TGF-β/SMAD3 Pathway Stimulates Sphingosine-1 Phosphate Receptor 3 Expression

IMPLICATION OF SPHINGOSINE-1 PHOSPHATE RECEPTOR 3 IN LUNG ADENOCARCINOMA PROGRESSION

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Jiawei Zhao†, Jingjing Liu‡, Jen-Fu Lee‡, Wenliang Zhang†, Mustapha Kandouz†, Garrett C. VanHecke§, Shiyou Chen*, Young-Hoon Ahn*, Fulvio Lonardo††, and Menq-Jer Lee***

From the Departments of †Pathology and §Chemistry, ‡Karmanos Cancer Institute, and **Cardiovascular Research Institute, Wayne State University School of Medicine, Detroit, Michigan 48201 and the ¶Department of Physiology and Pharmacology, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602

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Previously, we showed that levels of sphingosine-1 phosphate receptor 3 (S1PR3) are increased in a panel of cultured human lung adenocarcinoma cell lines, and that S1PR3-mediated signaling pathways regulate proliferation, soft agar growth, and invasion of human lung adenocarcinoma cells in vitro. In the present study, we examine S1PR3 levels in human lung adenocarcinoma specimens. cDNA array and tumor microarray analysis shows that mRNA and protein levels of S1PR3 are significantly increased in human lung adenocarcinomas when compared with normal lung epithelial cells. Promoter analysis shows 16 candidate SMAD3 binding sites in the promoter region of S1PR3. ChIP indicates that TGF-β treatment stimulates the binding of SMAD3 to the promoter region of S1PR3. Luciferase reporter assay demonstrates that SMAD3 transactivates S1PR3 promoter. TGF-β stimulation or ectopic expression of TGF-β up-regulates S1PR3 levels in vitro and ex vivo. Pharmacologic inhibition of TGF-β receptor or SMAD3 abrogates the TGF-β-stimulated S1PR3 up-regulation. Moreover, S1PR3 knockdown dramatically inhibits tumor growth and lung metastasis, whereas ectopic expression of S1PR3 promotes the growth of human lung adenocarcinoma cells in animals. Pharmacological inhibition of S1PR3 profoundly inhibits the growth of lung carcinoma in mice. Our studies suggest that levels of S1PR3 are up-regulated in human lung adenocarcinomas, at least in part due to the TGF-β/SMAD3 signaling axis. Furthermore, S1PR3 activity promotes the progression of human lung adenocarcinomas. Therefore, S1PR3 may represent a novel therapeutic target for the treatment of deadly lung adenocarcinomas.

Sphingosine-1-phosphate (S1P)³ is a serum-borne bioactive lipid mediator, which is generated by two sphingosine kinase isozymes, SphK1 and SphK2, using sphingosine as the substrate (1). S1P functions as an extracellular ligand or intracellular lipid mediator (2–5), and regulates various physiological and pathological functions (5–8). When S1P is functioning as an extracellular ligand, its activities are mediated by the S1P family of G protein-coupled receptors (S1PR1–S1PR5) (2, 9–11). Several lines of evidence suggest that S1P-mediated signaling pathways are closely linked to the tumorigenesis of various human cancers (12–16). However, the pathological link between the S1P-mediated signaling pathways and human lung adenocarcinoma is poorly understood. Previously, we showed that levels of sphingosine-1 phosphate receptor 3 (S1PR3) are significantly increased in cultured human lung adenocarcinoma cell lines (16). Moreover, we demonstrated that the S1PR3-activated signaling pathways play an important role in promoting the progression and invasiveness of human lung adenocarcinoma cells (11, 16).

TGF-β activates multiple signaling pathways to regulate various tumorigenic processes. For example, TGF-β regulates epithelial-mesenchymal transition, which is a critical process in cancer initiation and progression (17–20). Also, TGF-β stimulates the production of inflammatory cytokines in tumor microenvironments (21), and promotes tumor progression through extracellular matrix remodeling, cell adhesion, migration, and immune tolerance (17, 22, 23). Upon TGF-β ligation, TGF-β receptors phosphorylate SMAD (homolog of mothers against decapentaplegic) signaling molecules, leading to the nuclear translocation of SMADs. The nucleus-localized SMADs interact with specific transcriptional activators and repressors and regulate the expression of tumorigenic genes (24). In addition, TGF-β activates SMAD-independent pathways such as MAPK, JNK, NFκB, Ras/Raf/ERK, and Rho kinase pathways in a cell type-dependent manner (24, 25). Although both SMAD and non-SMAD pathways were reported to

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† Both authors contributed equally to this work.

‡ To whom correspondence should be addressed: Dept. of Pathology, Wayne State University School of Medicine, 540 East Canfield Ave., Scott Hall, Rm. 9215, Detroit, MI 48201. Tel.: 313-577-9473; Fax: 313-577-0798, E-mail: mengjer.lee@wayne.edu.

§ The abbreviations used are: S1P, sphingosine-1-phosphate; TGF-β, transforming growth factor beta; SBE, SMAD3 binding element; LLC, Lewis lung carcinoma; Ad, adenovirus; Ctrl, control; qPCR, quantitative PCR; TMA, tumor microarray; ESI, electrospray ionization; ANOVA, analysis of variance.
be involved in tumorigenic process, the mechanistic details remain to be elucidated.

Previous studies have suggested the cross-talk between TGF-β and S1P signaling pathways. TGF-β was shown to activate SphK1 and stimulate the production of S1P (26), which may be involved in extracellular matrix deposition and fibrosis. On the other hand, S1P transactivates the TGF-β pathway and regulates several TGF-β-mediated physiological and pathological functions (27, 28). Thus, a better understanding of the cross-talk between the S1P- and TGF-β mediated signaling pathways is expected to open new perspectives for the treatment of TGF-β-triggered pathologies such as inflammation, fibrosis, and cancer.

In the present study, we show that levels of S1PR3 are significantly increased in human lung adenocarcinoma specimens. Mechanistically, our data suggest that the TGF-β/SMAD 3 signaling pathway contributes to S1PR3 up-regulation in lung adenocarcinomas. Moreover, our study suggests that S1PR3 represents a novel therapeutic target for the treatment of human lung cancers.

Results

Up-regulation of S1PR3 in Human Lung Adenocarcinomas—Previously, we showed that levels of S1PR3 are significantly increased in a panel of cultured human lung adenocarcinoma cell lines when compared with normal lung epithelial cells (16). The pathological relevance of this in vitro observation was investigated by measuring mRNA levels of S1PR3 in cDNA microarrays of human lung adenocarcinoma specimens (OriGene, HLRT). Quantitative PCR analysis showed that mRNA levels of S1PR3 are significantly increased in human lung adenocarcinoma specimens when compared with normal lung tissues (Fig. 1A). We previously showed that S1PR2 levels are increased in endothelial senescence and inflammation (10, 29). However, we observed that levels of S1PR2 are decreased in human lung cancers (Fig. 1B).

Next, we utilized immunohistochemical staining to examine protein levels of S1PR3 in a paraffin-embedded tumor microarray of human lung adenocarcinoma specimens (Accumax). Anti-S1PR3 (Cayman) immunoreacted with plasma membrane-localized S1PR3 in HEK293 cells transiently transfected with S1PR3 vector. No immunoreactivity was observed in HEK293 transfected with pcDNA control vector (Fig. 1C). Immunohistochemical staining analysis showed that the intensity of anti-S1PR3 immunostaining is significantly increased in human lung adenocarcinomas when compared with their respective adjacent normal lung epithelial cells (Fig. 1, D–F). Moreover, levels of S1PR3 are increased in human lung squamous carcinoma specimens (Fig. 1, G and H). Oncogenic K-Ras mutation is found in more than 25% of non-small cell lung carcinomas and represents one of the most prevalent oncogenic drivers in non-small cell lung carcinomas (30, 31). We utilized a conditionally inducible knock-in K-RasG12D (Lox-Stop-Lox-K-RasG12D, LSL-K-RasG12D) mouse model (32, 33) to measure S1PR3 levels in lung adenocarcinomas and normal lung tissues. As shown in Fig. 2A, lung tumors were readily observed in heterozygous LSL-K-RasG12D mice following intratracheal injection of adenoviral particles carrying Cre recombinase (Ad-Cre). S1PR3 levels were increased ~20-fold in lungs of K-RasG12D-expressing mice when compared with that in mice treated with empty adenoviral particles (Ad-
Ctrl (Fig. 2B). A minimal increase of S1PR4 was observed in lungs of K-Ras<sup>G12D</sup>-expressing mice. There were no significant changes of S1PR1 and S1PR2, and S1PR5 was not detected in lungs of Ad-Cre-injected mice (Fig. 2B). In addition, immunohistochemical staining showed that protein levels of S1PR3 were markedly increased in lung carcinoma specimens of K-Ras<sup>G12D</sup> transgenic mice (Fig. 2C) when compared with normal lung tissues of wild-type mice. In control, no staining was detected in lung adenocarcinoma specimens of K-Ras<sup>G12D</sup> transgenic mice when immunohistochemical staining was performed without S1PR3 antibody (data not shown). These data suggest that S1PR3 levels are increased in lung adenocarcinomas.

**TGF-β/SMAD3 Signaling Pathway Stimulates S1PR3 Expression**—Promoter analysis suggested that the promoter region of S1PR3 contains 16 potential binding elements for the SMAD3 molecule (Fig. 3A, Table 1), a critical signal transducer downstream of TGF-β/SMAD3 receptor signaling. Also, it was shown that K-Ras mutant up-regulated TGF-β, which is required for tumor angiogenesis (34). Therefore, we examined whether the TGF-β/SMAD3 signaling contributes to oncogenic K-Ras mutant-stimulated S1PR3 up-regulation. Ectopic expression of oncogenic K-Ras<sup>G12V</sup> mutant significantly increased S1PR3 (Fig. 3B and C). Expression of K-Ras<sup>G12V</sup> did not alter levels of other S1P receptor subtypes. In agreement with a previous study (34), levels of TGF-β were increased in K-Ras<sup>G12V</sup>-expressing cells (Fig. 3D). Treatments with TGF-β antibody (Fig. 3E) and inhibition of TGF-β receptor I and SMAD3 using compound SB-431542 and SIS3 (Fig. 3F), respectively, abrogated the S1PR3 up-regulation in K-Ras<sup>G12V</sup>-expressing cells.

Next, we investigated whether TGF-β treatment stimulates S1PR3 expression in lung epithelial cells. HBEC2-KT cells, an immortalized normal human lung epithelial cell line (16), were treated with TGF-β for various times. Quantitative analysis of the expression of S1P receptor subtypes by qPCR analysis showed that TGF-β treatment increased mRNA levels of S1PR3 in a time-dependent manner (Fig. 4A). TGF-β treatment did not affect levels of other subtypes of S1PRs such as S1PR1, S1PR2, and S1PR5. S1PR5 was not detected in HBEC2-KT cells. Also, TGF-β treatment increased protein levels of S1PR3 in HBEC2-KT cells (Fig. 4B). Validation of the specificity of anti-S1PR3 for Western blotting analysis showed that anti-S1PR3 specifically immunoreacted with S1PR3 (Fig. 4C).

Moreover, transduction with adenoviral particles carrying an active form of the TGF-β vector (35–37) effectively increased S1PR3 expression in HBEC2-KT cells when compared with transduction with control adenoviral particles, in ex vivo mouse lung minces (Fig. 4D). Furthermore, TGF-β treatment time-dependently increased levels of Spk1 (Fig. 4E) and S1P production (Fig. 4F) in HBEC2-KT normal lung epithelial cells. TGF-β treatment did not alter levels of Spk2 in HBEC2-KT cells.

Next, we used selective pharmacological inhibitor to investigate the role of SMAD3 in TGF-β-stimulated S1PR3 up-regulation. Inhibition of TGF-β receptor I and SMAD3 using compound SB-431542 and SIS3, respectively, abrogated the TGF-β-stimulated S1PR3 up-regulation (Fig. 4G). In contrast, inhibition of other signaling molecules downstream of TGF-β...
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**TGF-β ChIP assay** showed that TGF-β SMAD3 binding elements in the promoter region of S1PR3. We designed 16 pairs of primers (Table 1) that amplify these candidate sites in HBEC2-KT lung epithelial cells. Subsequently, we designed allel control experiment, treatment with inhibitor effectively diminished the TGF-β signaling (e.g. NFκB, JNK, and p38 kinase) did not significantly diminish the TGF-β-stimulated S1PR3 up-regulation. In a parallel control experiment, treatment with inhibitor effectively diminished the activation of their respective target following TGF-β stimulation (Fig. 4H). These data suggest that the TGF-β receptor I/Samd3 signaling pathway contributes to the TGF-β-stimulated S1PR3 expression in lung epithelial cells.

Treatment of HBEC2-KT cells with TGF-β markedly stimulated the nuclear accumulation of phosphorylated SMAD3 (Fig. 5A, arrows), indicating that TGF-β treatment activates SMAD3 in HBEC2-KT lung epithelial cells. Subsequently, we designed 16 pairs of primers (Table 1) that amplify these candidate SMAD3 binding elements in the promoter region of S1PR3. ChIP assay showed that TGF-β treatment significantly increased the binding of phospho-SMAD3 to P13, P14, and P15 sites in the promoter region of S1PR3 (Fig. 5B). No specific binding was observed when ChIP assays were performed using irrelevant normal IgG as a control, suggesting that bindings of phospho-SMAD3 are specific.

Next, we used a luciferase reporter assay to examine whether SMAD3 transactivates those candidate SMAD3 binding sites present in the S1PR3 promoter region. As shown in Fig. 5C, SMAD3 activates PGL3-promoter luciferase vector carrying P14, whereas SMAD3 did not activate PGL3-promoter luciferase vector carrying P13 and P15. The luciferase reporter assay is specific, because SMAD3 was unable to activate scrambled P14 (Fig. 5D).

**S1PR3 in Lung Adenocarcinoma**

**TABLE 1**

| SBE | Sequence | Position | Primer name | Primer sequence |
|-----|----------|----------|-------------|-----------------|
| 1   | GCCAGA   | (−2344) to (−2399) | SBE1 F | CCAAGTGGACGAGATTAG |
| 2   | TACAGA   | (−2200) to (−2195) | SBE2 F | GACACCCACTATGGCAAC |
| 3   | TGCAGA   | (−2113) to (−2108) | SBE3/4 F | GTTTTAGGTGTCGATTC |
| 4   | AACAGA   | (−2092) to (−2087) | SBE3/4 R | CACCTCTTCTCCACACCTCC |
| 5   | GTCAGA   | (−2011) to (−2006) | SBE5/6 F | GAGGTTGAGGAGAGATAG |
| 6   | AGCAGA   | (−1969) to (−1964) | SBE5/6 R | TCCCAACACAGGGCTTCT |
| 7   | TCAGA    | (−1849) to (−1845) | SBE7 F | GCCATGAGGAGAGATAG |
| 8   | CAGACT   | (−1765) to (−1760) | SBE7 R | GCCATAAACCTAGAGGCC |
| 9   | ACAGA    | (−1610) to (−1606) | SBE9 F | CAACCTTCCAAGTATCCC |
| 10  | ACAGA    | (−1433) to (−1429) | SBE10/11 F | GTAGGTTCCAACAAAGGG |
| 11  | CAGA     | (−1418) to (−1415) | SBE10/11 R | CACTTCGCTGGCTACTGTC |
| 12  | CCAGA    | (−1299) to (−1295) | SBE12/13 F | CACAGTGGCACAGGGAGG |
| 13  | CAGA     | (−1267) to (−1264) | SBE12/13 R | GCCCTCAGAGGTGGCTG |
| 14  | AGACAGA  | (−1084) to (−1078) | SBE14 F | GCCCTTCTCTCAGAGAGAG |
| 15  | CAGA     | (−644) to (−641) | SBE14 R | GGCGGAGGAGTGGCAGC |
| 16  | CCAGAC   | (−350) to (−345) | SBE16 F | GAATCCGCCCAAACAAAAAC |
|     |          |          | SBE16 R | GAATAGGTCGACAGCAACAG |

**S1PR3 Promotes Lung Adenocarcinoma Progression**—We previously showed that S1PR3 activation promotes proliferation, soft agar growth, and invasion of human lung adenocarcinoma cells in vitro (11, 16). Therefore, we utilized animal models to examine the role of S1PR3 in human lung adenocarcinoma progression. Human H1793 lung adenocarcinoma cells, abundantly expressing S1PR3 (16), were stably transfected with sh-S1PR3 or sh-control vectors. Expression of sh-S1PR3 effectively knocked down ~67% of S1PR3 in H1793 cells (Fig. 6A). Moreover, S1PR3 knockdown significantly inhibited tumor growth in a subcutaneous xenograft mouse model (Fig. 6, B and C). Similarly, S1PR3 knockdown diminished lung colonization of H1793 cells, which were injected via the tail vein route (Fig. 6, D and E). In contrast, H1299 human lung adenocarcinoma cells express very low levels of S1PR3 among human lung adenocarcinoma cell lines (16) and are poorly tumorigenic in athymic mice. Ectopic expression of S1PR3 profoundly promoted tumor growth in athymic mice (Fig. 6F). These results suggest that S1PR3 activity promotes tumorigenesis of human lung adenocarcinomas.

**Pharmacological Inhibition of S1PR3 Diminishes Lung Adenocarcinoma Growth**—Next, we investigated whether treatment with S1PR3 antagonist diminishes the growth of human lung adenocarcinoma cells. C57BL/6 mice were subcutaneously implanted with murine Lewis lung carcinoma (LLC) cells. 1 week after tumor implantation, mice were intraperitoneally injected every 3 days with VPC23019, an antagonist of S1PR1.
and S1PR3 receptors (38). Administration of VPC23019 significantly inhibited tumor growth (Fig. 7A). Lewis lung carcinoma cells predominantly express S1PR3, and S1PR1 is barely detected (Fig. 7B). Thus, the effect of VPC23019 on inhibition of tumor growth is most likely due to its antagonistic activity on S1PR3 present in LLC cells. Indeed, treatment with TY-52156, a highly selective antagonist of S1PR3 (39–41) (Fig. 7C), significantly suppressed the growth of Lewis lung carcinoma cells (Fig. 7, D and E). These results suggest that S1PR3 represents a novel therapeutic target for the treatment of lung carcinomas.

Discussion

We previously showed that levels of S1PR3 are increased in a panel of cultured human lung adenocarcinoma cell lines when compared with normal lung epithelial cells (16). In this report, we observed that mRNA and protein levels of S1PR3 are significantly up-regulated in human lung adenocarcinoma specimens. Our observation is supported by the analysis of Oncomine data sets (42–45) showing that S1PR3 expression correlates with clinical stages (42, 44), EML4-ALK gene fusion (42), lymphatic and perineural invasion (44), metastasis to bone (44), vascular invasion (44), BCL amplification (45), and APC deletion and family history (43) of human lung adenocarcinomas. These data suggest that S1PR3 is up-regulated in human lung adenocarcinomas, and S1PR3 expression correlates with the aggressiveness of lung adenocarcinomas.

Oncogenic K-Ras mutation is found in more than 25% of non-small cell lung cancers (30, 31). In the LS-L-K-RasG12D transgenic mouse model, we found that the expression of K-RasG12D mutant triggered the development of lung cancers and concurrently stimulated the expression of S1PR3. In agreement with our study, Oncomine data sets analysis showed that S1PR3 up-regulation correlates with K-Ras mutation status in human lung cancers (42, 43, 48, 50, 51) (see Genomic Data Commons (https://gdc.cancer.gov)). Mechanistically, our data suggest that the oncogenic K-Ras mutant-stimulated S1PR3 expression is mediated by an autocrine TGF-β/SMAD3 axis in lung epithelial cells. In supporting our observations, it was shown that oncogenic K-Ras mutant stimulated the expression of TGF-β, which plays a critical role in tumor angiogenesis in K-Ras mutant-driven cancers (34). It should be noted that lung cancers driven by K-Ras mutant are generally refractory to chemotherapy as well as targeted agents (31, 52). To date, the identification of drugs to therapeutically inhibit K-Ras mutant has been unsuccessful, suggesting that other approaches are required. We showed that oncogenic K-Ras mutant stimulates S1PR3 expression, suggesting that S1PR3 represents a novel
therapeutic target for the treatment of K-Ras mutant-driven lung cancers.

Previously, we showed that S1PR3 regulates the proliferation, colony formation, and invasiveness of human lung adenocarcinoma cells in vitro (11, 16). In the present study, we utilized animal models to examine the role of S1PR3 in the progression of human lung adenocarcinomas. H1793 human lung adenocarcinoma cells abundantly express S1PR3, and S1PR3 knockdown profoundly abrogated proliferation, colony formation in soft agar, and invasion of tumor cells in vitro (11, 16). Similarly, S1PR3 knockdown significantly inhibited tumor growth in a xenograft model, as well as lung colonization of adenocarcinoma cells in a tail vein implantation model. In contrast, H1299 human lung adenocarcinoma cells express very low levels of S1PR3 among lung adenocarcinoma cell lines (16). Expression of S1PR3 significantly promoted growth of tumor xenograft. These results suggest that the S1PR3-mediated signaling pathways play an important role in promoting the progression of lung adenocarcinoma cells. We previously characterized two S1PR3-mediated signaling pathways that may have functional implications in promoting lung adenocarcinoma progression. We found that S1PR3 activation transcriptionally up-regulates EGFR levels and greatly potentiates the effect of EGF on the proliferation of lung adenocarcinoma cells (16). Moreover, we characterized a novel signaling pathway, namely S1PR3/JNK/AP-1/ETS-1/CD44 axis, which critically regulates the invasiveness of human lung adenocarcinoma cell in vitro (11). Collectively, our studies suggest that S1PR3 represents a potential therapeutic target for the treatment of human lung adenocarcinomas. Indeed, our study using pharmacological inhibitors supports this notion. We found that administration of VPC23019 (an antagonist of S1PR1 and S1PR3 receptors (38)) and TY-52156 (a selective inhibitor of S1PR3 (39–41)) of VPC23019 (an antagonist of S1PR1 and S1PR3 receptors (38)) and TY-52156 (a selective inhibitor of S1PR3 (39–41)) significantly diminished lung tumor growth in xenograft mouse model.

Mechanistically, we showed that TGF-β/SMAD3 signaling pathway transactivates S1PR3/S1PR3 axis in lung epithelial cells. A previous study showed that TGF-β activates sphingosine kinase via a non-SMAD signaling pathway and that the TGF-β/sphingosine kinase axis is important for the migration and invasion of esophageal cancer cells in vitro (53). However, the role of the TGF-β signaling axis on the regulation of S1PRs was not investigated in that study. Moreover, in agreement with our observation, Cencetti et al. (54) showed that TGF-β stimulated S1PR3 expression in C2C12 myoblasts. In contrast to their study, we precisely defined the SMAD3 binding sites on the promoter region of S1PR3 and demonstrated that the TGF-β-stimulated S1PR3 up-regulation is dependent on the SMAD3 signaling molecule. Furthermore, we found that TGF-β con-
comitantly stimulated SphK1 expression and increased S1P production in lung epithelial cells. Collectively, our results suggest that TGF-β activates an autocrine S1P/S1PR3 signaling axis in lung epithelial cells, which may contribute to lung adenocarcinoma progression.

Several tumors, including lung cancers, express high levels of TGF-β (55–57), which correlates with tumor progression and clinical prognosis (58–63). Thus, our observation of the TGF-β-mediated S1PR3 up-regulation in lung cancers is pathologically relevant. In addition, TGF-β plays an important role in regulating the tumorigenic processes including epithelial-mesenchymal transition (17, 20, 64–66) and tumor inflammation (67–72). For example, TGF-β stimulates the expression of pro-inflammatory and pro-tumorigenic cytokine IL-6 (71, 72). Elevated systemic and pulmonary productions of IL-6 are commonly observed in lung adenocarcinoma patients and correlate with poor patient survival (73, 74). Moreover, the TGF-β/IL-6 axis was recently shown to mediate the chemo-resistance in lung cancer (71). Our results show that TGF-β activates the

**Experimental Procedures**

**Reagents**—Sphingosine-1 phosphate (Biomol) and VPC23019 (Cayman Chemical) were prepared as micelles by sonication in aqueous solution of fatty acid-free bovine serum albumin (0.4 mg/ml, Sigma). TY-52156 was chemically synthesized as described (39). TGF-β was from R&D Systems. Anti-S1PR3 and anti-phospho-SMAD3 were from Cayman and Abcam, respectively. SB-431542 and SIS3 were purchased from Sigma. Unless specified, other reagents are from Sigma.

**Cell Cultures**—Immortalized normal human lung epithelial cells (HBEC2-KT and HBEC3-KT) were cultured using keratinocyte-serum free medium (Invitrogen) (75). H1793 human lung adenocarcinoma cells were cultured using HITES medium (RPMI 1640 medium supplemented with hydrocortisone (10 nm), insulin (5 μg/ml), transferrin (100 μg/ml), 17 β-estradiol (10 nm), sodium selenite (30 nm), and 5% fetal bovine serum) (75). H1299 and mouse Lewis lung carcinoma cells were cultured essentially as we described previously (16). Cells were cultured in a humidified atmosphere of 5% CO₂ at 37 °C.

**Real-time PCR Analysis**—Total RNA was isolated using TRIzol reagent (Invitrogen) and was reverse-transcribed with an oligo(dT) primer (Promega) by Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Promega) for first-strand cDNA synthesis. For real-time PCR quantitation, 50 ng of reversely transcribed cDNAs were amplified with the ABI 7500 system (Applied Biosystems) in the presence of TaqMan DNA polymerase. The qPCR reaction was performed by using a universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. The sense and antisense primers used for qPCR analysis are: human and mouse S1PR1, sense, 5′-ATC ATG GCC TGG AAC TGC ATC A-3′, antisense, 5′-CGA GTC CTG ACC AAG GAG TAG AT-3′; human and mouse S1PR2, sense, 5′-CAG ACG GTA CCA CCT CTC AAG A-3′, antisense, 5′-TAG TGG GCT TTG TAG AGG A-3′; human and mouse S1PR3, sense, 5′-CAA ACC GCA TGT ACT TTT TCA T-3′, antisense, 5′-TAC TGC CCT CCC TGA GGA ACC A-3′; human S1PR4, sense, 5′-GGG CCA TCT TCC GCC TGG TG-3′, antisense, 5′-TGC CCC GCA GAT CCT ACT GG-3′; human S1PR5, sense, 5′-GGC GCC CAC CTG TCC TGT AC-3′, antisense, 5′-TCG GGT CTC TGC TG-3′; human and mouse S1PR3, sense, 5′-AAC CCC CTG TGT AGC CTC CC-3′, antisense, 5′-AGC AGG TTG ATG GGT GAC AG-3′; human and mouse SphK1, sense, 5′-AAA CCC CTG TGT AGC CTC CC-3′, antisense, 5′-AGC AGG TTG ATG GGT GAC AG-3′; human and mouse SphK2, sense, 5′-GCA CAGCAA CAG TGA GCA-3′, antisense, 5′-GAG CCT GAG TAG GGA G-3′; porcine TGF-β, sense, 5′-GCA CGT GGA GCT ATA CCA GAA-3′, antisense, 5′-CAT CAA AGG ACA GCA CCT CC-3′; human GADPH, sense, 5′-GAA GGT GAA GGT CGG AGT-3′, antisense, 5′-GAA GAT GAT GAT GGG TTT C-3′; and mouse GADPH, sense, 5′-CAC CTT CGA TGC CGG GGC TG-3′, antisense, 5′-GGC CAT GAG TGC CGG GGC TG-3′, antisense, 5′-GGC CAT GAG TGC CGG GGC TG-3′, antisense, 5′-GGC CAT GAG TGC CGG GGC TG-3′.

cDNA array analysis of mRNA levels of S1PR3 was performed using TissueScan qPCR arrays (HLRT101 and HLRT105, OriGene) following the manufacturer’s instructions. The

**FIGURE 7.** Inhibition of S1PR3 diminishes lung carcinoma growth. A, C57BL/6 mice were subcutaneously inoculated with LLC cells (1 × 10⁶ cells). 1 week later, mice were intraperitoneally administered with VPC23019 (1.5 mg/kg of body weight) or control vehicle every 3 days. B, S1PR1 and S1PR3 levels in Lewis lung carcinoma cells. –ve, PCR reactions were performed without cDNA. **, p < 0.01, n = 6, ANOVA. C, CHO cells were transduced with adenoviral particles carrying S1PR1, S1PR2, S1PR3, or pcDNA control vector. Cells were serum-starved for 24 h. Subsequently, cells were treated with TY-52156 (10 μM) for 10 min, followed by stimulating with S1P (200 nM, 10 μM). ERK1/2 activation (p-ERK) was measured by Western blotting analysis. D, C57BL/6 mice were subcutaneously inoculated with LLC cells (1 × 10⁶ cells). 1 week later, mice were intraperitoneally administered with TY-52156 (10 mg/kg of body weight) or control vehicle every 2 days. **, p < 0.01, n = 6, ANOVA. E, tumor weights were measured 24 days after implantation. **, p < 0.01, n = 6, ANOVA.
results of adenocarcinomas were extracted, and then analyzed by Student’s t test.

**Immunofluorescence Microscopy**—Cells were fixed with 4% paraformaldehyde for 30 min, followed by permeabilization with PBS containing 0.05% Triton X-100. After washing three times with PBS, cells were incubated with primary antibody at room temperature overnight. Cells were then washed three times with PBS, and incubated with FITC-conjugated secondary antibody for 1 h. Fluorescence images were captured by the Leica TCS SP5 confocal system (Leica, Wetzlar, Germany).

**Immunohistochemical Staining**—Human lung carcinoma tumor microarray (TMA) was purchased from Accumax (Accumax 306). Immunohistochemical staining was performed using VECTASTAIN ABC kit (Vector Laboratories, catalog number PK-6200) following the manufacturer’s instructions. Briefly, TMA sections were deparaffinized and dehydrated. Antigen retrieval was performed by microwave irradiation (two cycles of 5 min each) in 10 mM citrate buffer (pH 6.0). TMA was incubated with rabbit polyclonal S1PR3 antibody (1:200, Cayman) for 60 min, and then with biotinylated secondary antibody solution for 30 min and VECTASTAIN ABC Reagent for 30 min at room temperature. Subsequently, sections were incubated with peroxidase substrate (ImmPACT DAB (3,3’-diaminobenzidine), Vector Laboratories, catalog number SK-4105) until the desired stain intensity develops. Levels of S1PR3 were visualized by light microscopy (Leica DMI3000B).

**Western Blotting Analysis**—Protein extraction and Western blotting were performed as described (11). Briefly, cells were collected in ice-cold PBS using cell scrapers followed by centrifugation (500 × g, 5 min). Cell extracts were prepared with radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Calbiochem) with constant agitation at 4 °C for 30 min. After centrifugation at 15,000 × g for 20 min, supernatant was collected and protein concentration was measured using a bicinchoninic acid protein assay kit with BSA as standard. 50 μg of protein extracts were dissolved in 2 × Laemmli sample buffer, heated at 95 °C for 5 min, and resolved on a 10% SDS-PAGE gel. After electrophoresis, gels were transferred to nitrocellulose membranes. Subsequently, membranes were blocked in 5% nonfat dry milk (Lab Scientific) in TBST buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, and 0.05% Tween 20). Membranes were washed and incubated with indicated primary antibodies (1:1000 dilution) on a rotary shaker at 4 °C overnight. The blots were then incubated with peroxidase-conjugated secondary antibody for 1 h at room temperature and developed with enhanced chemiluminescent reagent (Thermo Scientific).

**ChIP Analysis**—The ChIP assay was performed using Pierce Agarose ChIP Kit, following the manufacturer’s instructions. Briefly, 1 × 10⁷ cells were cross-linked with 1% formaldehyde for 10 min. Following the addition of glycine quenching solution, cells were scraped and resuspended in 1× PBS with protease inhibitor cocktails (Calbiochem). Cells were then lysed in lysis buffer, and nuclear lysates were treated with micrococcal nuclease. Lysates were immunoprecipitated with anti-phospho-SMAD3 (Thermo Scientific) at 4 °C overnight. Immunoprecipitation with irrelevant normal IgG was used as a control. Immune complexes were isolated with protein A/G-Sepharose beads at 4 °C for 1 h. After washings, DNA fragments contained in immune complexes were purified, and then amplified by qPCR reactions. Sequences of primer pairs used for ChIP assay of SMAD3 binding to S1PR3 promoter are shown in Table 1.

**Luciferase Reporter Assay**—Oligonucleotides of candidate SMAD3 binding sites in the S1PR3 promoter region were synthesized, with an overhanging Nhel and SacI restriction site sequence at the 5’-end and 3’-end, respectively, of the antisense strand. Synthesized oligonucleotides are: P13 sense, 5’-GTC AGC AGG CAG AGT CAT TCG C-3’; P13 antisense, 5’-CTA GGC AAG TGA CTC TGC CTG ACA GCT-3’; P14 sense, 5’-GGG CAA AAG ACA GAA GTT AAC C-3’; P14 antisense, 5’-CTA GGG TTA CTT TCT GTC TTT TCG CCA GCT-3’; P15 sense, 5’-GTG CAC CAG CAG AGG CTG GGG C-3’; P15 antisense, 5’-CTA GGC CCC AGC CTC TGC TGG TGC ACA GCT-3’; Scramble P14 sense, 5’-GGG CAA ATG GCG AAA AGT AAC C-3’; Scramble P14 antisense, 5’-CTA GGG TTA CTT TCT GCC ATT TGC CCA GCT-3’. Equimolar amounts of sense and antisense oligonucleotides were mixed at 95 °C for 5 min, followed by cooling to room temperature. Annealed double-strand oligonucleotides were ligated with Nhel- and SacI-digested pGL3-promoter luciferase reporter vector (Promega). Recombinant luciferase vectors were verified by DNA sequencing.

HEK293 cells were co-transfected with recombinant pGL3 luciferase vector, pcDNA-SMAD3 (47) or empty pcDNA plasmids, and pRL-null vector (Promega) carrying the Renilla luciferase gene (5:5:1) by using Lipoctamine 2000 reagent (Life Technologies). 24 h after transfection, both firefly and Renilla luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega) using a SpectraMax M3 Multi-mode Microplate Reader (Molecular Devices). Firefly luciferase activities (M1) were normalized to Renilla luciferase activities (M2).

**Sphingolipid Measurement by LC-MS/MS**—Sphingolipids were extracted from culture medium as we described previously (29, 46). Samples were filtered through 0.45-μm nylon filters directly into auto sampler vials for LC-MS/MS analysis. Reverse phase HPLC was performed using BDS HYPERSIL C8 columns (100 × 2.1 mm, 2.4 μm, Thermo Scientific) and gradient elution on Waters Alliance 2695 system (Waters Corp.). The mobile phase consisted of methanol, water, and ammonium formate. Solvent A was 2 mM ammonium formate in methanol with 0.2% formic acid. The column was equilibrated with solvent A for 5 min. Samples were injected using the autosampler (an integral part of the Waters Alliance 2695 system) maintained at 10 ± 2 °C. The injection volumes were 80 μl for each sample. A complete injection of each sample took 7 min including column equilibration. The flow rate was 0.3 ml/min. The HPLC eluent was directly introduced to Quattro LC mass spectrometer (Micromass, Waters), equipped with an electrospray ion source that was used for ESI-MS/MS. The ESI-MS/MS experiments for the quantitation of sphingolipids were carried out in the positive ion mode with ESI needle voltage, 2.8 kV; source block temperature, 120 °C; desolation temperature, 350 °C; desolation gas flow, 540 liters/h; nebulizer gas flow, 80 liters/h; and collision gas pressure, 3.2 × 10⁻⁴ bars. Cone volt-
age and collision energy for each multiple reaction monitoring transition were optimized. Chromatographic data were analyzed by the QuanLynx module of the MassLynx software (Waters) to integrate the chromatograms for each multiple reaction monitoring transition.

**Tumor Growth and Lung Colonization in Mice**—All animal procedures were performed according to the National Institutes of Health and institutional guidelines, and were approved by the Wayne State University Animal Use and Care Committee. For subcutaneous implantation, lung carcinoma cells were adjusted to $1 \times 10^7$ cells/ml. Mice were injected with 0.1 ml of cell suspension into the subcutaneous dorsa in the proximal midline. Alternatively, $1 \times 10^6$ cells (in 50 µl) were injected via the tail vein route. NOD-Scid mice (8 weeks old, female, Harlan) were used for H1299 cells, and C57BL/6 mice (8 weeks old, female, The Jackson Laboratory) were used for Lewis lung carcinoma cells. Tumor volume was measured in two dimensions using calipers, and volume was determined using the formula $\text{width}^2 \times \text{length} \times 0.52$ (49). For VPC23019 treatment, mice were randomized into two groups (six animals per group) 1 week after inoculation of tumor cells. One group of mice was intraperitoneally injected with VPC23019 (1.5 mg/kg of body weight), and the other was injected with 100 µl of 0.4% BSA (vehicle control) every 3 days. For TY-52156 treatment, mice were randomized into two groups (six animals per group) every 2 days.

**Statistical Analysis**—Results are shown as mean ± S.D. Differences between paired samples were analyzed by Student’s t test. ANOVA analysis was performed to analyze tumor progression in mouse experiments. $p$ value < 0.01 is considered highly significant, and $p < 0.05$ is considered statistically significant.

**Author Contributions**—J. Z., J. L., J. F. L., W. Z., and M. K. designed the study and conducted experiments. G. C. V. and Y. H. A. chemically synthesized TY-52156. S. C. prepared research reagents, F. L. conducted experiments. G. C. V. and Y. H. A. chemically synthesized TY-52156. S. C. prepared research reagents, F. L. conducted experiments. G. C. V. and Y. H. A. chemically synthesized TY-52156. S. C. prepared research reagents, F. L. conducted experiments.

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