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S1P Receptor 2 Signals Through the RGS-RhoGEF, LARG, To Promote Smooth Muscle Cell Differentiation

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Abstract

Objective—The goals of this study were to identify the signaling pathway by which S1P activates RhoA in SMC and to evaluate the contribution of this pathway to the regulation of SMC phenotype.

Methods and Results—Using a combination of receptor-specific agonists and antagonists we identified S1PR2 as the major S1P receptor sub-type that regulates SMC differentiation marker gene expression. Based upon the known coupling properties of S1PR2 and our demonstration that over-expression of $G\alpha_{12}$ or $G\alpha_{13}$ increased SMC specific promoter activity, we next tested whether the effects of S1P in SMC were mediated by the RGS-RhoGEFs (LARG, PRG, p115). Although each of the RGS-RhoGEFs enhanced actin polymerization, MRTF-A nuclear localization, and SMC-specific promoter activity when over-expressed in 10T1/2 cells, LARG exhibited the most robust effect and was the only RGS-RhoGEF activated by S1P in SMC. Importantly, siRNA-mediated depletion of LARG significantly inhibited the activation of RhoA and SMC differentiation marker gene expression by S1P. Knockdown of LARG had no effect on SMC proliferation, but promoted SMC migration as measured by scratch wound and transwell assays.

Conclusion—These data indicate that S1PR2-dependent activation of RhoA in SMC is mediated by LARG and that this signaling mechanism promotes the differentiated SMC phenotype.

Introduction

Smooth muscle cell (SMC) differentiation is critical during vascular development, and alterations in SMC phenotype contribute to a number of cardiovascular pathologies including atherosclerosis, hypertension, and restenosis [1]. Although our understanding of SMC differentiation has been complicated by the plasticity of this cell-type and the fact that SMC originate from multiple locations within the embryo [2], the transcription mechanisms involved are starting to become clear. Extensive evidence indicates that Serum Response Factor (SRF) regulates nearly all of the SMC differentiation marker genes by binding to CArG cis elements within their promoters [1]. The discovery of the SRF co-factor, myocardin, was an extremely important advance because this cardiac and smooth muscle selective transcription factor strongly activates SMC-specific transcription in many cell-types and is required for SMC differentiation in the developing aorta [3]. Two Myocardin-Related Transcription Factors, MRTF-A and MRTF-B, have also been identified. Although expressed more widely than myocardin [4], studies have demonstrated that the MRTFs up-regulate SMC-specific transcription and are required for endogenous SMC differentiation marker gene expression in at least some SMC subsets [5, 6]. Importantly, genetic disruption of MRTF-B in the mouse

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resulted in defective SMC differentiation of the cardiac neural crest cells that populate the brachial arches [7, 8], while loss of MRTF-A inhibited the expression of SM α-actin that occurs in mammary myoepithelial cells during lactation [9, 10].

The identification of the signaling mechanisms that regulate the myocardin factors will be important for our understanding of the control of SMC phenotype. The Treisman laboratory was the first to demonstrate that MRTF nuclear localization is regulated by the small GTPase, RhoA [11], and we and others have shown that RhoA/MRTF signaling is a critical determinant of SMC-specific transcription [5, 6, 12, 13]. Furthermore, RhoA activity was shown to be required for the induction of SMC differentiation marker gene expression by angiotensin II, TGF-β, intracellular calcium, and mechanical stretch suggesting that this pathway plays an integral role in the regulation of SMC phenotype [14-18].

RhoA activity is tightly regulated by GTPase Activating Proteins (GAPs) that facilitate RhoA's intrinsic GTPase activity (inhibiting RhoA), Guanine Exchange Factors (GEFs) that facilitate exchange of GDP for GTP (activating RhoA), and Rho GDP-dissociation inhibitors (RhoGDIs) that sequester RhoA into an inactive fraction. However, the major regulators of RhoA activity in SMC are not completely clear, and even less is known about the signaling mechanisms by which these proteins are activated. We were the first to demonstrate that the lipid agonist, sphingosine 1-phosphate (S1P), up-regulates SMC-specific gene expression by activating RhoA [5]. The goal of the current study was to identify the signaling pathway that mediates the effects of S1P on RhoA in SMC and to evaluate the contribution of this pathway to the regulation of SMC phenotype.

Materials and Methods

Plasmids and Reagents

S1P, FTY-720, SEW2871, and JTE-013 were purchased from Cayman chemical. LARG, PRG, and P115 cDNAs were kind gifts from Dr. T. Kozasa (University of Tokyo). $G\alpha_{12}$ and $G\alpha_{13}$ cDNAs were kind gifts from Dr. P. Gierschik (Ulm University, Germany). The $G\alpha_q$ cDNA was a kind gift from Dr. G. Johnson (University of North Carolina, Chapel Hill, NC). All cDNAs were sub-cloned into pcDNA 3.1. Antibodies to LARG and PRG were kind gifts of Dr. Keith Burridge (University of North Carolina, Chapel Hill, NC). The p115RhoGEF Ab was purchased from Santa Cruz.

Transient Transfections and Reporter Gene Assays

The 10T1/2 and SMC cell cultures, transient transfections, and promoter luciferase assays have been previously described [5, 13]. Statistical comparisons between groups were made using the Student's *t* test with statistical significance accepted at $p<0.05$.

GST G17A pull-downs

GST-Rho(G17A) was a kind gift of Dr. K. Burridge and pull-downs were performed as previously described [19]. In brief, SMCs were plated and starved for 24 hours and then treated with S1P (10uM) or 10% serum for 3.5 or 9 minutes. Protein lysates were incubated with 20ug GST-Rho(G17A) beads at 4 °C for 3 hours. Complexes were washed 3× in lysis buffer prior to analysis by Western blot.

Immunoflourescence

Phalloidin staining and measurements of MRTF localization were described previously [13].

siRNA knockdowns

siRNA oligos were purchased from Invitrogen and transfections were performed using the Dharmafect transfection reagent as per protocol. RGS-RhoGEF knockdown was confirmed in all experiments by Westerns Blotting. See supplemental methods for additional details.

RhoA Activity Assays

The G-LISA RhoA activation assay kit (Cytoskeleton) was used as per protocol.

Quantitative RTPCR

The Trizol reagent was used to extract RNA from control and LARG K/D cells. SM22, SM α-actin, and 18S mRNAs were measured as previously described. See supplemental methods for additional details.

Migration Assays

Transwell assays were preformed as previously described [20]. For wound healing assays, confluent cultures of Control and LARG knockdown SMC were scraped with a P200 tip and then placed on an inverted microscope equipped with a heated, humidified, and O_2/CO_2 perfused stage. Pictures taken every 3.5 minutes for 9 hours were assembled into movies using Quicktime. At the end of each experiment cell lysates were subjected to Western Blot to ensure LARG knockdown. See supplemental methods for additional details.

BRDU incorporation

Assays in control and LARG knockdown SMC were conducted as previously described [21].

Results

S1P signals through S1PR2 to activate SMC differentiation marker gene expression

As shown in figure 1, treatment of serum-starved mouse SMC with S1P strongly up-regulated the expression of multiple SMC differentiation marker genes including smooth muscle myosin heavy chain (SM MHC), SM22, SM α-actin and calponin. These results are in excellent agreement with previous studies from our lab and others [5, 22, 23]. S1P signals through a family of G-protein coupled receptors (S1PR1-5) [24]. Because S1P receptor expression levels and coupling properties can vary significantly, it is often difficult to identify the receptor sub-type that mediates a specific S1P-dependent response. RT PCR analysis revealed that our aortic SMC cultures express S1PR1, S1PR2, S1PR3, and low levels of S1PR5 (data not shown). To help determine which of these receptor subtypes was responsible for activating SMC-specific transcription, we utilized several receptor sub-type specific agonists and antagonists. SMC differentiation marker gene expression in SMC was not enhanced by SEW2871, an S1PR1-specific agonist, or FTY720, an S1P agonist that activates all S1P receptors but S1PR2 (Fig 1b). However, as expected by the known coupling patterns of the S1P receptors (see below), these agonists did activate MAPK. To examine the role of S1PR2 more directly, we pre-treated SMC cells with the S1PR2-specific antagonist, JTE-013. Importantly, JTE-013 inhibited the effects of S1P on SM α-actin and SM22 protein expression in SMC (Fig 1c). To determine whether these effects were mediated transcriptionally we also tested JTE-013 on S1P-dependent SMC-specific promoter activity in multi-potential 10T1/2 cells. This SMC precursor line is frequently used to study SMC-specific gene activation [25-28] and our RT PCR analyses indicated that 10T1/2 cells express all 5 S1P receptors (data not shown). As shown in Figure 1d JTE-013 dose-dependently inhibited SM22 and SM a-actin promoter activity. JTE-013 did not affect the expression of house keeping genes or the activity of a minimal thymidine kinase promoter in these studies.

$G\alpha_{12/13}$ and the RGS-RhoGEFs stimulate SMC-specific transcription

The G-protein-dependent signaling pathways activated by S1PR1-3 have been fairly well-characterized in a number of heterologous cell culture systems (see [24] for review). S1PR1 couples almost exclusively to Gαi and regulates MAPK activity and cell growth while S1PR2 and S1PR3 have been shown to couple somewhat promiscuously to $G\alpha_{12/13}$ and Gαq. Since both $G\alpha_{12/13}$ and Gαq have been associated with RhoA activation (see [29] for review), we tested whether over expression of these G-proteins was sufficient to stimulate SMC-specific transcription. As shown in figure 2, over-expression of $G\alpha_{12}$ (Q231L) or $G\alpha_{13}$ (Q226L) in SMC activated SM22 and SM α-actin promoter activity approximately 2-3 fold while expression of Gαq (Q209L) had no effect. In contrast, over-expression of $G\alpha_{12}$ (Q231L) or $G\alpha_{13}$ (Q226L) did not activate the c-fos promoter while Gαq (Q209L) did suggesting at least some specificity between the transcriptional responses to these G-protein sub-types.

Previous studies have shown that RhoA activation by $G\alpha_{12/13}$-coupled receptors is mediated by the Regulator of G-protein Signaling (RGS) sub-family of RhoGEFs that includes p115RhoGEF (p115), PDZ-RhoGEF (PRG), and Leukemia Associated Rho GEF (LARG) [29]. The RGS-RhoGEFs bind specifically to $G\alpha_{12/13}$ (through the RGS domain) and function as GAPs for these G-proteins. Importantly, this interaction also stimulates the GEF activity of RGSRhoGEFs providing a direct mechanistic link between $G\alpha_{12/13}$-coupled receptors and RhoA activation. All three RGS-RhoGEFs are expressed in rat aortic SMC [30, 31] and interestingly, Becknell et. al. demonstrated that LARG expression in the lung and GI tract of the mouse was specific for the SMC layers in those organs [31]. Of additional importance to our studies, the RGS-RhoGEFs, unlike most other GEFs, specifically activate RhoA but not the closely-related small GTPases, Rac and Cdc42. Based on these observations and our data implicating S1PR2 and $G\alpha_{12/13}$ in the activation of SMC-specific gene expression, we hypothesized that one or more of the RGS-RhoGEFs mediated the effects of S1P on RhoA in SMC and that this family of RhoA activators were important regulators of SMC phenotype.

To begin to examine the role of the RGS-RhoGEFS in SMC, we tested whether ectopic expression of these proteins in 10T1/2 cells could up-regulate SMC-specific promoter activity. As shown in figure 3 over-expression of LARG strongly increased (8-16 fold) the activities of the SM MHC, SM α-actin, and SM22 promoters. The effects of PRG were somewhat less robust (~4-7 fold), but this difference may reflect slightly lower PRG expression levels in our experiments (see inset). Somewhat surprisingly, expression of p115 had only minor effects on SM MHC and SM22 promoter activity and no significant effect on SM α-actin promoter activity. Co-expression of C3 toxin, completely inhibited the transcriptional effects of all three RGS-RhoGEFS strongly supporting the involvement of RhoA in this response (data not shown). Since the effects of RhoA signaling on SMC-specific transcription are thought to be due to alterations in actin polymerization that regulate MRTF nuclear localization, we also tested the effects of the RGS-RhoGEFs on these parameters. As shown in figure 3, over-expression of either LARG, PRG, or p115 in 10T1/2 cells enhanced actin polymerization (Fig. 3b) and localization of GFP-MRTF-A to the nucleus (Fig. 3c).

The effects of S1P were mediated by LARG

To identify the RGS-RhoGEFs that were activated by S1P in SMC, we used an assay described by the Burridge lab that employs a nucleotide free variant of RhoA (G17A) to precipitate activated RhoGEFs from cell lysates [19]. As shown in figure 4a, treatment of SMC with S1P resulted in a dramatic increase in the amount of LARG present in GST-RhoA(G17A) precipitates suggesting that S1P activates LARG in this model. LARG was also activated by 10% serum to a somewhat lesser extent. We observed little to no activation of PRG or p115 by S1P or serum.

We next used a siRNA approach to test whether LARG was required for S1P-mediated activation of RhoA in SMC. In parallel experiments we also knocked-down all three RGS-RhoGEFs in combination to examine potential compensatory effects within this family. We consistently achieved greater than 90% knock-down of LARG when compared to SMC transfected with a control siRNA that targets GFP, and we observed no up-regulation of either PRG or p115 in LARG knock-down cells (figure 4b). As measured by the Rhotekin-based assay (Cytoskeleton inc.), knockdown of LARG in SMC inhibited S1P-dependent RhoA activation by approximately 65% (Figure 4c). In addition, depletion of LARG was as effective as the triple knock-down providing further evidence that LARG is the major GEF within this family that mediates the activation of RhoA by S1P. LARG knockdown had no effect on S1P- or PDGF-BB mediated activation of MAPK (Fig 4d).

Importantly, knockdown of LARG in SMC significantly inhibited S1P-dependent activation of the SM22 and SM α-actin promoters (Fig 5a) and S1P-dependent activation of endogenous SMC differentiation marker gene expression as measured by quantitative RT-PCR (figure 5b). Western Blotting also demonstrated that LARG knockdown significantly inhibited S1P mediated increases in SM22, MHC, and SM α-actin protein levels by 45%, 52%, and 27% respectively (see Fig 5c). In good agreement with the results from the RGSRhoGEF and RhoA activity assays, single knockdowns of PRG or p115 had no effect on S1P-dependent SMC differentiation marker gene expression (data not shown). Taken together these results strongly indicate that S1P activates RhoA in SMC by activating LARG and that this signaling mechanism promotes SMC differentiation marker gene expression.

LARG inhibited SMC migration

Phenotypically modulated SMC exhibit increased migration, and it is well known that RhoA plays an important, yet complicated role in the regulation of this process. RhoA stimulates the formation of the stress fibers and focal adhesion complexes that promote firm cell adhesion. However, RhoA also regulates actin-myosin based contractility that is required for trailing edge retraction, and RhoA activity has been detected at the leading edge where it likely contributes to membrane protrusion by stimulating linear actin polymerization (see [32] for review). The mechanisms that regulate this shifting balance between cellular adhesion and cell movement are incompletely understood, and studies have shown that RhoA can inhibit or promote cell migration depending upon cell context [33-35].

Given our demonstration that LARG promoted the differentiated SMC phenotype, we hypothesized that it may also inhibit SMC migration. To test this directly, we measured serum-induced migration in control and LARG knock-down SMC using a transwell assay. As shown in figure 6, migration was significantly increased in LARG knock-down SMC cells suggesting that LARG is a limiting factor in this assay. We also assessed the effects of LARG on SMC migration in a scratch wound assay using live cell imaging. As shown in figure 6c (and in supplemental data), wound closure by SMC transfected with control siRNA was much slower than that in LARG knock-down SMC which was virtually complete by 9h. Since wound closure can also be affected by changes in cell proliferation we used BrdU incorporation assays to determine cell proliferation indices in control and LARG knock-down SMC. As shown in figure 6b knock-down of LARG did not affect SMC proliferation strongly suggesting that the effects of LARG observed in the scratch wound model were due to decreased SMC migration.

Discussion

A growing body of evidence indicates that S1P regulates vascular function by controlling the growth, migration, contraction, and cell-cell interactions of endothelial cells and SMC (see [36] for review). The identification of the S1P-dependent signaling mechanisms involved has been complicated by the expression of multiple S1P receptors in the vessel wall and the

promiscuous coupling of those receptors to G-proteins that have dramatically different effects on cell function. We were the first to demonstrate that S1P increased SMC differentiation in a RhoA dependent manner [5], and the current study supports previous studies implicating S1PR2 in this response [22, 23]. More importantly, we show that S1PR2-dependent activation of RhoA in SMC is mediated by LARG and that this RGSRhoGEF is a key component in the control of SMC phenotype. The identification of this signaling mechanism provides significant insight into the control of SMC function and into the vascular/SMC phenotypes observed in a variety of knockout models.

The analysis of S1P receptor sub-type-specific knockout mouse models has yielded important information on the role of S1P receptor signaling in the vasculature. S1PR1-deficient mice die around E13.5 and have a defect in SMC investment of the dorsal aorta [37]. However, because a similar phenotype was observed in EC-specific S1PR1 knockouts [38], this effect was likely secondary to defects in EC tube maturation and not to defects in SMC differentiation per se. Vascular abnormalities were not observed in S1PR2 deficient mice [39], but the earlier lethality and increased hemorrhage observed in S1PR1/S1PR2 and S1PR2/S1PR3 double knockouts [40] suggests that S1PR2 may have an independent role in the establishment and maintenance of a mature vasculature. Although it has been difficult to determine whether SMC differentiation during development was affected by the loss of S1PR2, Shimizu et. al. have directly implicated S1PR2 in the regulation of SMC phenotype in adult animals [41]. Using a carotid artery ligation model these authors demonstrated that S1PR2 deficient mice had larger neointimas [41] and reduced SM α-actin expression following vessel injury [22].

Our results also implicate $G\alpha_{12/13}$ in the regulation of SMC differentiation. When coupled with the branchial arch SMC defect observed in MRTF-B deficient mice [7, 8], it is intriguing to postulate that $G_{12/13}$ signaling to RhoA plays a critical role in the differentiation of cardiac neural crest cells into SMC. Interestingly, neural crest cell-specific deletion of $G\alpha_{12/13}$ signaling resulted in proximal outflow tract defects and the development of an aneurysm-like structure in the septal branch of the left coronary artery [42]. Because cell tracing analyses demonstrated that neural crest cell migration to these structures was not impaired, these phenotypes may have resulted from defects in the differentiation/maturation of these cells into SMC. In addition, deletion of endothelin receptor A, another $G\alpha_{12/13}$-coupled receptor that activates SMC contraction and SMC differentiation marker gene expression [43, 44], also resulted in defective outflow tract development [45].

Our demonstration that S1PR2 signals through LARG may have important implications on the control of vascular tone. Extensive evidence indicates that RhoA regulates SMC contraction by inhibiting myosin phosphatase (see [46] for review), and several in vitro studies have shown that S1P constricts vessels by a RhoA dependent mechanism [47, 48]. Adult S1PR2 deficient mice have relatively normal systemic blood pressure, but flow measurements showed decreased resistance in mesenteric and renal vascular beds especially in the presence of adrenergic stimulation [49]. It has also been postulated that the deafness observed in S1PR2 deficient mice may be due to dilation of the spiral modiolar artery that supplies blood to the inner ear [50]. LARG knock-out mice also have relatively normal blood pressure, but were shown to be less susceptible to salt-sensitive hypertension [51]. Thus, we feel that S1PR2-dependent activation of LARG could serve a critical role in blood pressure regulation and that targeting this pathway could be beneficial in the treatment of hypertension. S1P also affects vascular tone by increasing in intracellular calcium, an effect most likely mediated by S1P3 [39]. Interestingly, recent studies have demonstrated that calcium may also be important for SMC differentiation marker gene expression and that cross-talk between calcium and RhoA may be involved [16, 23]. In our model over-expression of LARG in SMC did not activate the calcium/calcineurin-dependent IL-2 promoter suggesting that LARG does not significantly activate

calcium signaling (see supplemental figure 1). However, it is possible that these two pathways act in parallel to regulate SMC-specific transcription or that they intersect further downstream.

The residual S1P-dependent RhoA activity observed in LARG deficient SMC could be due to incomplete knockdown of LARG expression but could also reflect the contributions of S1PR3 coupling to Gαq. Gαq has been shown to activate RhoA and SRF-dependent transcription through a separate family of RhoGEFs that includes Trio, Duet, and p63RhoGEF [52]. In support of this idea, Ishii et al. detected residual S1P-dependent RhoA activity in S1PR2 deficient fibroblasts but no S1P-dependent RhoA activity in fibroblasts isolated from S1PR2/S1PR3 double knockouts [39]. The increased lethality and hemorrhage observed in the S1PR2/S1PR3 double knockout mice also supports this concept [40]. Surprisingly, Gq did not stimulate SMC-specific transcription in SMCs, but it is possible that these RhoGEFs are not highly expressed in our these cells. Compensatory RhoA activation may also explain the lack of a significant SMC phenotype in S1PR2 and LARG knockout mice during development and why SMC phenotypes have been revealed only under conditions of vascular stress (i.e. artery ligation or salt-induced hypertension).

Although all three RGS-RhoGEFs are expressed in SMC and are sufficient to increase actin polymerization and EGFP-MRTF-A localization when over-expressed in 10T1/2 cells, we detected little to no RGS-RhoGEF activity in serum-starved SMC, and only LARG was activated by S1P. The latter result supports previous studies demonstrating that specific $G\alpha_{12/13}$-coupled agonists activate specific RGS-RhoGEFs. For example, activation of RhoA by thrombin in PC-3 prostate cancer cells was primarily mediated by LARG [53] while activation of RhoA by lysophosphatidic acid in HEK293 cells was mediated by PRG [54]. Although the molecular interactions within the agonist-receptor-$G\alpha_{12/13}$ complex that mediate this specificity are currently unknown, agonist-specific RGS-RhoGEF activation could provide an additional level of control over RhoA activity and could help integrate multiple RhoA-dependent signals. Further supporting differential activation of the RGS-RhoGEFs, Guilluy et. al. recently demonstrated in SMC (using the G17ARhoA pull down assay) that angiotensin II treatment specifically activated p115 [55]. These authors also described a novel mechanism of p115 activation that was mediated by JAK2-dependent tyrosine phosphorylation. In our experiments p115 did not activate SMC-specific promoter activity as strongly as LARG or PRG but did result in increased actin polymerization and MRTF-A nuclear localization. These data suggest that additional signals might be required to fully activate MRTF-A dependent transcription or that the RGS-RhoGEFs might act on separate pools of RhoA that differentially affect MRTF-A activity. Interestingly, qualitative assessment of p115-expressing cells revealed a more cortical pattern of actin polymerization. p115 lacks the PDZ domain present in LARG and PRG, but whether this difference significantly affects p115 activation, function, and/or localization has not been directly tested in our model.

Our demonstration that LARG decreases SMC migration provides mechanistic insight into the control of cell migration by RhoA and helps explain the anti-migratory effects of S1PR2-dependent signaling observed in several of other models [56, 57]. Importantly, our results are in excellent agreement with the previous demonstration that S1PR2 deficient SMC exhibit increased migration [41], and strongly suggest that this effect is due, at least in part, to decreased LARG activity. Ong et al. have recently shown that expression of LARG in breast and colorectal cancer cells markedly inhibited migration [58] providing further evidence that LARG inhibits this process. The precise mechanisms for the anti-migratory effects of LARG are not completely understood but may involve increased cell adhesion. For example, Dubash et.al. have shown that siRNA depletion of LARG decreases stress fiber and focal adhesion formation [59]. Clearly, additional studies will be necessary to identify the spatial and temporal patterns of RhoA activation that are necessary for cell migration and to determine whether LARG (and other RhoGEFs) activate RhoA within specific cellular compartments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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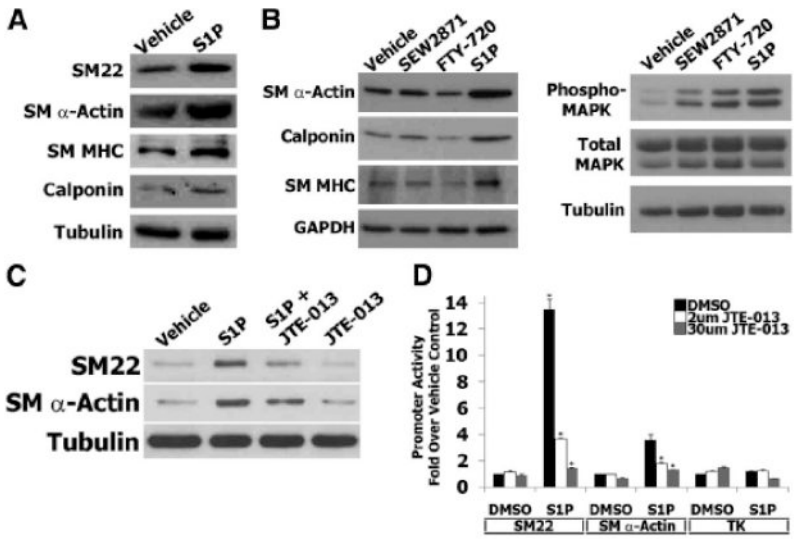

Figure 1. S1P stimulated SMC differentiation marker gene expression through S1PR2

A. Mouse aortic SMC were serum-starved for 24h and then treated with S1P (10uM) for 24 hours. Cell lysates were separated on an SDS-page gel, transferred, to nitrocellulose and probed with antibodies to the indicated SMC differentiation marker genes. **B.** Western Blots were performed on SMCs that were serum-starved and then treated for with S1P [10μm], SEW2871 [5μm] (stimulates only S1PR1), or FTY-720 [10μm] (stimulates all S1P receptors except S1PR2). SMC marker expression represent 24 hours agonist treatment. MAPK activation control represent 12.5 minute agonist treatment **C.** Western Blots were performed on SMC treated with S1P for 24 hours +/- pre-treatment with JTE-013. **D.** 10T1/2 cells transfected with the indicated promoter luciferase construct were treated with S1P +/- pretreatment with the S1PR2 antagonist, JTE-013. Luciferase activity was measured 24 after treatment and is expressed relative to untreated cells. * $p < 0.05$ vs S1P-treated in the absence of JTE-013.

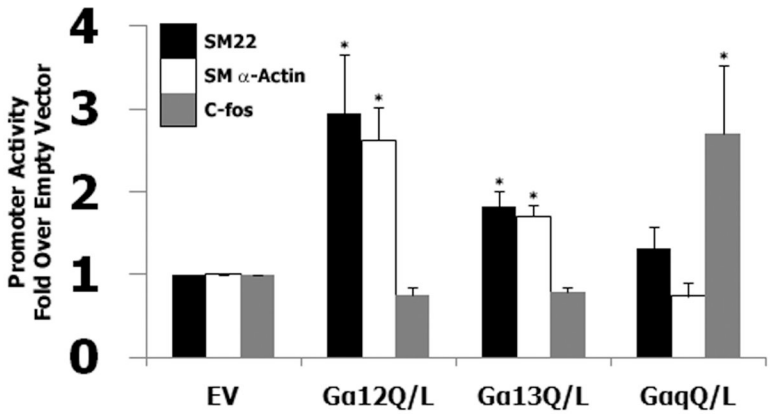

Figure 2. $G\alpha_{12}$ and $G\alpha_{13}$ increased SMC transcription

SMCs were co-transfected with the indicated promoter-luciferase construct along with $G\alpha_{12}$(Q231L), $G\alpha_{13}$(Q226L), Gαq(Q209L), or empty expression vector (EV). Luciferase activity was measured 24h after transfection. * $p<0.05$ vs EV

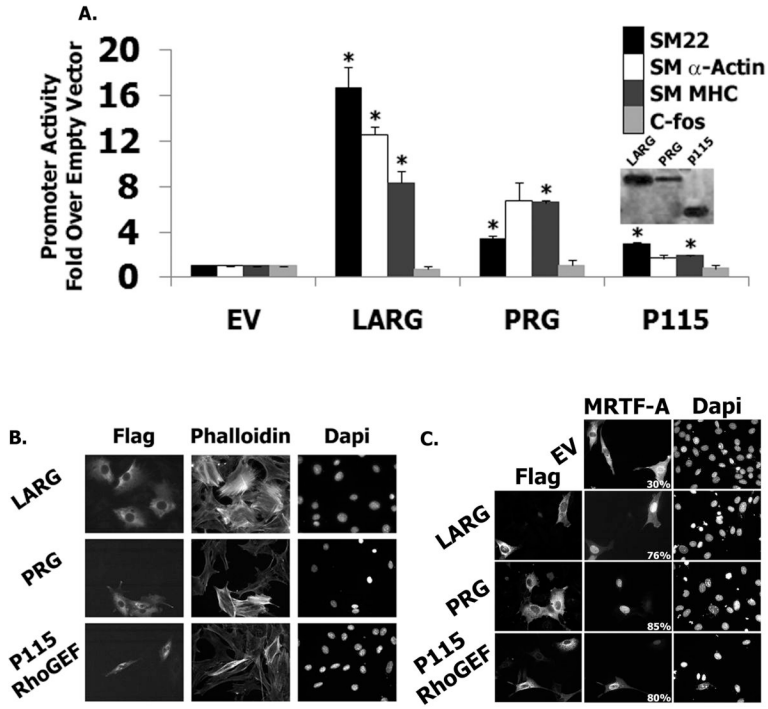

Figure 3. RGS-RhoGEF over-expression increased SMC transcription, stress fiber formation, and MRTF-A localization

A. 10T1/2 cells were co-transfected with the indicated promoter-luciferase construct along with LARG, PRG, p115, or empty expression vector (EV). Luciferase activity was measured 24h after transfection. * $p<0.05$ vs EV. **B.** 10T1/2 cells expressing the indicated flag-tagged RGS-RhoGEF were stained with phalloidin to visualize actin polymerization. **C.** 10T1/2 cells were co-transfected with the indicated RGS-RhoGEF and GFP-MRTFA. Cell counts (>100 cells per condition) demonstrated that RGS-RhoGEF expression increased the percentage of cells that exhibited mainly nuclear MRTF-A localization (see lower right hand corner of micrographs in the middle column).

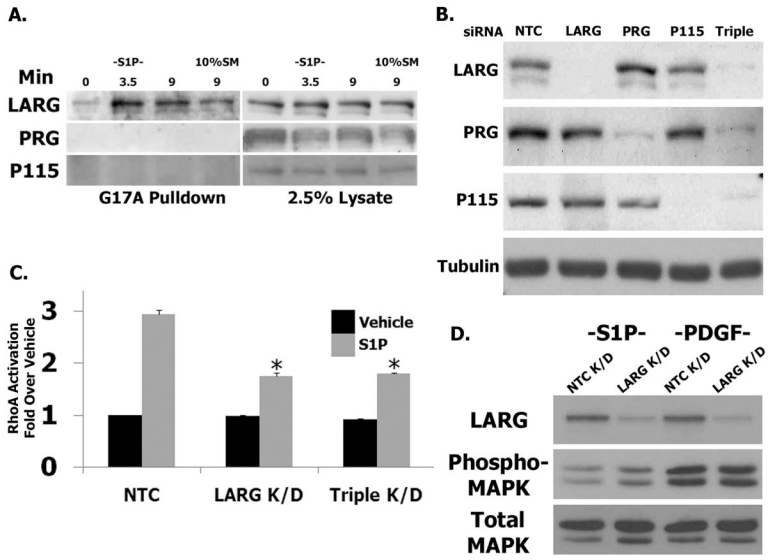

Figure 4. S1P induced RhoA activation in SMC was mediated by LARG

A. SMCs were starved for 24 hours and then treated with S1P or 10% serum for 0, 3.5, or 9 min. Cell lysates were incubated with GST-Rho(G17A) coated beads and precipitates were analyzed for the presence of the indicated RGS-RhoGEF by Western Blot. The amount of RGS-RhoGEF protein present in cell lysates is presented at right. **B.** Western Blot demonstrating significant knockdown of all three RGS-RhoGEFs using our siRNA transfection protocol. (NTC = Non-Targeted Control siRNA) **C.** RhoA activation was measured in control, LARG, and triple knockdown cells using the G-LISA™ assay (Cytoskeleton) and is expressed relative to vehicle-treated cells. At least 90% RGS-RhoGEF knockdown was ensured by Western Blot in all experiments (not shown). * $p < 0.05$ vs S1P-treated NTC. **D.** Western blot of Control and LARG knockdown SMCs serum starved for 24 hours, treated for 12.5 min, and probed for phosphorylated and total MAPK.

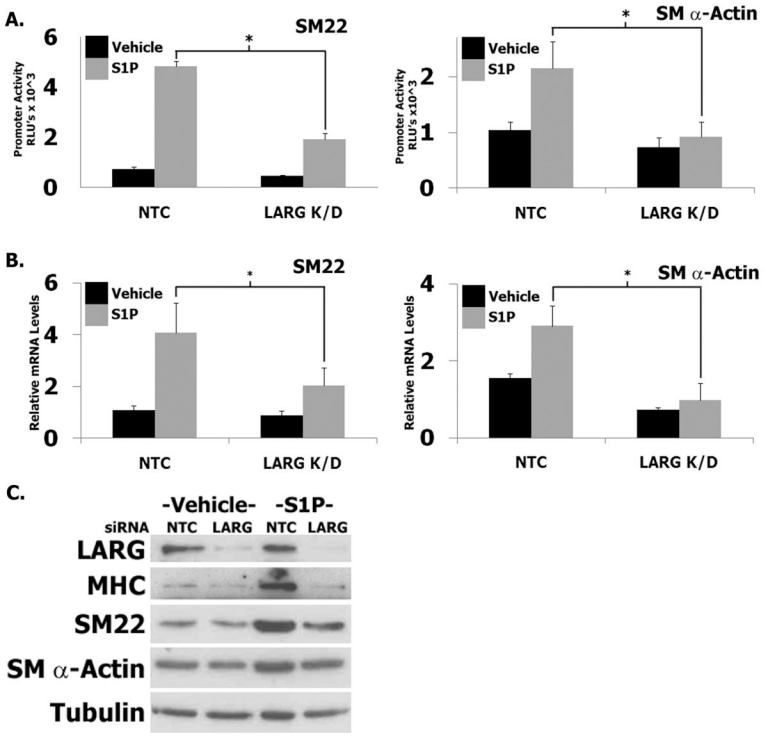

Figure 5. Knockdown of LARG inhibited S1P-induced up-regulation of SMC differentiation marker gene expression

A. Control and LARG knockdown SMC were transfected with SM22 or SM α-actin specific promoter luciferase constructs and then serum-starved for 24 hours. Luciferase activity was measured after 8h of S1P treatement. LARG knockdown was confirmed by Western Blot in all experiments (not shown). * $p<0.05$ vs. S1P-treated NTC. **B and C.** Control and LARG knockdown SMC were serum-starved for 24 hours and then treated with S1P for an additional 24 h. Expression of the indicated SMC differentiation marker genes was measured by quantitative RT PCR (B) and Western Blotting (C).

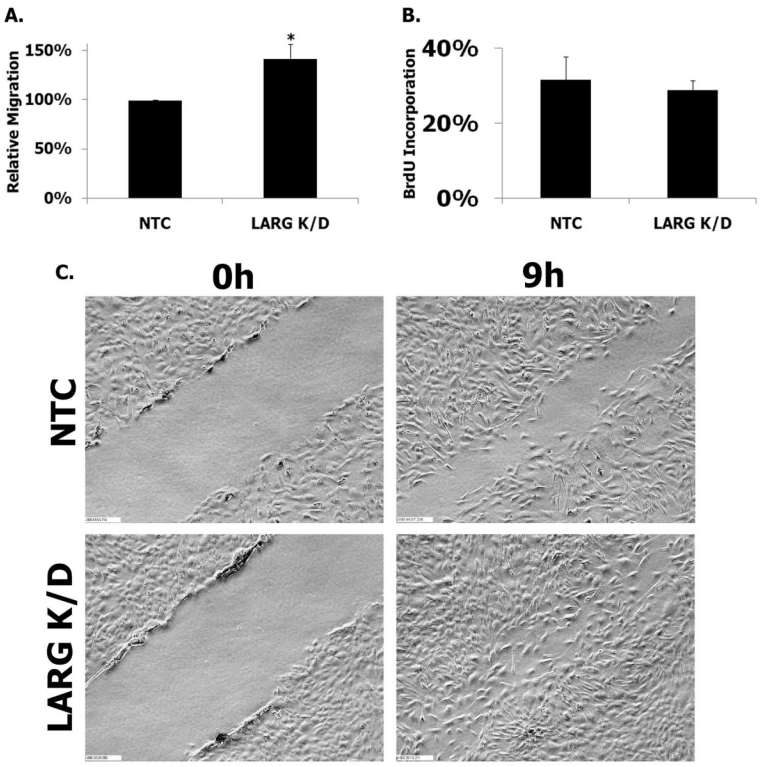

Figure 6. Knockdown of LARG increased SMC migration

A. Equal numbers of Control and LARG knockdown cells were plated on fibronectin coated transwell inserts and were allowed to migrate toward 10% serum-containing media for 18 h. Following fixation the number of cells that had migrated to the bottom chamber were visualized by crystal violet staining. SMC migration was evaluated in 3 separate experiments and is expressed relative to migration of control cells set to 1. * $p<0.05$ versus control. **B.** Control and LARG knockdown SMC were treated with BrdU for 18 h prior to fixation. The percentage of BrDu-containing nuclei in each group was determined by immunohistochemistry. **C.** Confluent cultures of Control and LARG knockdown SMC were scraped with a P200 tip and then placed on an inverted microscope equipped with a heated, humidified, and O_2/CO_2 perfused stage. Pictures taken every 3.5 minutes for 9 h were assembled into movies using Quicktime (0h and 9h frames shown).