. Marasa, J. Chen, D. J. Turner, A. Passaniti and J. Y. Wang: Induced JunD in intestinal epithelial cells represses CDK4 transcription through its proximal promoter region following polyamine depletion. *Biochem J*, 403(3), 573-81 (2007) DOI: 10.1042/BJ20061436
3. M. Maceyka, S. G. Payne, S. Milstien and S. Spiegel: Sphingosine kinase, sphingosine-1-phosphate, and apoptosis. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1585(2–3), 193-201 (2002) DOI: 10.1016/S1388-1981(02)00341-4
4. M. Adada, D. Canals, Y. A. Hannun and L. M. Obeid: Sphingosine-1-phosphate receptor 2. *FEBS J*, 280(24), 6354-66 (2013) DOI: 10.1111/febs.12446
5. Y. Takuwa, Y. Okamoto, K. Yoshioka and N. Takuwa: Sphingosine-1-phosphate signaling in physiology and diseases. *Biofactors*, 38(5), 329-37 (2012) DOI: 10.1002/biof.1030
6. G. M. Strub, M. Maceyka, N. C. Hait, S. Milstien and S. Spiegel: Extracellular and intracellular actions of sphingosine-1-phosphate. *Adv Exp Med Biol*, 688, 141-55 (2010) DOI: 10.1007/978-1-4419-6741-1_10
7. W. L. Santos and K. R. Lynch: Drugging sphingosine kinases. *ACS Chem Biol*, 10(1), 225-33 (2015) DOI: 10.1021/cb5008426
8. G. M. Strub, M. Paillard, J. Liang, L. Gomez, J. C. Allegood, N. C. Hait, M. Maceyka, M. M. Price, Q. Chen, D. C. Simpson, T. Kordula, S. Milstien, E. J. Lesnefsky and S. Spiegel: Sphingosine-1-phosphate produced by sphingosine kinase 2 in mitochondria interacts with prohibitin 2 to regulate complex IV assembly and respiration. *FASEB J*, 25(2), 600-12 (2011) DOI: 10.1096/fj.10-167502
9. J. J. Aarthi, M. A. Darendeliler and P. N. Pushparaj: Dissecting the role of the S1P/S1PR axis in health and disease. *J Dent Res*, 90(7), 841-54 (2011) DOI: 10.1177/0022034510389178
10. T. Hla and V. Brinkmann: Sphingosine 1-phosphate (S1P): Physiology and the effects of S1P receptor modulation. *Neurology*, 76(8 Suppl 3), S3-8 (2011) DOI: 10.1212/WNL.0b013e31820d5ec1
11. J. Greenspon, R. Li, L. Xiao, J. N. Rao, R. Sun, E. D. Strauch, T. Shea-Donohue, J. Y. Wang and D. J. Turner: Sphingosine-1-phosphate regulates the expression of adherens junction protein E-cadherin and enhances intestinal epithelial cell barrier function. *Dig Dis Sci*, 56(5), 1342-53 (2011) DOI: 10.1007/s10620-010-1421-0
12. J. Greenspon, R. Li, L. Xiao, J. N. Rao, B. S. Marasa, E. D. Strauch, J. Y. Wang and D. J. Turner: Sphingosine-1-phosphate protects

intestinal epithelial cells from apoptosis through the Akt signaling pathway. *Dig Dis Sci*, 54(3), 499-510 (2009)
DOI: 10.1007/s10620-008-0393-9

13. M. N. Bavaria, S. Jin, R. M. Ray and L. R. Johnson: The mechanism by which MEK/ERK regulates JNK and p38 activity in polyamine depleted IEC-6 cells during apoptosis. *Apoptosis*, 19(3), 467-79 (2014)
DOI: 10.1007/s10495-013-0944-1
14. S. Bhattacharya, R. M. Ray and L. R. Johnson: Prevention of TNF-alpha-induced apoptosis in polyamine-depleted IEC-6 cells is mediated through the activation of ERK1/2. *Am J Physiol Gastrointest Liver Physiol*, 286(3), G479-90 (2004)
DOI: 10.1152/ajpgi.00342.2003
15. R. Liu, R. Zhao, X. Zhou, X. Liang, D. J. Campbell, X. Zhang, L. Zhang, R. Shi, G. Wang, W. M. Pandak, A. E. Sirica, P. B. Hylemon and H. Zhou: Conjugated bile acids promote cholangiocarcinoma cell invasive growth through activation of sphingosine 1-phosphate receptor 2. *Hepatology*, 60(3), 908-18 (2014)
DOI: 10.1002/hep.27085
16. A. Quaroni, J. Wands, R. L. Trelstad and K. J. Isselbacher: Epithelioid cell cultures from rat small intestine. Characterization by morphologic and immunologic criteria. *J Cell Biol*, 80(2), 248-65 (1979)
DOI: 10.1083/jcb.80.2.248
17. W. Hu, J. Huang, S. Mahavadi, F. Li and K. S. Murthy: Lentiviral siRNA silencing of sphingosine-1-phosphate receptors S1P1 and S1P2 in smooth muscle. *Biochem Biophys Res Commun*, 343(4), 1038-44 (2006)
DOI: 10.1016/j.bbrc.2006.03.079
18. M. Maceyka, K. B. Harikumar, S. Milstien and S. Spiegel: Sphingosine-1-phosphate signaling and its role in disease. *Trends Cell Biol*, 22(1), 50-60 (2012)
DOI: 10.1016/j.tcb.2011.09.003
19. M. Nagahashi, N. C. Hait, M. Maceyka, D. Avni, K. Takabe, S. Milstien and S. Spiegel: Sphingosine-1-phosphate in chronic intestinal inflammation and cancer. *Adv Biol Regul*, 54, 112-20 (2014)
DOI: 10.1016/j.jbior.2013.10.001
20. E. Studer, X. Zhou, R. Zhao, Y. Wang, K. Takabe, M. Nagahashi, W. M. Pandak, P. Dent, S. Spiegel, R. Shi, W. Xu, X. Liu, P. Bohdan, L. Zhang, H. Zhou and P. B. Hylemon: Conjugated bile acids activate the sphingosine-1-phosphate receptor 2 in primary rodent hepatocytes. *Hepatology*, 55(1), 267-76 (2012)
DOI: 10.1002/hep.24681
21. K. Chen, W. Xie, B. Luo, W. Xiao, D. H. Teitelbaum, H. Yang, K. Zhang and C. Zhang: Intestinal mucosal barrier is injured by BMP2/4 via activation of NF-kappaB signals after ischemic reperfusion. *Mediators Inflamm*, 2014, 901530 (2014)
DOI: 10.1155/2014/901530
22. H. Ouyang, H. S. Yang, T. Yu, T. D. Shan, J. Y. Li, C. Z. Huang, W. Zhong, Z. S. Xia and Q. K. Chen: MEK/ERK pathway activation by insulin receptor isoform alteration is associated with the abnormal proliferation and differentiation of intestinal epithelial cells in diabetic mice. *Mol Cell Biochem*, 413(1-2), 165-78 (2016)
DOI: 10.1007/s11010-015-2650-5
23. V. Pomerleau, M. Landry, J. Bernier, P. H. Vachon and C. Saucier: Met receptor-induced Grb2 or Shc signals both promote transformation of intestinal epithelial cells, albeit they are required for distinct oncogenic functions. *BMC Cancer*, 14, 240 (2014)
DOI: 10.1186/1471-2407-14-240
24. K. Zhang, M. W. Hornef and A. Dupont: The intestinal epithelium as guardian of gut barrier integrity. *Cell Microbiol*, 17(11), 1561-9 (2015)
DOI: 10.1111/cmi.12501
25. E. N. Elliott and K. H. Kaestner: Epigenetic regulation of the intestinal epithelium. *Cell Mol Life Sci*, 72(21), 4139-56 (2015)
DOI: 10.1007/s00018-015-1997-9
26. E. Salvo Romero, C. Alonso Cotoner, C. Pardo Camacho, M. Casado Bedmar and M. Vicario: The intestinal barrier function and its involvement in digestive disease. *Rev Esp Enferm Dig*, 107(11), 686-96 (2015)
DOI: 10.17235/reed.2015.3846/2015
27. C. Tincati, D. C. Douek and G. Marchetti: Gut barrier structure, mucosal immunity and intestinal microbiota in the pathogenesis and treatment of HIV infection. *AIDS Res Ther*, 13, 19 (2016)
DOI: 10.1186/s12981-016-0103-1
28. J. H. Suh and J. D. Saba: Sphingosine-1-phosphate in inflammatory bowel disease and

colitis-associated colon cancer: the fat's in the fire. *Transl Cancer Res*, 4(5), 469-483 (2015)

29. C. T. Murphy, K. Nally, F. Shanahan and S. Melgar: Shining a light on intestinal traffic. *Clin Dev Immunol*, 2012, 808157 (2012) DOI: 10.1155/2012/808157
30. J. Kunisawa, Y. Kurashima, M. Higuchi, M. Gohda, I. Ishikawa, I. Ogahara, N. Kim, M. Shimizu and H. Kiyono: Sphingosine 1-phosphate dependence in the regulation of lymphocyte trafficking to the gut epithelium. *J Exp Med*, 204(10), 2335-48 (2007) DOI: 10.1084/jem.20062446
31. Y. Takuwa, Y. Okamoto, K. Yoshioka and N. Takuwa: Sphingosine-1-phosphate signaling and biological activities in the cardiovascular system. *Biochim Biophys Acta*, 1781(9), 483-8 (2008) DOI: 10.1016/j.bbalip.2008.04.003
32. D. H. Nguyen-Tran, N. C. Hait, H. Sperber, J. Qi, K. Fischer, N. Ieronimakis, M. Pantoja, A. Hays, J. Allegood, M. Reyes, S. Spiegel and H. Ruohola-Baker: Molecular mechanism of sphingosine-1-phosphate action in Duchenne muscular dystrophy. *Dis Model Mech*, 7(1), 41-54 (2014) DOI: 10.1242/dmm.013631
33. S. Calise, S. Blescia, F. Cencetti, C. Bernacchioni, C. Donati and P. Bruni: Sphingosine 1-phosphate stimulates proliferation and migration of satellite cells: role of S1P receptors. *Biochim Biophys Acta*, 1823(2), 439-50 (2012) DOI: 10.1016/j.bbamcr.2011.11.016
34. S. Spiegel and S. Milstien: The outs and the ins of sphingosine-1-phosphate in immunity. *Nat Rev Immunol*, 11(6), 403-15 (2011) DOI: 10.1038/nri2974
35. M. H. Graler: Targeting sphingosine 1-phosphate (S1P) levels and S1P receptor functions for therapeutic immune interventions. *Cell Physiol Biochem*, 26(1), 79-86 (2010) DOI: 10.1159/000315108
36. T. Sanchez and T. Hla: Structural and functional characteristics of S1P receptors. *J Cell Biochem*, 92(5), 913-22 (2004) DOI: 10.1002/jcb.20127
37. Y. Yatomi, R. J. Welch and Y. Igarashi: Distribution of sphingosine 1-phosphate, a bioactive sphingolipid, in rat tissues. *FEBS Lett*, 404(2-3), 173-4 (1997) DOI: 10.1016/S0014-5793(97)00121-X
38. A. N. Anbazhagan, S. Priyamvada, A. Alakkam, A. Kumar, A. Borthakur, S. Saksena, R. K. Gill, W. A. Alrefai and P. K. Dudeja: Transcriptional Modulation of SLC26A3 (DRA) by Sphingosine-1-Phosphate. *Am J Physiol Gastrointest Liver Physiol*, ajpgi 00308 2015 (2016)
39. E. Germinario, S. Peron, L. Toniolo, R. Betto, F. Cencetti, C. Donati, P. Bruni and D. Danieli-Betto: S1P2 receptor promotes mouse skeletal muscle regeneration. *J Appl Physiol (1985)*, 113(5), 707-13 (2012) DOI: 10.1152/japplphysiol.00300.2012
40. M. H. Moon, J. K. Jeong and S. Y. Park: Activation of S1P2 receptor, a possible mechanism of inhibition of adipogenic differentiation by sphingosine 1phosphate. *Mol Med Rep*, 11(2), 1031-6 (2015)
41. M. Grimm, D. Tischner, K. Troidl, J. Albarran Juarez, K. K. Sivaraj, N. Ferreiros Bouzas, G. Geisslinger, C. J. Binder and N. Wettschureck: S1P2/G12/13 Signaling Negatively Regulates Macrophage Activation and Indirectly Shapes the Atheroprotective B1-Cell Population. *Arterioscler Thromb Vasc Biol*, 36(1), 37-48 (2016)
42. D. R. Herr, M. J. Reolo, Y. X. Peh, W. Wang, C. W. Lee, R. Rivera, I. C. Paterson and J. Chun: Sphingosine 1-phosphate receptor 2 (S1P2) attenuates reactive oxygen species formation and inhibits cell death: implications for otoprotective therapy. *Sci Rep*, 6, 24541 (2016) DOI: 10.1038/srep24541
43. R. Wiltshire, V. Nelson, D. T. Kho, C. E. Angel, S. J. O'Carroll and E. S. Graham: Regulation of human cerebro-microvascular endothelial baso-lateral adhesion and barrier function by S1P through dual involvement of S1P1 and S1P2 receptors. *Sci Rep*, 6, 19814 (2016) DOI: 10.1038/srep19814
44. R. Liu, X. Li, X. Qiang, L. Luo, P. B. Hylemon, Z. Jiang, L. Zhang and H. Zhou: Taurocholate Induces Cyclooxygenase-2 Expression via the Sphingosine 1-phosphate Receptor 2 in a Human Cholangiocarcinoma Cell Line. *J Biol Chem*, 290(52), 30988-1002 (2015) DOI: 10.1074/jbc.M115.668277

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Send correspondence to: Huiping Zhou, 1217 East Marshall Street, MSB#533, Richmond, VA 23298, USA, Tel: 804-828-6817, Fax: 804-828-0676, E-mail: Huiping.Zhou@vcuhealth.org