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Lentiviral siRNA silencing of sphingosine-1-phosphate receptors $S1P_1$ and $S1P_2$ in smooth muscle ☆

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Abstract

Sphingosine-1-phosphate regulates diverse biological processes through five receptor types, $S1P_{1-5}$. Two or more S1P receptors are usually co-expressed on target cells. We have previously shown that smooth muscle cells of the gut co-express $S1P_1$ and $S1P_2$ receptors that could mediate distinct functions. In the absence of selective agonists and antagonists, we developed siRNA constructs to silence each receptor separately. The constructs were based on identical sequences in several mammalian species. A lentiviral vector-based system was used to deliver siRNA into HEK293T cells and smooth muscle cells. One $S1P_1$ and two $S1P_2$ siRNA constructs specifically inhibited ectopic expression of $S1P_1$ and $S1P_2$ receptors, respectively, as determined by immunocytochemistry and Western blot, and endogenous expression of $S1P_1$ and $S1P_2$ receptors in smooth muscle cells, as determined by RT-PCR. Measurement of PLC-β and Rho kinase activities, which mediate initial and sustained muscle contraction, confirmed receptor silencing and showed that contraction is mediated exclusively by $S1P_2$ receptors.

Keywords: siRNA; Lentiviral vector; Sphingosine-1-phosphate; S1P receptors; Smooth muscle contraction; PLC-β; Rho kinase

The bio-active sphingolipid metabolite, sphingosine-1-phosphate (S1P), mediates a wide spectrum of biological processes, including cell growth, survival, migration, motility, immunity, inflammation, and neural signaling [1–3]. It functions predominantly as a ligand for five G protein-coupled receptors (GPCRs), now named $S1P_{1-5}$ that regulate various signaling pathways. Two or more receptors are often co-expressed in the same cells. For example, both $S1P_1$ and $S1P_2$ receptors are co-expressed in smooth muscle cells of the gut [4], whereas $S1P_1$, $S1P_2$, and $S1P_3$ are co-expressed in coronary arterial smooth muscle cells [5]. The lack of specific antagonist or agonist of S1P receptors makes it difficult to distinguish the role of individual S1P receptors.

Molecular approaches to knockout or knockdown individual receptors provide an alternative strategy to characterize the distinct function of S1P receptors. Knockout mice for several S1P receptors have been established [6–9]. The $S1P_1$ knockout, however, leads to embryonic death due to defects in vascular maturation [6]. Neither $S1P_2$ nor $S1P_3$ knockout induces an obvious phenotype [8,9], whereas a $S1P_2/S1P_3$ double knockout displays partial lethality and vascular abnormalities [7]. Anti-sense oligodeoxynucleotides have been reported to knockdown S1P receptors [10–12]. More recently, small interfering RNAs (siRNAs) have been used to induce sequence-specific post-transcriptional gene silencing, providing a novel approach to loss-of-function studies. Various siRNA sequences for $S1P_1$ [13–16] and $S1P_2$ [17,18] have been used to silence the corresponding receptors in endothelial cells and C2C12 myoblasts, but the effectiveness of these chemically synthesized siRNAs in silencing target genes was variable due to limited transfection efficiency. A vector-based

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siRNA for $S1P_1$ receptors has been applied to human endothelial cell lines [19], but none so far has been applied to native smooth muscle cells.

Several lentiviral vector systems have been developed to deliver short hairpin RNA (shRNA) in mammalian cells and animals [20,21]. In comparison with other systems, the lentiviral system produces long-term high efficiency gene delivery due to chromosomal gene integration in a wide range of host cells, without eliciting toxicity or an immune response [22,23]. We have successfully employed pLL3.7 lentiviral vector to silence NIBP, the NF-κB-inducing-kinase binding protein in mammalian cells [24]. Here, we describe the use of a modified lentiviral vector system for delivery of siRNA to silence the expression of ectopic $S1P_1$ and $S1P_2$ receptors in HEK293T cells and endogenous $S1P_1$ and $S1P_2$ receptors in gastric smooth muscle cells. This approach should provide a powerful tool to characterize the distinct functions of S1P receptors in smooth muscle cells.

Materials and methods

Design and preparation of siRNA expression cassettes encoding siRNA for $S1P_1$ and $S1P_2$ receptors. The sense sequence of the siRNA cassettes specifically targeting the nucleotides of $S1P_1$ (Accession No. BC051023) or $S1P_2$ (AK085114) was designed through siRNA Target Finder (Ambion, Austin, TX). Oligonucleotides bearing 100% identity among available cDNA sequences of target genes from various species, including mouse, rat, and human, were selected by BLAST homology search. The localization of selected three siRNA targets (sense) for $S1P_1$ and $S1P_2$ receptors is shown in Fig. 1A.

A two-step polymerase chain reaction (PCR) strategy was employed because the reverse primer used in the one-step PCR strategy containing the complementary sequence of whole shRNA cassette (sense-loop-antisense) plus terminator and digestion site (around 90 bp) increased the risk of false synthesis. In addition, the hairpin structure of the long oligo primer reduced the efficiency of PCR-based cloning and made it difficult to obtain the right clone for some targets. Accordingly, two consecutive PCRs were performed using two separate reverse primers to generate a siRNA expression cassette (SEC) consisting of human U6 promoter and a hairpin siRNA cassette plus terminator. The first reverse primer containing the complementary sequence of the 3′-end of U6 promoter element and the oligonucleotides encoding sense siRNA plus the loop sequence were used in the first PCR with the forward U6 primer at the 5′-end of the U6 promoter. A fraction (1:100) of the first PCR product (25 cycles of 94 °C, 30 s, 55 °C, 45 s, and 72 °C, 1 min) was used as template in the second PCR (25 cycles under the same conditions) with the same U6 forward primer and the second reverse primer containing the loop and antisense siRNA-encoding sequence plus terminator and cloning sites. PCR was executed with AmpliTag DNA polymerase (PE Applied Biosystems, Foster, CA) as standard protocol. The second PCR products carrying the whole SEC plus cloning sites on both ends were gel-purified with Qiagen kit. The sequences of the U6 forward primer and representative first and second reverse primers for one $S1P_2$ siRNA ($S1P_2$a) are shown in Fig. 1B. The hairpin siRNA structure of $S1P_2$a is shown in Fig. 1C.

Lentiviral vector cloning. In preliminary studies, we found that addition of one *Bam*HI site after *Xba*I and an *Eco*RI site before *Xho*I to the SEC ensured high efficiency (100%) of harvesting the right clone by digestion with *Bam*HI/*Eco*RI. The purified PCR product was digested by *Xba*I/*Xho*I and subcloned into pLL3.7 vector, which encodes the CMV-promoted EGFP (enhanced green fluorescent protein) marker as internal control (Fig. 1D, gift from Dr. Van Parijs' Lab, MIT Center for Cancer Research) [25]. The resulting lentiviral siRNA vector was confirmed by restriction enzyme digestion with *Bam*HI/*Eco*RI and DNA sequencing with Flap primer (5′-CAGTGCAGGGGAAAGAATAGTAGAC-3′).

Lentivirus packaging. Packaging, purification, and titer determination of the lentivirus were performed as described previously [25,26]. All recombinant lentiviruses were produced by calcium phosphate-mediated transient transfection of HEK293T cells according to standard protocols. Briefly, HEK293T cells from Clontech were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/ml). The subconfluent cells in a 10-cm culture dish were co-transfected with lentiviral vector (10 μg), and the lentiviral packaging vectors pRSV-REV (2 μg), pMDLg/pRRE (5 μg), and the vesicular stomatitis virus G glycoprotein (VSVG) expression vector pMD2G (3 μg). The viruses were collected from the culture supernatants

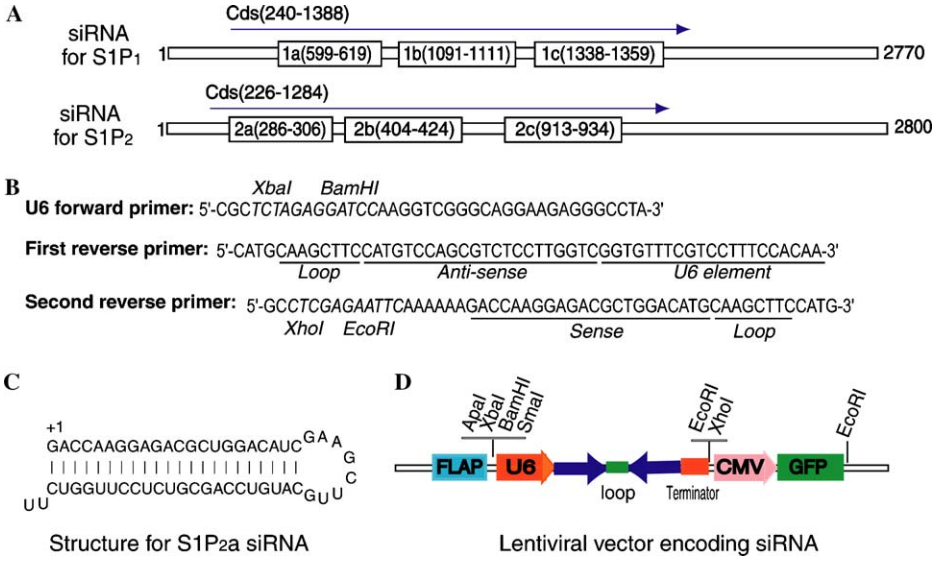

Fig. 1. Cloning of lentiviral siRNA vectors. (A) Locations of oligonucleotides of the genes encoding siRNA for $S1P_1$ and $S1P_2$ receptors. (B) Sequences for universal U6 forward primer and representative first and second reverse primers of $S1P_2$a construct. (C) Representative hairpin structure of $S1P_2$a. (D) Cloning sites and characteristics of lentiviral siRNA vector.

on days 2 and 3 post-transfection, concentrated by ultracentrifugation for 1.5 h at 25,000 rpm, and resuspended in phosphate-buffered saline (PBS). Titers were determined by infecting HEK293T cells with serial dilutions of concentrated lentivirus and counting EGFP-expressing cells after 48 h under fluorescent microscopy. For a typical preparation, the titer was approximately $4–10 \times 10^8$ infectious units per ml.

Lentiviral vector transduction in cultured smooth muscle cells. Cultured gastric smooth muscle cells were prepared from the circular muscle layer of rabbit distal stomach by sequential enzymatic digestion, filtration, and centrifugation as previously described [4,27]. Cultured cells were incubated with lentivirus at various 'multiplicity of infection' (MOI) for 24 h and cultured with DMEM plus 10% FBS.

Reverse transcription-PCR analysis in smooth muscle cells. Total RNA was isolated with the Trizol reagent and treated with TURBO DNase (Ambion, Austin, TX). Two micrograms of RNA was used to synthesize cDNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) with random hexanucleotide primer. PCR was performed on the cDNA using specific primers for $S1P_1$ and $S1P_2$ receptors as described previously [4]. Rabbit GAPDH primers (sense 5′-TCACCATCTTC CAGGAGCGA-3′, antisense 5′-CACAATGCCGAAGTGGTCGT-3′) were used as internal control. PCR products were purified and confirmed by sequencing.

Fluorescent immunocytochemistry. HEK293T cells were co-transfected with lentiviral siRNA vectors and Myc-tagged $S1P_1$ or $S1P_2$ plasmids (gift from Dr. Sarah Spiegel, Department of Biochemistry, Virginia Commonwealth University) by the calcium phosphate precipitation technique. After 2–3 days, cells were fixed with 4% paraformaldehyde for 30 min, permeated with 0.5% Triton X-100 PBS for 30 min, pre-blocked with 10% normal goat serum for 30 min and incubated with the primary mouse anti-Myc monoclonal antibody at 1:400 at room temperature for 2 h. After washing, Alexa Fluor 594 (red) secondary goat anti-mouse antibody (Invitrogen, Carlsbad, CA) 1:200 was applied for 30 min. Double fluorescence (red for Myc-$S1P_1$ or $S1P_2$ and green for EGFP) was analyzed under fluorescent microscopy.

Western blot analysis. Transfected HEK293T cells were solubilized in Triton X-100-based lysis buffer containing 20 mM Tris–HCl (pH 7.4), 1% Triton X-100, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Lysates were incubated at 4 °C for 30 min, and nuclear and cellular debris was cleared by centrifugation at 20,000*g* for 10 min at 4 °C. Equal amounts of protein (10 μg) were incubated for 1 h at 37 °C in SDS sample buffer, fractionated by SDS–PAGE in Tris–glycine buffer, and transferred to nitrocellulose membrane. The SeeBlue pre-stained standard (Invitrogen) was used as molecular weight reference. Blots were blocked in 5% nonfat dried milk/Tris-buffered saline (pH 7.6) plus 0.1% Tween 20 (TBS-T) for 1 h and then incubated overnight at 4 °C with anti-Myc monoclonal antibody (1:1000) in TBS-T. After incubation for 1 h with horseradish peroxidase-conjugated anti-mouse antibody (1/2000; 10 μg/ml) in TBS-T, immunoreactive proteins were visualized using SuperSignal West femto maximum sensitivity substrate kit (Pierce, Rockford, IL).

Assay for PLC-β activity. Phospholipase C (PLC)-β activity was determined from the formation of total inositol phosphates using ion-exchange chromatography as described previously [28]. Briefly, cultured smooth muscle cells were labeled with myo-[^{3}H]inositol (0.5 μCi/ml) for 24 h; the cells were washed with PBS and treated with S1P (1 μM) for 30 s in 1 ml of 25 mM Hepes buffer (pH 7.4). The reaction was terminated by addition of 940 μl chloroform–methanol–HCl (50:100:1). The samples were extracted with 310 μl chloroform and 340 μl H_2O, and the phases were separated by centrifugation at 1000*g* for 15 min. The upper aqueous phase was applied to DOWEX AG-1 column, and [^{3}H]inositol phosphates were eluted with 0.8 M ammonium formamate–0.1 M formic acid. Radioactivity was determined by liquid scintillation and expressed as counts per minute (cpm).

Assay for Rho kinase activity. Rho kinase activity was determined by immuno-kinase assay in cell extracts as described previously [29]. Immunoprecipitates with anti-Rho kinase-2 antibody were washed twice with a kinase buffer containing 10 mM $MgCl_2$ and 40 mM Hepes (pH 7.4) and then incubated for 5 min on ice with 5 μg of myelin basic protein. Rho kinase assay was initiated by the addition of 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) and 20 μM ATP, followed by incubation for 10 min at 37 °C. ^{32}P-labeled myelin basic protein was absorbed onto phosphocellulose disks, and free radioactivity was removed by repeated washings with 75 mM phosphoric acid. The amount of radioactivity on the disks was measured by liquid scintillation.

Results

Efficiency and specificity of lentiviral siRNA for $S1P_1$ and $S1P_2$ receptors

To determine the efficiency and specificity of lentiviral siRNA for $S1P_1$ and $S1P_2$ receptors, immunocytochemistry and Western blot analysis were performed in HEK293T cells after cotransfection of the lentiviral siRNA constructs with Myc-tagged $S1P_1$ or $S1P_2$ plasmid. As shown by both techniques in Fig. 2, $S1P_2$a and $S1P_2$b siRNAs were most effective, and $S1P_2$c less effective in silencing the ectopic expression of $S1P_2$ receptors. Expression of siRNAs for unrelated genes (e.g., NIBP and $S1P_1$ receptor) had no effect on the expression of Myc-tagged $S1P_2$ fusion protein. Similar studies were done for $S1P_1$ siRNA, but only one of three $S1P_1$ siRNA constructs, labeled $S1P_1$b, was effective in silencing the expression of $S1P_1$ receptors (Fig. 3). Here also, expression of $S1P_2$a siRNA had no effect on expression of Myc-tagged $S1P_1$ fusion protein. These data suggested that two $S1P_2$ and one $S1P_1$ siRNA constructs efficiently and specifically silenced corresponding gene expression.

Transduction efficiency of lentiviral siRNA in smooth muscle cells

A series of preliminary studies were done using pLL3.7 lentiviral vector encoding EGFP for convenient observation to determine whether a lentiviral vector can transduce rabbit cultured gastric smooth muscle cells. No difference in efficiency was observed with passage 1–4 and with infection at 6 or 24 h after cell plating. Transgene expression started at 20–24 h after addition of lentivirus and was maintained for over 3 months. The efficiency of pLL3.7 lentiviral vector transduction in smooth muscle cells was 40–50% at MOI of 100. Addition of polybrene (10 μg/ml) increased the efficiency to 70–80%. The lentiviral siRNAs for $S1P_1$ and $S1P_2$ receptors generated similar efficiencies in transducing smooth muscle cells (Fig. 4).

Stable silencing of endogenous $S1P_1$ and $S1P_2$ receptors in smooth muscle cells

In order to determine whether the lentiviral siRNA constructs for $S1P_1$ and $S1P_2$ receptors silenced endogenous $S1P_1$ or $S1P_2$ receptors in cultured smooth muscle cells, conventional RT-PCR analysis was performed using primers of rabbit $S1P_1$ and $S1P_2$ receptors as described previously [4]. As shown in Fig. 5, $S1P_1$b siRNA was effective in silencing endogenous expression of $S1P_1$ receptors and $S1P_2$a siRNA

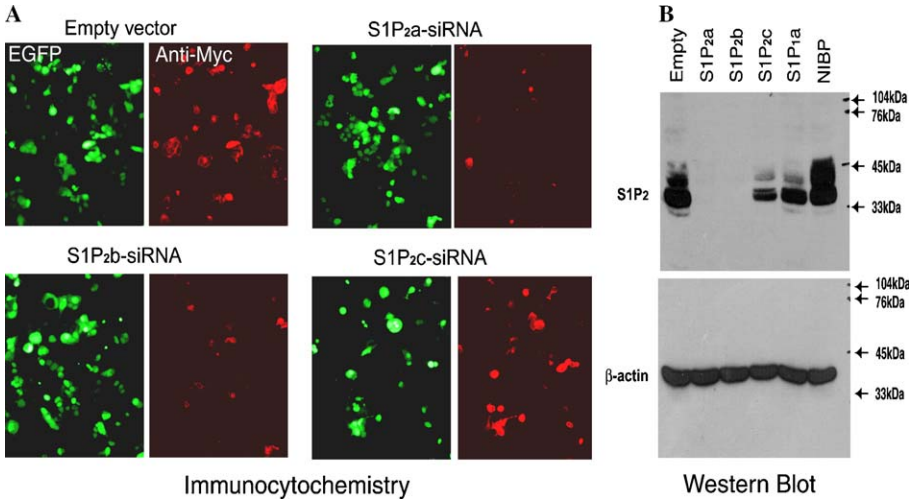

Fig. 2. Silencing of ectopic expression of $S1P_2$ receptors by two $S1P_2$ siRNA constructs ($S1P_2$a and $S1P_2$b). HEK293T cells were co-transfected with Myc-tagged $S1P_2$ plasmid and various lentiviral siRNA vectors in the ratio of 1:4. After 3 days, immunocytochemical staining (A) and Western blot analysis (B) were performed using anti-Myc monoclonal antibody. The green color denotes EGFP internal marker and the red denotes the expression level of Myc-tagged $S1P_2$ receptors. The empty lentiviral vector and additional siRNA lentiviral vectors of unrelated genes ($S1P_1$ and NIBP) were used as controls. Expression of β-actin was used as a loading control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

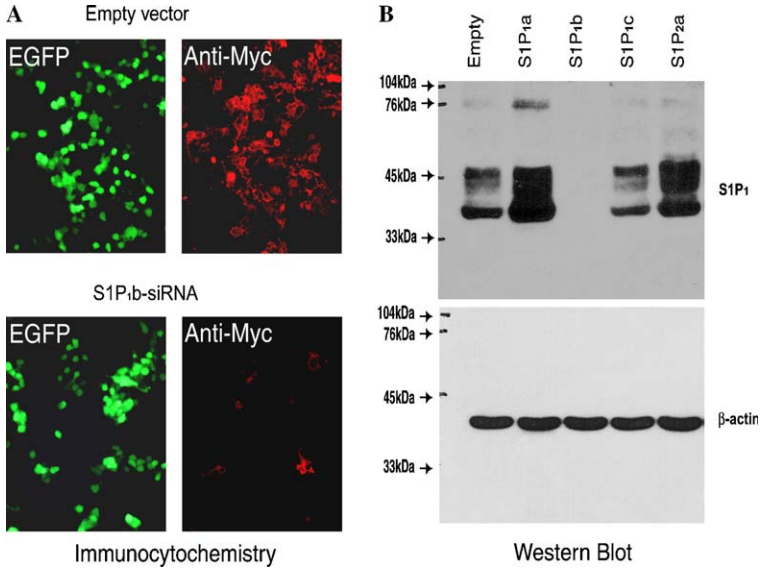

Fig. 3. Silencing of ectopic expression of $S1P_1$ receptors by one $S1P_1$ siRNA construct ($S1P_1$b). HEK293T cells were co-transfected with Myc-tagged $S1P_1$ plasmid and various lentiviral siRNA vectors in the ratio of 1:4. After 3 days, immunocytochemical staining (A) and Western blot analysis (B) were performed as described. The empty lentiviral vector and additional siRNA lentiviral vectors of unrelated genes ($S1P_2$) were used as controls. Expression of β-actin was used as a loading control.

was effective in silencing endogenous expression of $S1P_2$ receptors, while $S1P_1$c and $S1P_2$c siRNAs were less effective. As predicted, the lentiviral vector-mediated siRNA produced long-term inhibition (4 weeks shown in Fig. 5) of endogenous expression of $S1P_1$ and $S1P_2$ receptors.

Functional analysis of stable silencing for $S1P_1$ and $S1P_2$ receptors in smooth muscle cells

Two important biological activities, PLC-β and Rho kinase, were examined to determine the efficiency of silencing $S1P_1$ and $S1P_2$ receptors in smooth muscle cells. Our previous studies had shown that both PLC-β1 and PLC-β3 are activated by S1P in smooth muscle cells leading to IP_3-dependent Ca^{2+} release and a transient initial contraction [4]. The initial contraction was followed by a sustained contraction that was dependent on activation of RhoA/Rho kinase [4,30].

As shown in Fig. 6, 6 weeks after infection with siRNA lentivirus, stable silencing of $S1P_2$ receptors virtually abolished S1P-stimulated PLC-β activity measured at 30 s and Rho kinase activity measured at 5 min after treatment of

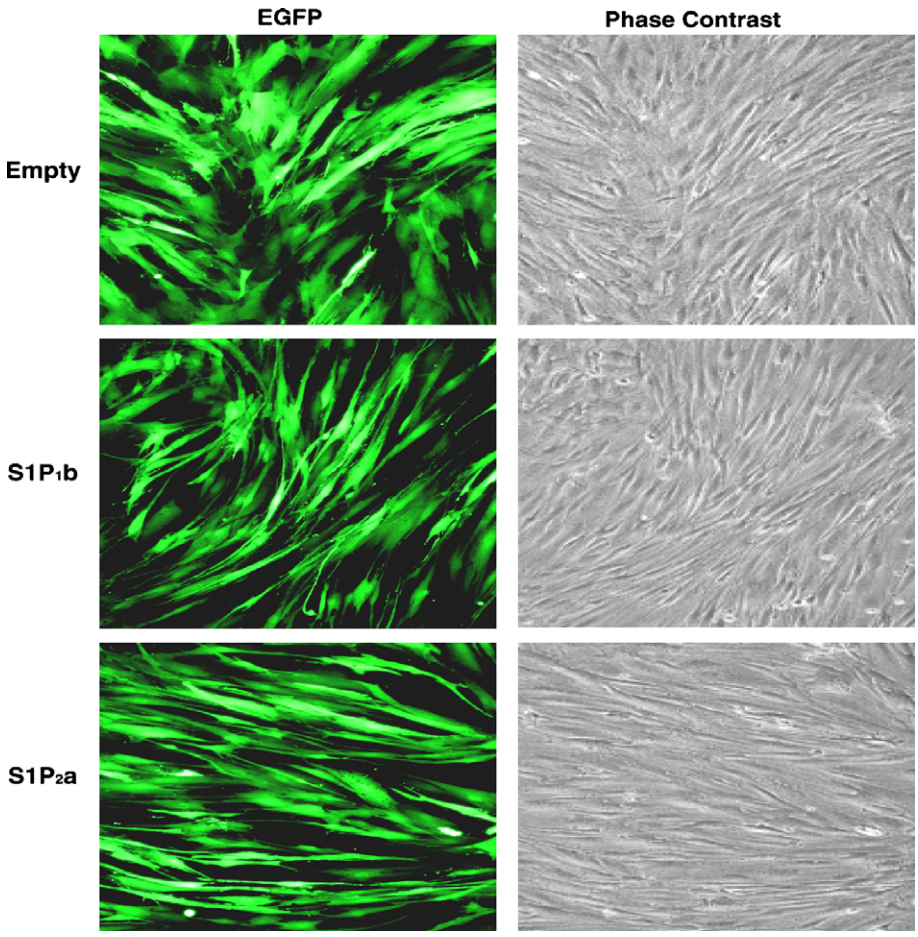

Fig. 4. Transduction efficiency of lentiviral siRNA vectors in rabbit gastric smooth muscle cells. Smooth muscle cells in first passage were infected with lentivirus carrying indicated siRNA expression vectors at MOI of 100. Fluorescence and phase-contrast micrographs (100×) were taken 7 days later.

cultured smooth muscle cells with S1P (1 μM). Silencing of $S1P_1$ receptors had no effect on either activity. These results confirmed functional silencing of the receptors and implied that activation of PLC-β and Rho kinase was mediated exclusively by $S1P_2$ receptors.

Discussion

Sphingosine-1-phosphate (S1P) regulates diverse biological processes through its receptors [1–3]. Functional characterization of individual S1P receptors is difficult because two or more receptors are usually co-expressed in the same cell, and selective agonists and antagonists are lacking except for $S1P_1$ receptors. The siRNA silencing of S1P receptors provides an alternative molecular approach in the absence of selective pharmacological tools. In the present study, we have identified two siRNAs for $S1P_2$ receptors and one siRNA for $S1P_1$ receptors capable of stable and selective silencing of these receptors. We have also developed a lentiviral system for efficient delivery of these siRNAs to silence ectopic expression of $S1P_1$ and $S1P_2$ in HEK293T cells and endogenous expression in rabbit smooth muscle cells. Effective silencing was demonstrated by immunocytochemistry and Western blot analysis of Myc-tagged receptors in HEK293T cells and by RT-PCR and functional analysis in smooth muscle cells. Silencing of $S1P_2$ but not $S1P_1$ receptors abolished S1P-stimulated PLC-β and Rho kinase activities. These activities are essential in mediating the initial and sustained phases of smooth muscle contraction, respectively [30].

The lentiviral vector-mediated gene delivery system has several advantages over other viral or nonviral gene delivery systems, such as the high efficiency of gene transfer into both dividing and non-dividing cells, long-term infection due to gene integration into the chromosome of host cells, and the absence of toxicity or immune response [22,23]. In other studies, vascular smooth muscle cells were shown to be efficiently transduced in vitro and in vivo by lentiviral vectors [31–33]. The present study showed that a modified lentiviral vector system efficiently delivered siRNAs for $S1P_1$ and $S1P_2$ receptors into visceral (gastric) smooth muscle cells.

The siRNAs used to silence $S1P_1$ and $S1P_2$ receptors in other studies were chemically synthesized [13–18], in vitro transcribed [34] or expressed via plasmid [19]. In the present study, we tested different siRNA sequences for $S1P_1$ or $S1P_2$ receptors based on identical sequences for the receptors in several mammalian species (mouse, rat,

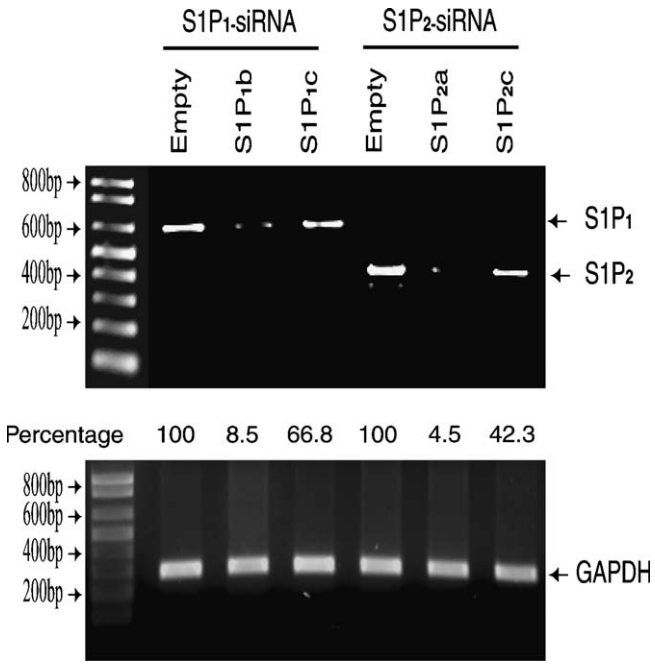

Fig. 5. Lentiviral siRNA silencing of endogenous $S1P_1$ and $S1P_2$ receptors in rabbit gastric smooth muscle cells. Total RNA was isolated from cultured smooth muscle cells 4 weeks after infection and conventional RT-PCR analysis for rabbit $S1P_1$ or $S1P_2$ receptors were performed. The percentage indicates the relative expression level compared with empty control lentivirus after normalization with GAPDH. Lentiviral siRNA $S1P_1$b and $S1P_2$a are most effective in silencing corresponding receptors.

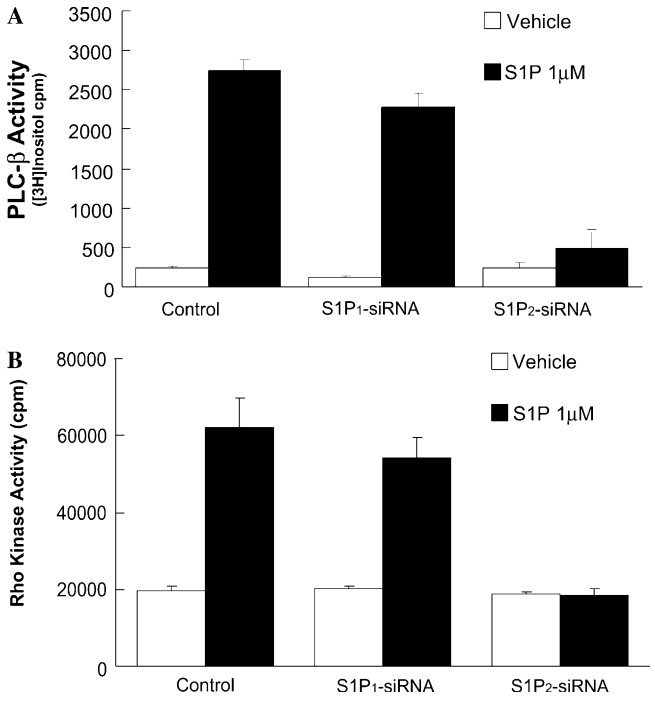

Fig. 6. Functional consequence of selective silencing of $S1P_1$ and $S1P_2$ by lentiviral siRNAs in rabbit gastric smooth muscle cells. Smooth muscle cells in first passage were infected with lentivirus carrying empty control, $S1P_1$b or $S1P_2$a siRNA expression vectors at MOI of 100 in the presence of polybrene (10 μg/ml). After a further 6-week culture period (third passage), the cells were treated with S1P (1 μM) for 30 s for measurement of PLC-β activity (A), and for 5 min for measurement of Rho kinase activity (B).

human, bovine, dog, rabbit, guinea pig, etc.) and used a lentiviral expression vector to demonstrate specific and stable knockdown of the endogenous receptors in smooth muscle cells. These siRNA constructs, therefore, should be effective in all these species. In this study, they were effective in silencing ectopic rat $S1P_1$ and $S1P_2$ receptors, as well as endogenous rabbit $S1P_1$ and $S1P_2$ receptors, for which only partial gene sequences are known.

In conclusion, we have used a lentiviral siRNA delivery system to efficiently transduce gastric smooth muscle cells and selectively silence $S1P_1$ and $S1P_2$ receptors. The results demonstrate that contraction by S1P is exclusively mediated by $S1P_2$ receptors.

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