Short Communications

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A Crosstalk between the Receptor Tyrosine Kinase-Like Orphan Receptors *ROR1/2* and S1P Signaling Pathways in Lung Cancer

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Abstract

Objective: Availability of multimodal treatment strategies, including targeted therapies and immunotherapies, have improved the survival of non-small cell lung carcinoma (NSCLC). However, some patients still progress or respond poorly due to inherent resistance, acquired resistance, or lack of druggable driver mutations. Sphingosine-1-phosphate (S1P) and receptor tyrosine kinase-like orphan receptor (*ROR1/2*) signaling pathways are activated during lung carcinogenesis. **Methods:** In this study, we have evaluated the crosstalk of S1P and *ROR1/2* signaling pathways in lung cancer cells. **Results:** S1P treatment of lung cancer cells decreases ROR1 and ROR2 transcript levels. While treatment with PF-543, a pharmacological SphK1 inhibitor or genetic knockdown of *SPHK1* by shRNA, raises *ROR1* and ROR2. Furthermore, simultaneous inhibition of SphK1 along with *ROR1* reduced the migration of lung cancer cells. **Conclusion:** These findings demonstrate the reciprocal regulation of both pathways, suggesting that both pathways have an inverse relation i.e, in the absence of one pathway, another pathway may take charge of the other pathway. Therefore, simultaneously targeting both pathways could serve as a potential therapeutic target for lung cancer treatment.

Keywords: *SPHK1- ROR1- ROR2-* Lung Cancer- NSCLC

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Introduction

Lung cancer is the most common type of cancer globally. Owing to tumor heterogeneity and drug resistance, patients with non-small cell lung cancer (NSCLC) have poor prognosis [1, 2]. Treatment modalities for NSCLCs include chemotherapy, radiation, molecular targeted therapy, and immunotherapy. Furthermore, several drugs targeting various oncogenic pathways in NSCLC have been introduced in the last decade [3]. Oncogenic pathways targeted by such pharmacological molecules include, epidermal growth factor receptor (EGFR), ROS proto-oncogene 1 (ROS1), cellular mesenchymal epithelial transition (c-MET), fibroblast growth factor receptor (FGFR), mammalian target of rapamycin (mTOR), insulin like growth factor 1 receptor (IGFR), rearranged during transfection (RET), proto-oncogene B-Raf (*BRAF*), and anaplastic lymphoma kinase (ALK). However, most of the patients with NSCLC are diagnosed in the advanced stages (III/IV) and the median overall survival (OS) for patients with metastatic NSCLC is only 4–5 months [4, 5]. Thus, further research identifying newer drug targets for NSCLC is required.

Sphingosine-1-phosphate (S1P), a potent signaling molecule, is involved in various aspects of carcinogenesis. Sphingosine Kinases (SphK) are two enzymes that catalyze the synthesis of S1P. Overexpression of sphingosine kinase (SphK1) in several malignancies, including lung cancer, correlates with metastasis and poor prognosis (Pyne and Pyne, 2020). S1P is a natural ligand for five G-protein coupled receptors (GPCR) known as S1P receptor 1-5 (S1PR1-5) [6]. It activates downstream signaling pathways, regulating various cellular and biological functions such as cell proliferation, lymphocyte trafficking, inflammation and neovascularization [6]. On the other hand, receptor tyrosine kinase (RTK)-like orphan receptors (RORs) are also overexpressed in various forms of cancer, including NSCLC [7].

Signal transducer and activator of transcription 3 (*STAT3*) is a point of convergence for numerous oncogenic signalling pathways and is constitutively activated in many types of cancers [8]. S1P-S1PR1 signalling induces persistent activation of *STAT3* and leads to chronic intestinal inflammation and development of colitis-associated cancer [8]. Interestingly, *ROR1* expression is induced by *STAT3* [9], which is in turn being activated

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by S1P. Additionally, S1PR1 trans-activates receptor tyrosine kinases [10]. Thus, we hypothesized that the two pathways may crosstalk to regulate cell growth, survival, and carcinogenesis.

In this study, we examined the crosstalk of S1P and *ROR1*/2 pathways in lung cancer cells and found an association between them. S1P treatment decreased *ROR1* and *ROR2* transcript levels in lung cancer cells, while treatment with PF-543, a pharmacological SphK1 inhibitor or genetic knockdown of SPHK1 by shRNA, raised *ROR1* and *ROR2* levels. These outcomes demonstrate the reciprocal regulation of both pathways, suggesting that both pathways have an inverse relation i.e. in the absence of one pathway, another pathway may compensate the other pathway. Therefore, simultaneously targeting both pathways could serve as a potential therapeutic target for lung cancer treatment.

Materials and Methods

Establishment of cell lines and cell culture

The lung cancer cell lines A549 and L132 were purchased from the National Center for Cell Science (NCCS), Pune India, while bronchial epithelial cells BEAS2B (ATCC# CRL-9609) was procured from the American Type Culture Collection (ATCC). Dulbecco's modified Eagle Medium (DMEM) was used to culture the cells (A549 and L132). Furthermore, BEAS-2B cells were grown in a BEGM medium (Lonza) containing 10% fetal bovine serum (FBS). All experiments were carried out on BEAS2B cells between passages 10 and 30, with passage 1 defining the frozen cells from the supplier. Cells were trypsinized using trypsin-EDTA (0.25%). Cell cultures were incubated in a humidified 95% air: 5% CO2 environment. In 75-cm culture flasks, 2 million cells were plated and sub-cultured at 90% confluence.

Gene knockdown by short hairpin RNA (shRNA)

Transfected knockdown cells were harvested for RNA extraction. The clones (shRNA_SPHK1, shRNA_SPHK2, shRNA_*ROR1*, and shRNA_*ROR2*) were generated by transfection of cells and protocol was used according to the manufacturer. Table 1 shows the shRNA target sequences.

Lentiviral Particle Preparation

To produce a lentivirus containing shRNA, HEK293T cells were seeded in a 10-cm culture dish and co-transfected with plasmids at the concentrations shown below using the calcium phosphate technique once they reached 60-70% confluency. The following plasmids were used: pMD2.G of 1.5 µg, ps PAX2 of 3.75µg, and shRNA plasmid of 5 µg. Spent media was collected 48 hours after the transfection and filtered using a 0.45 µm syringe filter before being preserved at -70°C for future use. pMD2.G and psPAX2 plasmids were obtained from Addgene, whereas shRNA-SPHK1 were purchased from Sigma Aldrich.

The preparation of a Puromycin Kill Curve and selection of stable clones

For the preparation of kill curve, cells were seeded in 24-well plates and treated with different concentrations of puromycin for seven days, and the minimum concentration at which all the cells died, was used for the selection of transformed clones. A549 and BEAS2B were infected with lentivirus containing small hairpin RNA (shRNA) specific to *ROR1* (*shROR1*), *ROR2* (*shROR2*), *SPHK1 (shSPHK1),* and pLKO (shControl) with 8 µg/ml polybrene containing media. Cells were selected using 2µg/ml puromycin for 7 days. After selection, cells were used for downstream experiments.

RNA Extraction

Lung cancer cell lines L132, A549, and BEAS2B were cultured in 100-mm^2 cell culture plates in a complete DMEM medium. The confluency of cells was assessed under an inverted microscope (Model: Ti-Eclipse, make Nikon) and after washing with sterile PBS, cells were harvested and pelleted down by centrifugation. Cells were lysed in cell lysis buffer and cells were passed 8-10 times through a syringe attached with 20-gauge needle. RNA extraction was performed using PureLink RNA mini kit (Ambion) and PureLink DNase (Ambion) as per manufacturer's protocol. Total RNA was quantified spectrophotometrically at 260 nm and 280nm using the Nanodrop (Thermo Fisher). cDNA synthesis was performed using BioRad iScript cDNA synthesis kit (Catalogue No. 17188), as per manufacturer's protocol.

Real-time polymerase chain reaction (qRT-PCR)

To quantitate *SPHK1, SPHK2*, *ROR1*, *ROR2*, and GAPDH mRNA levels, reverse transcription (RT) reactions were performed using the iScript cDNA synthesis kit (Biorad, Hercules, CA) on Nexus Gradient Thermal Cycler (Eppendorf). Real-time PCR reactions were carried out using iTaq Universal SYBR-Green mix (Bio-Rad Laboratories) on CFX-96 thermal cycler (Bio-Rad, Hercules, CA) by using following primer sets SPHK1 Forward 5′-GGGAACTGGGCCACTTGT-3′Reverse 5'-CAAAGCCAAGCCCGAACC-3'; SPHK2 Forward 5'-CCGGAAGAAAGGGATCTGGG-3', Reverse

Table 1. shRNA Target Sequences

ID	Gene	TRC No.	Targeted sequence	Region	Predicted knockdown level (%)
*shSPHK1*_B	*SPHK1*	TRCN0000333675	GCAGCTTCCTTGAACCATTAT	CDS	94
*shSPHK1*_A	*SPHK1*	TRCN0000036965	GCAGCTTCCTTGAACCATTAT	CDS	87
*shROR1*_A	*ROR1*	TRCN0000002026	GCACCGTCTATATGGAGTCTT	CDS	88
*shROR1*_B	*ROR1*	TRCN0000002028	CGGAGAGCAACTTCATGTAAA	CDS	60
*shROR2*_A	*ROR2*	TRCN0000010625	GCACAGCCCAAATCATAACTT	CDS	98
*shROR2*_B	*ROR2*	TRCN0000001492	CGACAAGCTGAACGTGAAGAT	CDS	88

5'-TTCAGCTCTCCAACACTGGG-3'; *ROR1* Forward 5'-CAACAAGAAGCCTCCCTAATGG-3', Reverse 5'-CCTGAGTGACGGCACCTAGAA-3' (reverse); *ROR2* Forward 5'–GGCAGAACCCATCCTCGTG-3', reverse 5'-CGACTGCGAATCCAGGACC-3'; GAPDH Forward 5'-AATCCCATCACCATCTTCCAG- 3'; Reverse 5'-AAATGAGCCCCAGCCTTC-3'. GAPDH was used as a reference gene.

MTT Assay

Post-puromycin selection, cells were seeded in 96-well culture plates (8 × 10^3 /well) for 12, 24, 36, 48, and 72 h (in triplicate for each condition). MTT (Sigma, Saint Louis, USA) solution 10 µl (50 mg/ml) was added to each well and incubated for 4h. After the incubation, formazan crystals formed in the cells were solubilized using dimethyl sulfoxide, and the optical density was recorded at 570 and 600 nm using plate reader BioTek Eon (BioTek, Winooski, USA).

Wound healing assay

Post puromycin selection, 5× 10^4 cells/well were seeded in 24-well plate, and at approximately 100% confluency, a wound (scratch) was created using a 200 µl pipette tip and washed with 1× PBS for two times to remove cellular debris. Wounds were visualized at 10× with an inverted microscope Eclipse Ti (Nikon India Pvt Ltd), and three random images were captured at various time points. Wound width was measured using Image J software, and the graph was plotted with GraphPad Prism9 (La Jolla, CA, USA).

Bioinformatics analysis

Correlation between the two pathways in patient samples was determined using the available online tool GePIA (http://gepia.cancer-pku.cn/) [11]. Pearson's correlation was used to assess the correlation between expressions of two genes in lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) tumor and normal.

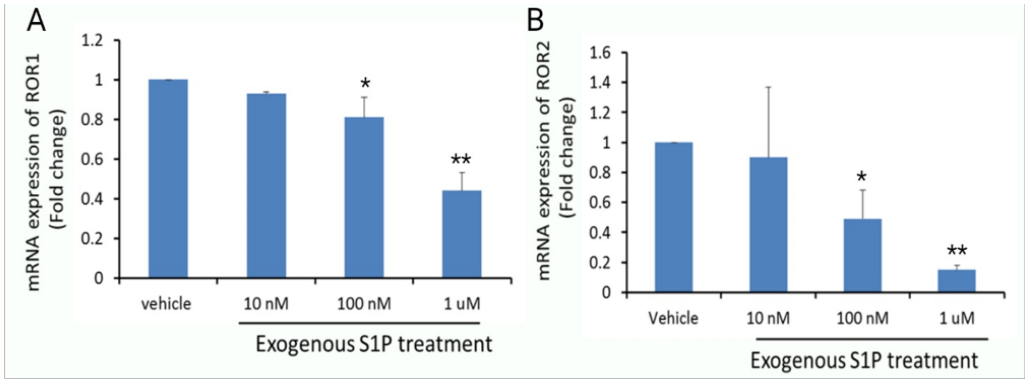
Statistical analysis

The statistical analysis was performed with GraphPad Prism 9 (La Jolla, CA, USA). The difference in the mean between the two groups was compared using student's t-test. Differences among multiple groups were compared using two-way-ANOVA with Sidak's multiple comparison test. The differences were considered statistically significant with $P < 0.05$.

Results

Treatment with S1P inhibits the expression of ROR1/ROR2

Lung cancer cells were treated with vehicle, 10 nM, 100 nM, and 1 µM concentrations of S1P for 24 hours. mRNA expression of *ROR1* and *ROR2* was measured using qRT-PCR. S1P decreased the mRNA expression in a dose-dependent manner, with a substantial reduction in



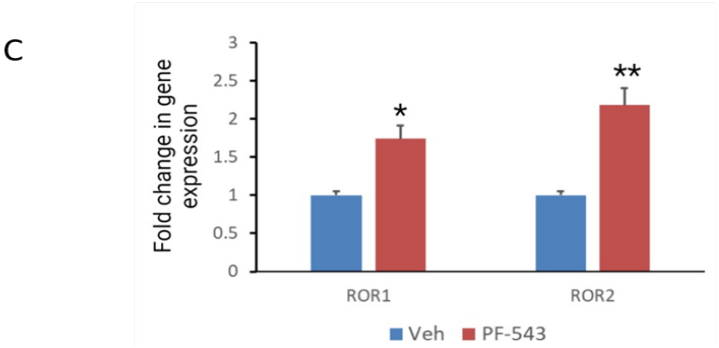


Figure 1. *ROR1* and *ROR2* mRNA Expression in L132 Cells Treated with S1P. L132 cells were serum starved for 48 hrs and treated with vehicle or indicated S1P for 24 hrs. mRNA expression of *ROR1* (A) and *ROR2* (B) was quantified by qRT-PCR. GAPDH was used as a reference gene. Results represent an average of three experiments (N=3). Data is presented at mean ±SDEV. Means were compared by ANOVA followed by posthoc test. *p<0.01 compared to vehicle. C) *ROR1* and *ROR2* mRNA expression in A549 cells treated with PF543. Cells were treated with vehicle or 1µM concentration of PF543 for 24 hrs. mRNA expression of *ROR1* and *ROR2* was quantified by qRT-PCR.GAPDH was used as a housekeeping gene. Means were compared by t-test.*p<0.05 compared to vehicle;**p<0.01 compared to vehicle.

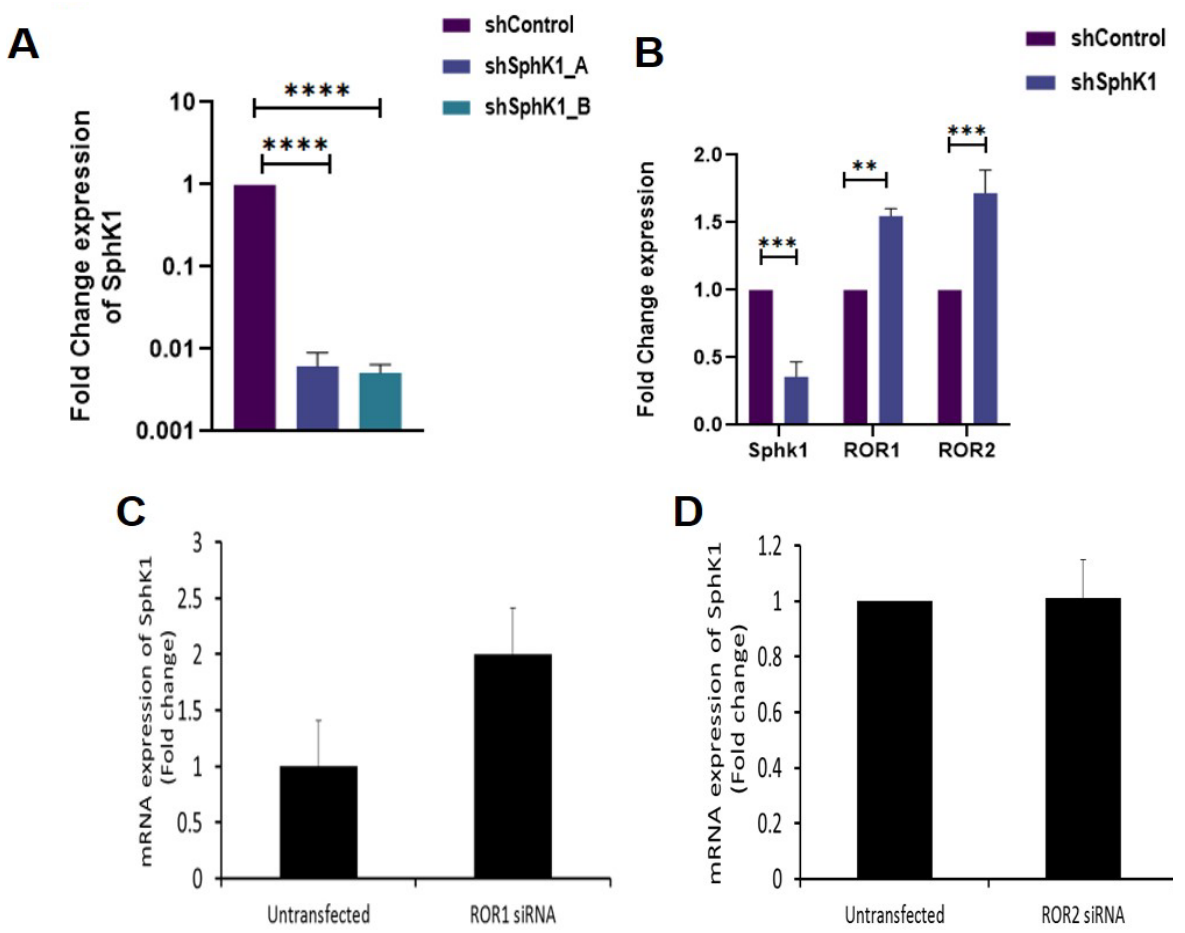


Figure 2. A-B) *ROR1* and *ROR2* mRNA Expression in *SPHK1* knockdown BEAS2B. A) SPHK1 mRNA levels in HEK293T cells transfected with shSPHK1 when compared with shControl.B) Expression of *ROR1* and *ROR2* in SPHK1 knockdown BEAS2B. Fold Change expression was calculated. The level of significance was calculated using Two-way ANOVA (Sidak's multiple comparison test). **P<0.01 compared to shControl. C-D) SPHK1 mRNA expression in *ROR1* and *ROR2* siRNA knocked down cells. L132 cells were transfected with *ROR1* (C) and *ROR2* (D), respectively. Forty-eight hours later, total RNA was isolated and mRNA expression of SPHK1 was quantified by qRT-PCR. GAPDH was used as a reference gene. Results represent an average of three experiments (N=3). Data is presented at the mean ±SDEV. **P<0.01 compared to untransfected.

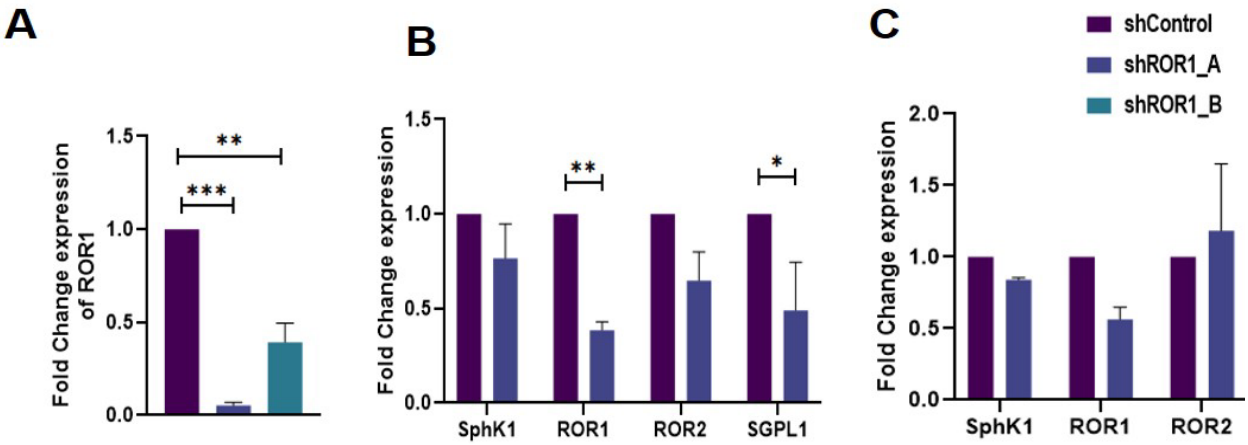


Figure 3. *SPHK1* mRNA Expression in *ROR1* Knockdown BEAS2B and A549 cells. A) *ROR1* mRNA levels in HEK293T cells transfected with sh*ROR1* when compared with shControl. B) Expression of *ROR2*, *SPHK1* and *SGLPL1* in *ROR1* knockdown in A549 cells; C) Expression of *ROR2*, and *SPHK1* in *ROR1* knockdown in BEAS2B. Fold Change expression was calculated. Level of significance was calculated using Two-way ANOVA (Sidak's multiple comparison test). **P<0.01 compared to shControl.

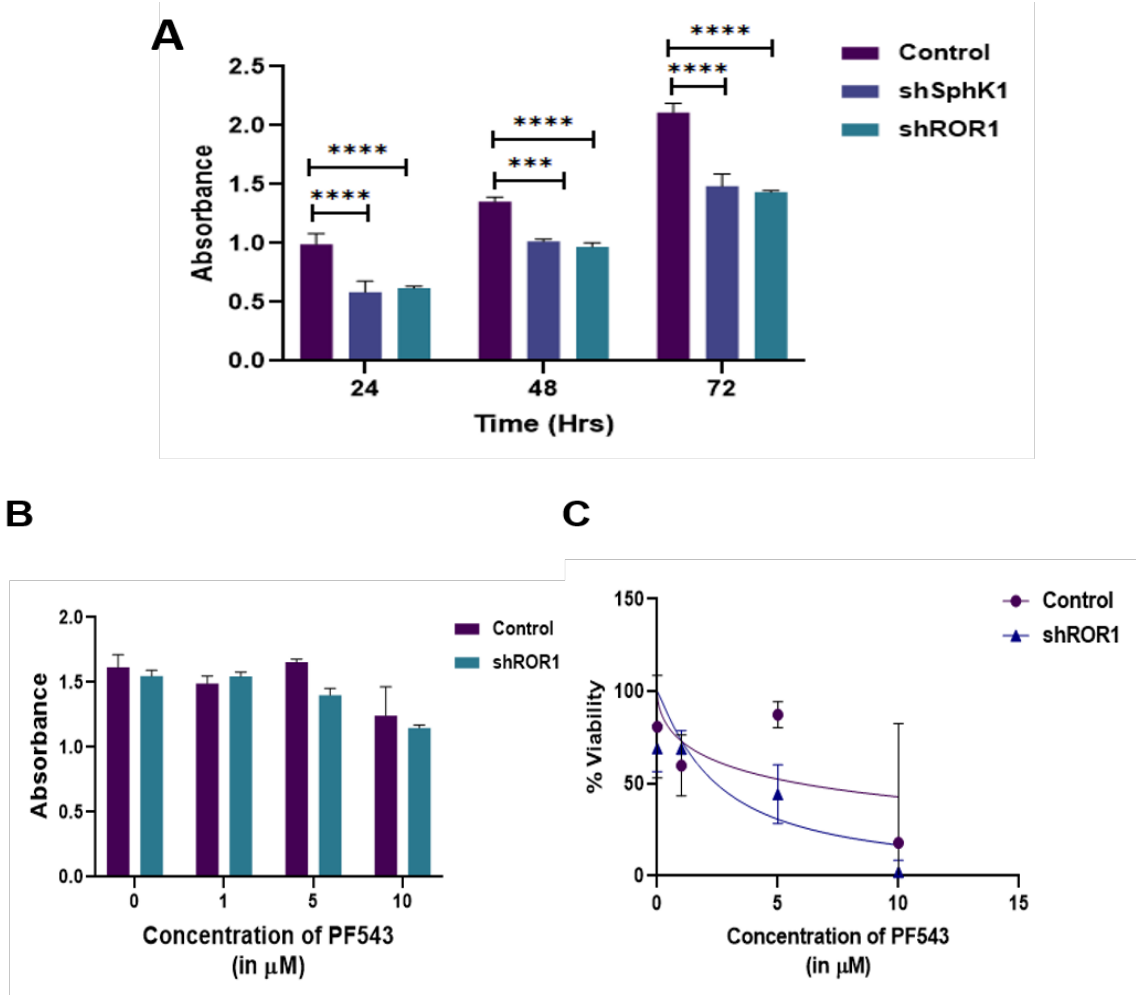


Figure 4. Cell Proliferation after *ROR1* and *SPHK1* Knockdown. A) 5000 cells of each (shControl, sh*SPHK1*, and shROR1) were seeded in 96 well plate. B-C) ROR1 knockdown A549 along with Control A549 were treated with various concentrations of PF543 for 48 hrs. MTT was used to assess the cell growth at different time points (24, 48, and 72 hrs). Graph showing B) Absorbance vs. PF543 concentration C) % viability vs PF543. Results represent an average of three experiments (N=3). Data is presented at mean ±SDEV. Tukey's multiple comparisons **P<0.01 compared to shControl.

mRNA transcript at 100 nM concentration (Figure 1A and B). However, the decrease in *ROR2* mRNA levels was more significant at 1 µM concentration (Figure 1A and B). To strengthen our findings, we employed an alternative approach in which we controlled intracellular S1P levels in lung cancer cells using PF-543, an SphK1 inhibitor. Lung cancer cells, A549 were treated with either a vehicle or a 1 µm dose of PF-543, and gene expression of *ROR1* and *ROR2* was evaluated using qRT-PCR. As shown in Figure 1C, we noticed that *ROR1* and *ROR2* expression elevated approximately twofold in PF-543-treated cells.

The preliminary results suggested that increasing intracellular S1P levels inhibits *ROR1* and *ROR2* transcription, whereas blocking S1P production using an SphK1 inhibitor, PF-543, induces *ROR1* and *ROR2* transcription. To confirm these findings, an shRNA was used to knockdown SPHK1 in HEK293T and BEAS2B cells, showing greater than 95% gene silencing efficiency (Figure 2A). Transient knockdown of SPHK1 in BEAS2B increased *ROR1* and *ROR2* expression, suggesting SPHK1 can alter *ROR1*/*ROR2* expression at the transcription level (Figure 2B).

ROR1/ROR2 knockdown promotes SPHK1 expression

Then, we asked whether *ROR1* and *ROR2* influence the expression of SPHK1 in lung cancer cells. *ROR1* and *ROR2* expression was silenced using siRNAs against *ROR1* and *ROR2*, and a 2-fold increase in SPHK1 expression was observed in *ROR1*-knocked down cells. However, SPHK1 expression was not changed in *ROR2*-knocked down cells (Figure 2C and 2D). Two shRNAs against the *ROR1* gene were used to knock down *ROR1* expression, with *shROR1*-A showing better efficacy than *shROR1*-B as shown in Figure 3A. Therefore, *shROR1*-A was employed for further experimental procedures. *ROR1* expression was reduced by approximately 65% in A549 cells with stable knockdown and by approximately 50% in BEAS2B cells (Figure 3B and 3C). However, decreased *ROR1* expression had no influence on SPHK1 mRNA expression, and a 50% decrease in SGPL1 mRNA expression in A549 cells as mentioned in Figure 3B. This suggests that *ROR1* and *ROR2* work to maintain intracellular S1P homeostasis, as *ROR1* lowers SGPL1 and thus increases S1P levels in the cells.

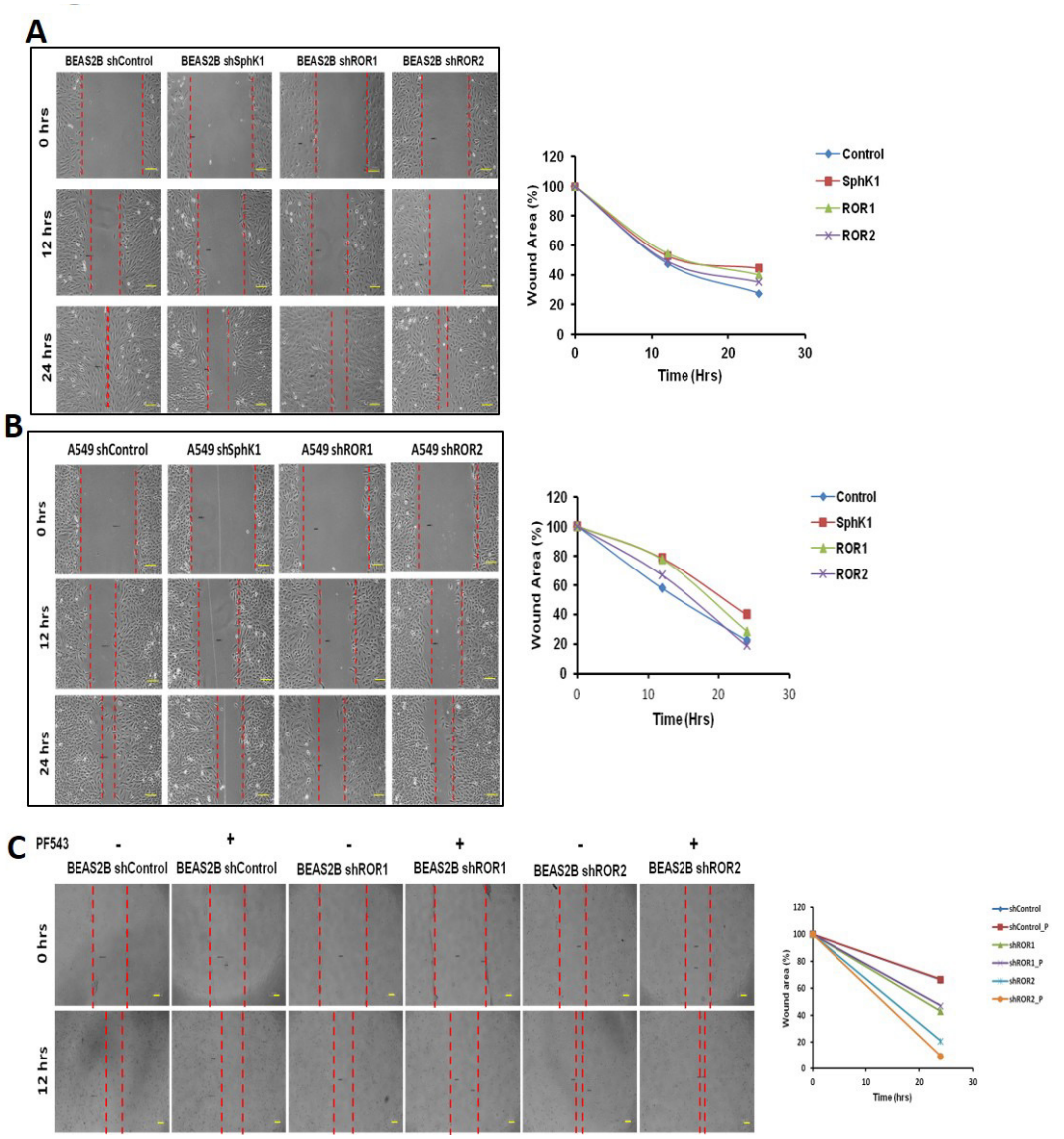


Figure 5. Wound Healing Assay. Change in migration ability of knockdown A) BEAS2B and B) A549 lung cancer cells. C) Change in migration ability of knockdown BEAS2B cells in the presence of PF543. Results represent an average of three experiments (N=3), Magnification, X100; Scale bar, 100 µm.

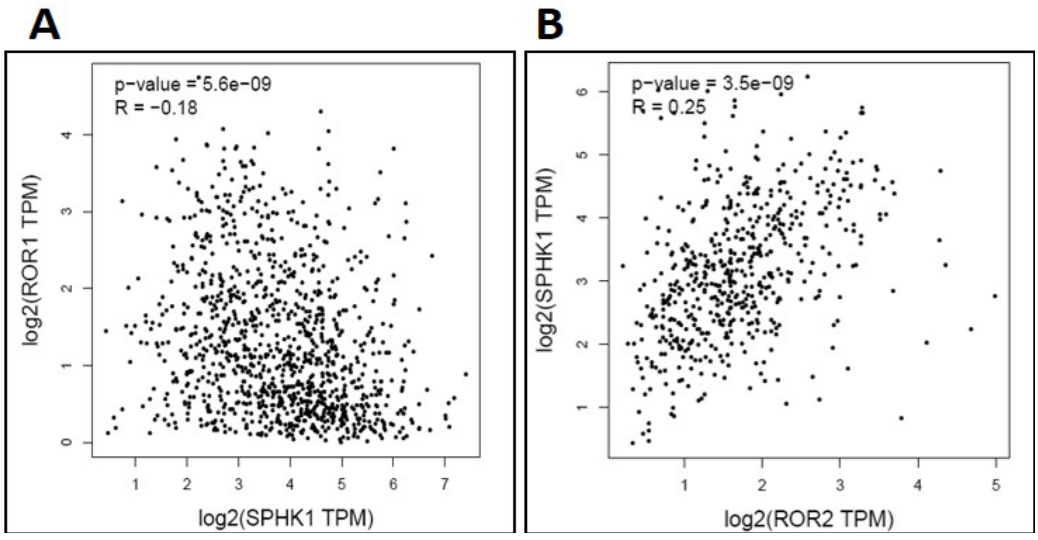


Figure 6. Correlation Analysis. Using GEPIA tool, correlation analysis was performed in lung cancer A) *SPHK1* vs. *ROR1*; B) *SPHK1* vs *ROR2*

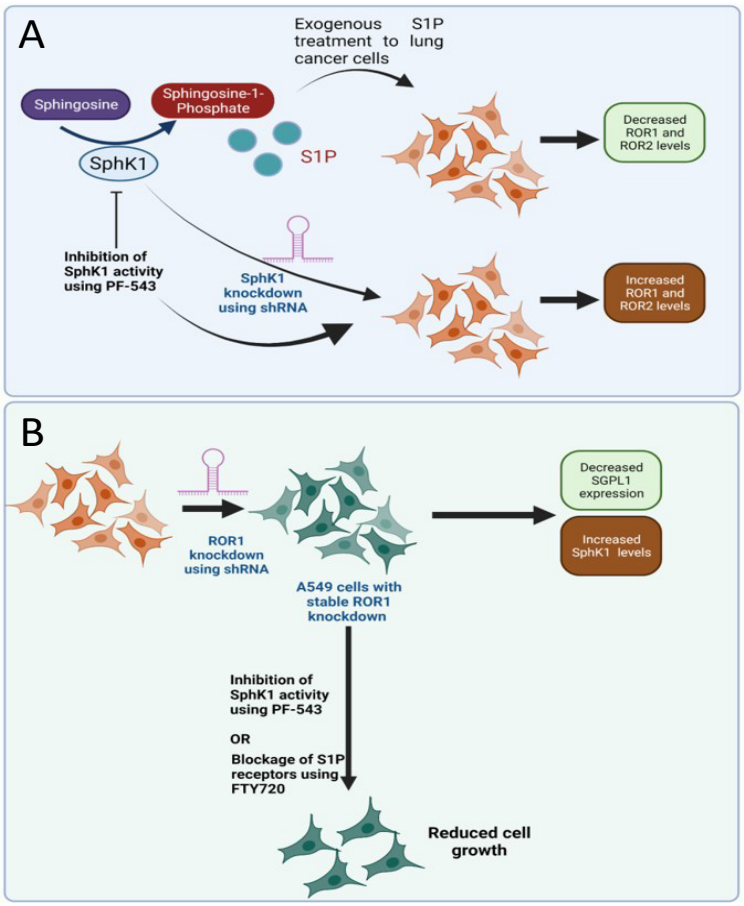


Figure 7. Scheme A: *SPHK1* catalyzes the synthesis of S1P, which promotes cell proliferation, cell survival, and angiogenesis. It mediates its actions through G-protein coupled receptors. An increase in the intracellular S1P results in a decrease in levels of *ROR1* and *ROR2*, whereas lowering the intracellular S1P either by treatment of *SPHK1* inhibitor or genetic knockdown increases *ROR1* and *ROR2* transcripts. Therefore, *ROR1* and *ROR2* may compensate for the decrease in the survival molecule (S1P). Scheme B: On the other side, *ROR1* knockdown cells increase *SPHK1* levels. *ROR1* knocked-down cells treated with *SPHK1* inhibitor and S1P receptor antagonist reduce cell growth in lung cancer cells

SPHK1 and ROR1 deficiency reduces the proliferation of lung cancer cells

To assess the role of two pathways individually on cell proliferation, *SPHK1* and *ROR1* knocked down cells were seeded on a 96-well plate, and cell proliferation at various time points was determined using an MTT assay. As shown in Figure 4A, SPHK1 and *ROR1*-knockdown cells respectively exhibited decreased cell growth compared to shControl cells.

Combinatorial inhibition of SphK1 and ROR1 significantly reduces cell proliferation

Since previous findings established the reciprocal role, the deficiency of one pathway can be compensated by the other. Therefore, to understand the effect of combined inhibition of both pathways, *ROR1* knocked-down cells or shControl cells were treated with PF543 at various doses. *ROR1* knockdown cells showed reduced viability than the shControl as well as untreated cells (Figure 4B-C). In addition to this, wound healing assay suggests that inhibiting both pathways individually decreases the migration efficiency of the cells (Figure 5A-B), whereas simultaneous inhibition of SPHK1 along with *ROR1* but not with *ROR2*, reduced the migration of lung cancer cells (Figure 5C). This finding suggests that simultaneous inhibition of both pathways might inhibit metastasis.

Furthermore, in order to assess the correlation between the two pathways in the patient samples, publicly available online tool GePIA was utilized. Correlation between the expression of two genes in lung adenocarcinoma and lung squamous cell carcinoma tumor was analyzed. SPHK1 was found to be negatively correlated with *ROR1* expression and positively correlated with *ROR2* expression (Figure 6A-B). Therefore, it becomes crucial to understand how the complex regulation of these pathways together can modulate the process of carcinogenesis.

Discussion

Here, we show that S1P signaling and *ROR1*/*ROR2* signaling have reciprocal regulation in lung cancer as well as normal lung epithelial cells (Figure 7). Treatment of the cells with S1P reduces *ROR1* and *ROR2* transcript levels, whereas inhibition of S1P synthesis either by a pharmacological inhibitor of SPHK1 (PF543) or genetic knockdown of SPHK1 by shRNA increases *ROR1* and *ROR2* (prominently). Thus, these findings indicate that both pathways have reciprocal regulation. *ROR1* and

ROR2 try to maintain intracellular S1P homeostasis, as the knockdown of *ROR1* decreases SGPL1 (an S1P catabolizing enzyme) and increases SPHK1 protein levels in the cells, thereby increasing S1P levels. Both pathways are known to promote cell growth and are involved in oncogenesis. Also, *ROR1* is known to mediate its effect via Wnt signaling [12], which is also one of the downstream targets of SPHK1 [13]. Therefore, it is plausible that deficiency of one pathway may compensate for the other pathway, whereas the excess of intracellular S1P (as it occurs in the exogenous treatment) reduces the other oncogenic pathway (*ROR1* and *ROR2*). However, simultaneous obstruction is more efficient at preventing cell growth.

To improve efficacy, minimize off-target toxicity, and provide a therapeutic benefit, targeted therapies require cellular protein expression that meets specific requirements. Lung cancer in India is a major health problem that is unfortunately diagnosed at an advanced stage, contributing to a poor prognosis. Therefore, to develop novel and efficient chemotherapeutic drugs, it is pertinent to fully understand the mechanism of lung carcinogenesis. Previous research found that *ROR1* protein expression was significantly higher in lung ADC tissues than adjacent non-tumor tissues [14]. SphK1 has been shown to be overexpressed in the NSCLC tumors. *SphK1* expression predicts the survival of NSCLC patients [15] and we had shown that NSCLC patients with high *SPHK1* expression had a shorter OS [16]. In addition, the expression of PLPP1, PLPP3, and S1PR1 has been shown to decrease in NSCLC tumors as compared to normal tissues and serve predictive biomarkers [16].

Patients in advanced stages and those with positive lymph node metastases had greater *ROR1* levels [17], and S1P signaling might be explored as targets for treatments that fulfill these requirements. Our findings revealed that when *ROR1* and *ROR2* knockdown cells were treated with PF-543, an inhibitor of SPHK1, cell growth was reduced, and when the knockdown cells were treated with FTY720 (fingolimod), a first-in-class S1P receptor modulator, cell growth was also reduced. Validation of our lung cancer result might provide an additional therapeutic feature. RORs and S1P signaling have recently been shown to be expressed in human tumors as part of a huge effort in target identification. Safingol, a SPHK1 inhibitor along with cisplatin, has been tested in a Phase I clinical trial for treating advanced-stage solid tumors [18]. Similarly, a *ROR1* monoclonal antibody has shown promise in a variety of cancers, including lung cancer [19]; however, its efficacy in solid tumors has yet to be determined. Combinatorial targeting of both pathways might lead to better efficacy for tumor cell killing and might increase the survival outcome in lung cancer patients.

Author Contribution Statement

AK: Conception, study design, interpretation of data, and critical reading and intellectual assessment of manuscript. RN: Study design, analysis and interpretation of data, preparation of the manuscript. RP: Analysis and interpretation of data, preparation of the manuscript.

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Funding statement

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Conflicts of interest

The authors declare that they have no competing interests.

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