

SmgGDS Regulates Cell Proliferation, Migration, and NF- κ B Transcriptional Activity in Non-small Cell Lung Carcinoma^{*[S]}

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Non-small cell lung carcinoma (NSCLC) is promoted by the increased activities of several small GTPases, including K-Ras4B, Rap1A, Rap1B, RhoC, and Rac1. SmgGDS is an unusual guanine nucleotide exchange factor that activates many of these small GTPases, and thus may promote NSCLC development or progression. We report here that SmgGDS protein levels are elevated in NSCLC tumors, compared with normal lung tissue from the same patients or from individuals without cancer. To characterize SmgGDS functions in NSCLC, we tested the effects of silencing SmgGDS expression by transfecting cultured NSCLC cells with SmgGDS small interfering RNA (siRNA). Cells with silenced SmgGDS expression form fewer colonies in soft agar, do not proliferate in culture due to an arrest in G₁ phase, and exhibit disrupted myosin organization and reduced cell migration. The transcriptional activity of NF- κ B in NSCLC cells is diminished by transfecting the cells with SmgGDS siRNA, and enhanced by transfecting the cells with a cDNA encoding SmgGDS. Because RhoA is a major substrate for SmgGDS, we investigated whether diminished RhoA expression mimics the effects of diminished SmgGDS expression. Silencing RhoA expression with RhoA siRNA disrupts myosin organization, but only moderately decreases cell proliferation and does not inhibit migration. Our finding that the aggressive NSCLC phenotype is more effectively suppressed by silencing SmgGDS than by silencing RhoA is consistent with the ability of SmgGDS to regulate multiple small GTPases in addition to RhoA. These results demonstrate that SmgGDS promotes the malignant NSCLC phenotype and is an intriguing therapeutic target in NSCLC.

Lung cancer is the leading cause of cancer death for both men and women in the United States (1). More than 80% of all cases of lung cancer are caused by non-small cell lung carcinoma (NSCLC),³ which is represented mainly by squamous cell lung

carcinoma and adenocarcinoma of the lung. NSCLC is the major cause of lung cancer mortality (1).

Numerous members of the Ras and Rho families of small GTPases have been implicated in the development or progression of lung cancer (2–16). These small GTPases are inactive in the GDP-bound state and become activated by associating with proteins known as guanine nucleotide exchange factors (GEFs). When a GDP-bound small GTPase associates with a GEF, the GEF induces the small GTPase to release GDP. The small GTPase then binds GTP, dissociates from the GEF, and interacts with its specific effectors to elicit a variety of cellular responses. Signaling by the GTP-bound small GTPase is terminated when the small GTPase hydrolyzes the bound GTP to GDP. Abnormalities in this GDP/GTP exchange cycle in NSCLC may generate high levels of activated, GTP-bound Ras and Rho family members, resulting in excessive signaling by the activated small GTPases.

The increased activity of several Ras family members, including K-Ras4B, Rap1A, and Rap1B, is believed to contribute to NSCLC development or progression. NSCLC cells often express K-Ras4B that is mutated at codon 12, which slows the ability of the mutant K-Ras4B to hydrolyze GTP (reviewed in Refs. 8 and 9). When this mutant K-Ras4B is activated by a GEF, the mutant K-Ras4B stays in the active GTP-bound form longer than usual due to its diminished ability to hydrolyze the bound GTP to GDP. This hyperactive K-Ras4B may promote NSCLC by stimulating the proliferation of NSCLC progenitor cells (12–15). The small GTPases Rap1A and Rap1B may be abnormally activated in NSCLC due to overexpression of C3G, which is a GEF that activates both Rap1A and Rap1B (6). It was recently reported that over 50% of NSCLC tumors tested express abnormally high levels of C3G (6). Activated Rap1A and Rap1B may promote NSCLC metastasis by regulating cell spreading (16), cell-cell adhesion (17), and cell migration (18, 19).

Rho family members reported to have a role in NSCLC include RhoB (2, 3), RhoC (4, 5), and Rac1 (10, 11). RhoB suppresses the aggressive NSCLC phenotype (2, 3), whereas RhoC enhances the malignant characteristics of NSCLC cells (4, 5). Rac1 activates unique c-Jun NH₂-terminal kinase (JNK)-dependent signaling pathways that stimulate the proliferation of NSCLC cells (10, 11). Surprisingly, the specific functions of RhoA in NSCLC have not been previously reported, although RhoA is known to promote tumorigenesis and metastasis in

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³ The abbreviations used are: NSCLC, non-small cell lung carcinoma; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein;

NHBE, normal human bronchial epithelial; siRNA, small interfering RNA; TNF- α , tumor necrosis factor- α ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

other forms of cancer (reviewed in Refs. 20 and 21). RhoA and other Rho family members may promote cancer cell invasion and migration by regulating the organization of the actin/myosin cytoskeleton (reviewed in Refs. 20 and 21).

Several Ras and Rho family members that participate in the development or promotion of NSCLC are able to regulate the activity of the transcription factor NF- κ B (22–26). Active NF- κ B promotes survival of NSCLC cells (27–29), and suppressing NF- κ B activity may be a promising treatment strategy for NSCLC (30–32). NF- κ B activity is stimulated by K-Ras through a Raf-dependent pathway (23), and is stimulated by both K-Ras and Rac1 through a Pak-dependent pathway (24). Interestingly, inhibition of the Rho effector ROCK inhibits NF- κ B activity in some cells (26), but stimulates NF- κ B activity in other cell types (25).

Activation of NF- κ B transcriptional activity occurs when the p65 RelA subunit of NF- κ B translocates to the nucleus and binds DNA in association with the appropriate co-activators and DNA-binding partners (reviewed in Refs. 33 and 34). These functions of RelA are modulated by post-translational modifications of RelA and its protein partners, including phosphorylation and acetylation (reviewed in Refs. 33 and 34). Signals that induce these modifications in RelA and its protein partners in a coordinated and synchronized manner stimulate RelA-dependent transcription. In contrast, signals that promote these events in an uncoordinated and asynchronous manner can actually repress RelA-dependent transcription (Refs. 27–29 and 35–37, reviewed in Ref. 33). Multiple small GTPases may act together to activate NF- κ B by coordinately regulating RelA nuclear translocation, phosphorylation, acetylation, DNA binding, and interaction with co-activators and DNA-binding partners.

The participation of multiple Ras and Rho family members in NSCLC makes these small GTPases important therapeutic targets in lung cancer. However, the significant overlap in the signaling pathways utilized by these different small GTPases, including pathways that converge on NF- κ B, potentially limits the efficacy of therapeutic approaches that target only one or a few small GTPases within the same family. Due to this limitation, NSCLC cell proliferation and metastasis may best be controlled by suppressing the activities of multiple small GTPases simultaneously in NSCLC. The GEF SmgGDS (also known as smg GDS, RAP1GDS1, and GDS) is an excellent candidate for such a strategy, because SmgGDS is the only known GEF that activates multiple small GTPases in both the Ras and Rho families (reviewed in Refs. 38 and 39). SmgGDS increases GTP binding by many small GTPases, including RhoA (40–43), Rac1 (41, 42), K-Ras4B (40, 42), Rap1A (40, 44, 45), and Rap1B (42, 44). SmgGDS also extracts these small GTPases from membranes and thus regulates their membrane localization (reviewed in Refs. 39 and 46). SmgGDS enhances DNA synthesis induced by Rap1A (47) and enhances the transforming activity of K-Ras4B (48) in several cell types. The ability of SmgGDS to act as a master regulator of multiple small GTPases makes SmgGDS a very unique and promising candidate to regulate the malignant phenotype.

Despite the obvious rationale for investigating the participation of SmgGDS in the development or progression of NSCLC,

the functions of SmgGDS in NSCLC or any other cancer have not been reported. A previous study examined mRNA transcripts that are up-regulated in squamous cell lung carcinoma, and identified mRNA for SmgGDS as one of the transcripts that are overexpressed in this type of lung cancer (7). Although this finding supports a role for SmgGDS in NSCLC, this previous study did not characterize SmgGDS protein levels in the lung tumors, nor did it test the functional significance of SmgGDS expression in NSCLC (7).

To test the expression and function of SmgGDS in NSCLC, we examined SmgGDS protein levels in NSCLC tumors, and determined the functional consequences of silencing SmgGDS expression in NSCLC cell lines. Because SmgGDS preferentially activates RhoA in comparison to other small GTPases that interact with SmgGDS (42, 43), we also examined the effects of silencing RhoA expression in NSCLC cell lines. Diminished RhoA expression should mimic the effects of diminished SmgGDS expression if the phenotypic effects induced by silencing SmgGDS expression are mainly due to reduced RhoA activity. Our results demonstrate that SmgGDS protein expression is higher in NSCLC tumors than it is in normal lung tissue. We found that the proliferation and migration of cultured NSCLC cells are inhibited significantly by silencing SmgGDS expression, but not to the same extent by silencing RhoA expression. This finding indicates that SmgGDS most likely mediates its effects by regulating multiple small GTPases in addition to RhoA. Silencing SmgGDS induces nuclear translocation of RelA, but profoundly inhibits NF- κ B transcriptional activity, suggesting that SmgGDS is needed to coordinate the signaling pathways that maintain NF- κ B activity in NSCLC. This study indicates that SmgGDS expression may promote NSCLC tumor progression and metastasis, and identifies SmgGDS as a new therapeutic target in NSCLC.

EXPERIMENTAL PROCEDURES

Cell Culture and siRNA Transfection—The NSCLC cell lines NCI-H23, NCI-H520, NCI-H522, and NCI-H1703 were obtained from the American Type Tissue Collection (Bethesda, MD), and cultured in complete NSCLC medium (RPMI 1640, 10% heat-inactivated fetal bovine serum, and antibiotics). The normal human bronchial epithelial (NHBE) cells and the medium in which they were cultured (Bronchial Epithelial Cell Medium) were purchased from Cambrex (San Diego, CA). All siRNA duplexes were purchased from Dharmacon (Lafayette, CO). Two siRNAs targeting different sequences in SmgGDS were used in the study. The first siRNA targeting SmgGDS is designated as siRNA I1, and targets the SmgGDS mRNA sequence 5'-GCAAAGAUGUUAUCAGCUG-3'. The second siRNA targeting SmgGDS is designated as siRNA I2, and targets the SmgGDS mRNA sequence 5'-GUUAAUAGAU-GCACAAGAA-3'. The siRNA used to silence RhoA expression targets the RhoA mRNA sequence 5'-AUGGAAAGCAG-GUAGAGUU-3'. A control siRNA, designated as Scramble siRNA, was designed by the manufacturer to not target any human genes (Dharmacon siControl number 1 siRNA). The cells were transfected with the different siRNAs at the indicated concentrations using Dharmafect-3 transfection reagent (Dharmacon) according to the manufacturer's instruction.

Immunohistochemistry—Multiple arrays of formalin-fixed, paraffin-embedded lung tumors and matched or unmatched normal lung tissue were obtained from U.S. Biomax, Inc. (Rockville, MD). The arrays consisted of product number LC1001, which is an array of 45 NSCLC tumors with matched normal lung tissue and additional samples of unmatched normal lung tissue, product number LC801, which is an array of 78 lung tumors (including 30 squamous cell carcinoma, 23 adenocarcinoma, and 9 small cell carcinoma samples) and unmatched normal lung tissue, and product BS04011, which is an array of 63 squamous cell lung carcinoma tumors. The tissue arrays were deparaffinized, incubated in peroxidase blocking buffer (3% H₂O₂ in methanol) and heated in citrate buffer (pH 6.0, 95 °C). After incubating in protein blocking buffer (3% bovine serum albumin in phosphate-buffered saline), the sections were incubated with SmgGDS antibody (BD Transduction Laboratories 612511) followed by incubation with the peroxidase labeling system (Dakocytomation Envision + Dual Link Peroxidase System). The sections were incubated in peroxidase substrate solution (Dakocytomation Liquid DAB + Substrate Chromagen System) and counterstained with hematoxylin (Dakocytomation). The specimens were dehydrated and cleared in xylene. For negative controls, the tissue sections were stained in the same manner except that the primary antibody to SmgGDS was omitted. All specimens were mounted in aqueous medium and examined using an Olympus BX41 microscope and Olympus DP70 camera. Antibody reactivity was assessed independently by two investigators without knowledge of the identities of the tissues being examined. The relative immunohistochemical staining of each tissue specimen was ranked by the examiners using the following scale: 0 = undetectable staining, +1 = weak staining, +2 = moderate staining, +3 = strong staining (Fig. 1).

To examine the immunohistochemical reactivity of SmgGDS antibody with cultured NSCLC cells, NCI-H1703 cells were transfected in the absence or presence of the indicated siRNAs and cultured for 72 h. The cells were then fixed with 10% buffered formalin, mixed with preheated Histogel (Richard-Allen Scientific, Kalamazoo, MI), and embedded in a paraffin block using our previously described techniques (49). The paraffin block containing the embedded cells was cut with a microtome to generate 4- μ m thick sections. The sections of NCI-H1703 cells embedded in paraffin were deparaffinized, immunohistochemically stained with the SmgGDS antibody, and counterstained with hematoxylin using the same methods described above for the immunohistochemical staining of the tumor tissues.

Enhanced Chemiluminescence (ECL) Western Blotting—Equal numbers of transfected cells were boiled in Laemmli sample buffer and subjected to SDS-PAGE. The proteins were transferred to polyvinylidene difluoride and immunoblotted using doubling dilutions of antibody to SmgGDS (BD Transduction Labs 612511), RhoA (Santa Cruz Biotechnology sc-418), or GAPDH (Santa Cruz Biotechnology sc-32233). Bound antibodies were visualized using horseradish peroxidase-linked anti-mouse IgG (Amersham Biosciences) and ECL reagents (PerkinElmer Life Sciences), as described previously (46).

[³H]Thymidine Uptake—Cell proliferation was assessed by measuring [³H]thymidine uptake by the cells, as we previously described (50). Cells were transfected in 96-well microtiter plates and then cultured for 72 h in complete NSCLC medium with [³H]thymidine present for the last 3 h of culture. The cells were collected on filters using an automatic cell harvester (Skatron, Sterling, VA) and radioactivity on the filters was measured by β -scintillation counting.

Cell Cycle Analysis—The percentage of the cell population in each phase of the cell cycle was measured using flow cytometry, as previously described (51). Transfected cells were fixed in 50% ethanol, incubated with RNase mixture (500 units/ml RNase A, 20,000 units/ml RNase T1) for 30 min, and stained with propidium iodide (100 μ g/ml). The cells were subjected to flow cytometry to measure propidium iodide staining of the cells. The results were analyzed using the ModFit program.

Cell Migration—The migration of cells in a monolayer was examined by culturing transfected cells for 72 h in complete medium to generate a confluent monolayer. The monolayer was scratched with a p-2 pipette tip (Eppendorf), and the medium was replaced with NSCLC medium containing 1% fetal calf serum. Digital images of the cells were collected immediately and after culturing the cells for an additional 24 h.

The migration of individual cells was examined as previously described (52). Transfected cells were plated at a density of 3×10^3 cells/600 μ l of complete medium in wells of a 24-well plate coated with collagen Type 1 (10 μ g/ml in phosphate-buffered saline) and colloidal gold. Digital images of the cells were collected after 24 h, and the areas of the migration trails were analyzed using the NIH Image J software program. Cells that were in contact with other cells, or whose migration trails crossed those of other cells, were not included in the analysis.

Immunofluorescence Assays—To examine myosin organization, the cells were transfected in the absence of siRNA (mock transfection) or in the presence of different siRNAs and cultured in complete medium for 72 h. The cells were fixed in ice-cold acetone (5 s) and incubated with 1% bovine serum albumin (30 min) to block nonspecific protein-binding sites, followed by incubation with the SA-2 human IgM antibody to the myosin heavy chain (53) and fluorescein isothiocyanate-labeled anti-human IgM. The washed cells were mounted in 90% glycerol, 0.1% *p*-phenylenediamine and examined using a Nikon Eclipse E600 fluorescence microscope.

To examine NF- κ B RelA localization, mock-transfected or siRNA-transfected cells were cultured in complete NSCLC medium for 48 h, and then cultured in serum-free medium for 24 h before incubation with or without TNF- α (100 ng/ml, 30 min) in serum-free medium. Cells were fixed with 3% formaldehyde (10 min), followed by incubation with 50 mM NH₄Cl (15 min), 0.2% Triton X-100 (10 min), and 1% bovine serum albumin (30 min). The cells were incubated with NF- κ B RelA antibody (Santa Cruz Biotechnology sc-109), followed by fluorescein isothiocyanate-labeled anti-rabbit IgG. The cells were mounted as described above and examined using a fluorescence microscope (Nikon Eclipse E600).

NF- κ B Transcriptional Activity—The cells were transfected in the absence of siRNA (mock transfection) or in the presence of different siRNAs and cultured in complete medium for 48 h.

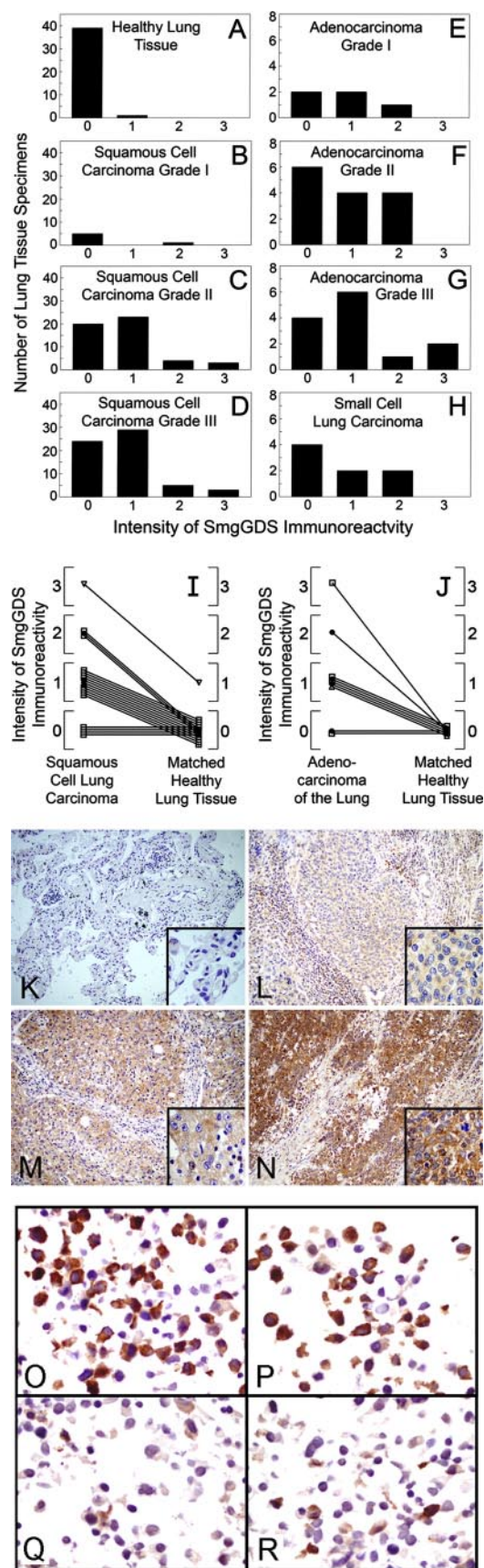


FIGURE 1. SmgGDS is expressed at high levels in NSCLC tumors. Commercial tissue arrays of normal lung tissue (A), squamous cell lung carcinoma tumors (B–D), lung adenocarcinoma tumors (E–G), and small cell lung carcinoma

The cells were then transfected with the pNifty-Luc NF- κ B reporter plasmid (Invivogen, San Diego, CA) using Lipofectamine (Invitrogen) according to the manufacturer's protocol. The pNifty-Luc NF- κ B reporter plasmid contains five NF- κ B sites in a promoter driving luciferase production. After transfection of this reporter plasmid, the cells were cultured in complete medium for 24 h, and then incubated in the absence or presence of TNF- α (100 ng/ml, 3 h) in complete medium. The cells were incubated with D-luciferin (150 μ g/ml, 2 min) and luminescence in the living cells was measured using a Wallac 1420 Victor-3 plate reader (PerkinElmer). Luminescence is reported as relative light units normalized to protein content in the samples, as determined using the Pierce BCA Protein Assay Kit (Pierce Biotechnology). As a control experiment, we co-transfected the cells with both the pNifty-Luc NF- κ B reporter plasmid and the pEGFP-C1 plasmid, which encodes green fluorescent protein (GFP), and then normalized the level of luminescence generated by the expressed luciferase to the level of fluorescence generated by the expressed GFP. Similar results were obtained when luminescence was normalized to protein content or to GFP fluorescence in the cell samples.

Statistical Analyses—The means of the measured values of each treatment group were compared by using Student's *t* test. Means were considered to be significantly different from one another if *p* was <0.05 in the Student's *t* test.

RESULTS

Immunohistochemical staining of commercial tissue arrays of lung tumors and normal lung tissue indicated greater SmgGDS expression in the lung tumors compared with normal lung tissue from the same patients or from individuals without lung cancer (Fig. 1). Among 40 normal lung tissue specimens examined, only 1 specimen (2.5%) had detectable SmgGDS expression, as indicated by a score of +1 or greater in the analysis of immunohistochemical staining with SmgGDS antibody (Fig. 1A). In contrast, SmgGDS expression was detectable (as indicated by an immunohistochemical score of +1 or greater) in 68 of 117 (58.1%) of the squamous cell lung carcinoma tumors examined (Fig. 1, B–D), in 20 of 32 (62.5%) of the adenocarcinoma lung tumors examined (Fig. 1, E–G), and in 4 of 8 (50%) of the small cell lung carcinoma tumors examined (Fig. 1H). When we compared SmgGDS expression between lung tumors and the matched normal lung tissue in the same patients (Fig. 1, I and J), we found that 15 of 19 (78.9%) of the patients with

tumors (H) were immunohistochemically stained with SmgGDS antibody, and ranked on a scale of 0, +1, +2, or +3 for SmgGDS antibody immunoreactivity. Commercial tissue arrays of squamous cell lung carcinoma tumors and matched normal lung tissue from the same patients (I) or lung adenocarcinoma tumors and matched normal lung tissue from the same patients (J) were similarly scored for SmgGDS antibody immunoreactivity. Sections of normal lung tissue (K), and squamous cell lung carcinoma tumors (L–N) were imaged by light microscopy to illustrate examples of tissue sections that were assigned a score of 0 (K), a score of +1 (L), a score of +2 (M), or a score of +3 (N) for SmgGDS antibody immunoreactivity. Insets in K–N are higher magnification views of the same tissue section shown in each panel containing the inset. The SmgGDS antibody was used to immunohistochemically stain sections of paraffin-embedded NCI-H1703 cells that had been transfected in the absence of siRNA (O), or in the presence of 100 nM Scramble siRNA (P), 25 nM SmgGDS siRNA (Q), or 100 nM SmgGDS siRNA (R) 72 h prior to fixation and imbedding in paraffin.

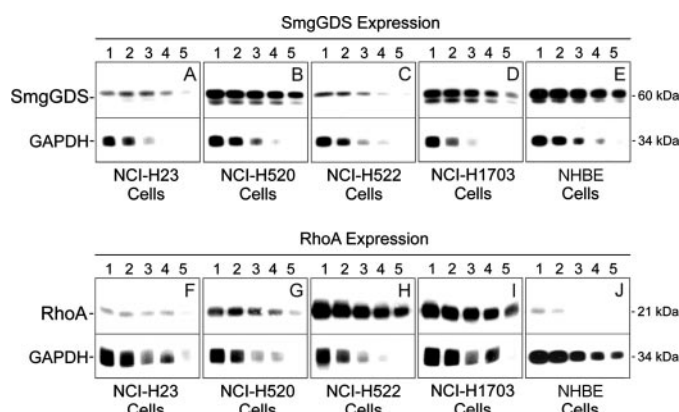


FIGURE 2. Expression levels of SmgGDS and RhoA differ between different NSCLC cell lines. Lysates of equal numbers of proliferating cells from the indicated NSCLC cell lines (A–D and F–I) or NHBE cells (E and J) were subjected to ECL-Western blotting using serial doubling dilutions of an antibody to SmgGDS (A–E) or RhoA (F–J), starting at a 1:50 dilution in lane 1. The same immunoblots were probed with serial doubling dilutions of an antibody to GAPDH, starting at a 1:1000 dilution in lane 1. Results are representative of three independent experiments.

squamous cell lung carcinoma had higher SmgGDS expression in their tumors than in their matched normal lung tissue (Fig. 1I), and 7 of 9 (77.7%) of the patients with adenocarcinoma of the lung had higher SmgGDS expression in their tumors than in their matched normal lung tissue (Fig. 1J). Examples of tissue sections that were assigned scores of 0, +1, +2, or +3 for SmgGDS antibody immunoreactivity are shown in Fig. 1, K–N.

To confirm that the SmgGDS antibody specifically recognizes SmgGDS in the immunohistochemical assays, we tested the reactivity of the SmgGDS antibody with paraffin-embedded NCI-H1703 cells that were previously treated with or without SmgGDS siRNA I1 (Fig. 1, O–R). The SmgGDS antibody reacts strongly with NCI-H1703 cells that were transfected either in the absence of siRNA (Fig. 1O) or transfected with 100 nM Scramble siRNA (Fig. 1P). In contrast, the SmgGDS antibody reacts weakly with NCI-H1703 cells transfected with SmgGDS siRNA I1 at concentrations of 25 (Fig. 1Q) or 100 nM (Fig. 1R). These results provide evidence that the SmgGDS antibody specifically recognizes SmgGDS in the paraffin-embedded cells and tissues.

The elevated expression of SmgGDS in NSCLC tumors suggests that SmgGDS promotes the development or progression of NSCLC. To begin to characterize the roles of SmgGDS in NSCLC, we examined the expression and functions of SmgGDS in four independent NSCLC cell lines. Western blotting of whole cell lysates indicated that SmgGDS is expressed in all four NSCLC cell lines tested (Fig. 2, A–D), with highest SmgGDS expression in the NCI-H520 (Fig. 2B) and NCI-H1703 (Fig. 2D) cells. SmgGDS is also expressed at high levels in NHBE cells (Fig. 2E). We also examined the expression of RhoA, which is the major small GTPase activated by SmgGDS (42, 43). Western blotting indicated that all four NSCLC cell lines also express RhoA (Fig. 2, F–I), with highest RhoA expression in the NCI-H522 (Fig. 2H) and NCI-H1703 (Fig. 2I) cells. RhoA expression is relatively low in the NHBE cells (Fig. 2J).

To test the participation of SmgGDS in NSCLC cell proliferation, we examined the effects of transfecting the cells with SmgGDS siRNA to silence SmgGDS expression in the cells.

Control groups included cells transfected without siRNA (mock transfection), and cells transfected with 100 nM Scramble siRNA. Transfection of 100 nM SmgGDS siRNA I1 silences SmgGDS expression to different extents in the different cell lines, as indicated by ECL-Western blotting of the cell lysates 72 h after transfection of the cells (Fig. 3, A–H). This difference is most noticeable by comparing the optical density (O.D.) of SmgGDS relative to the O.D. of GAPDH detected in the Western blots of cells transfected with SmgGDS siRNA (Fig. 3, E–H). Transfection with SmgGDS siRNA decreases the O.D. of SmgGDS relative to the O.D. of GAPDH by 87% in NCI-H23 cells (Fig. 3E) and by 76% in NCI-H1703 cells (Fig. 3H), indicating significant silencing of SmgGDS expression in NCI-H23 and NCI-H1703 cells. In contrast, SmgGDS siRNA decreases the O.D. of SmgGDS relative to the O.D. of GAPDH by only 30% in NCI-H520 cells (Fig. 3F) and by 60% in NCI-H522 cells (Fig. 3G), indicating less effective silencing of SmgGDS expression in NCI-H520 and NCI-H522 cells. Cells exhibiting the greatest loss of SmgGDS expression exhibit the greatest loss of cell proliferation, as indicated by the significant reduction in [³H]thymidine uptake by NCI-H23 (Fig. 3I) and NCI-H1703 cells (Fig. 3L) after SmgGDS siRNA transfection, compared with the more modest reduction in [³H]thymidine uptake by NCI-H520 (Fig. 3J) and NCI-H522 cells (Fig. 3K) after SmgGDS siRNA transfection. These results demonstrate that loss of SmgGDS diminishes NSCLC cell proliferation, indicating that SmgGDS promotes NSCLC cell proliferation.

We also examined the effects of transfecting the cells with RhoA siRNA to determine whether diminished RhoA expression mimics the effects of diminished SmgGDS expression. Transfection of RhoA siRNA silences RhoA expression almost completely in NCI-H522 (Fig. 3, C and G) and NCI-H1703 cells (Fig. 3, D and H), but has little or no effect on cell proliferation in these cell lines (Fig. 3, K and L). Transfection of RhoA siRNA inconsistently silences RhoA expression in NCI-H23 cells (Fig. 3, A and E) and modestly silences RhoA expression in NCI-H520 cells (Fig. 3, B and F), and has little or no effect on cell proliferation in these cell lines (Fig. 3, I and J). These results demonstrate that loss of RhoA does not inhibit cell proliferation to the same extent that loss of SmgGDS inhibits cell proliferation.

To further test the roles of SmgGDS and RhoA in NSCLC cell proliferation, we repeated the assays shown in Fig. 3 using NCI-H1703 and NCI-H23 cells, but modified the protocol by using 25 nM siRNA instead of 100 nM siRNA, and by testing an additional siRNA targeting SmgGDS, which we designated SmgGDS siRNA I2. Transfection with 25 nM SmgGDS siRNA I1 or 25 nM SmgGDS siRNA I2 significantly diminishes SmgGDS expression in NCI-H1703 (Fig. 4A) and NCI-H23 cells (Fig. 4D) and significantly diminishes the proliferation of the cells (Fig. 4, C and F). In contrast, even though transfection with 25 nM RhoA siRNA silences RhoA expression in both cell lines (Fig. 4, B and E), transfection with 25 nM RhoA siRNA only modestly diminishes cell proliferation in NCI-H1703 cells (Fig. 4C) and does not inhibit cell proliferation in NCI-H23 cells (Fig. 4F).

Silencing SmgGDS expression only minimally diminished the proliferation of NHBE cells, in comparison to the significant

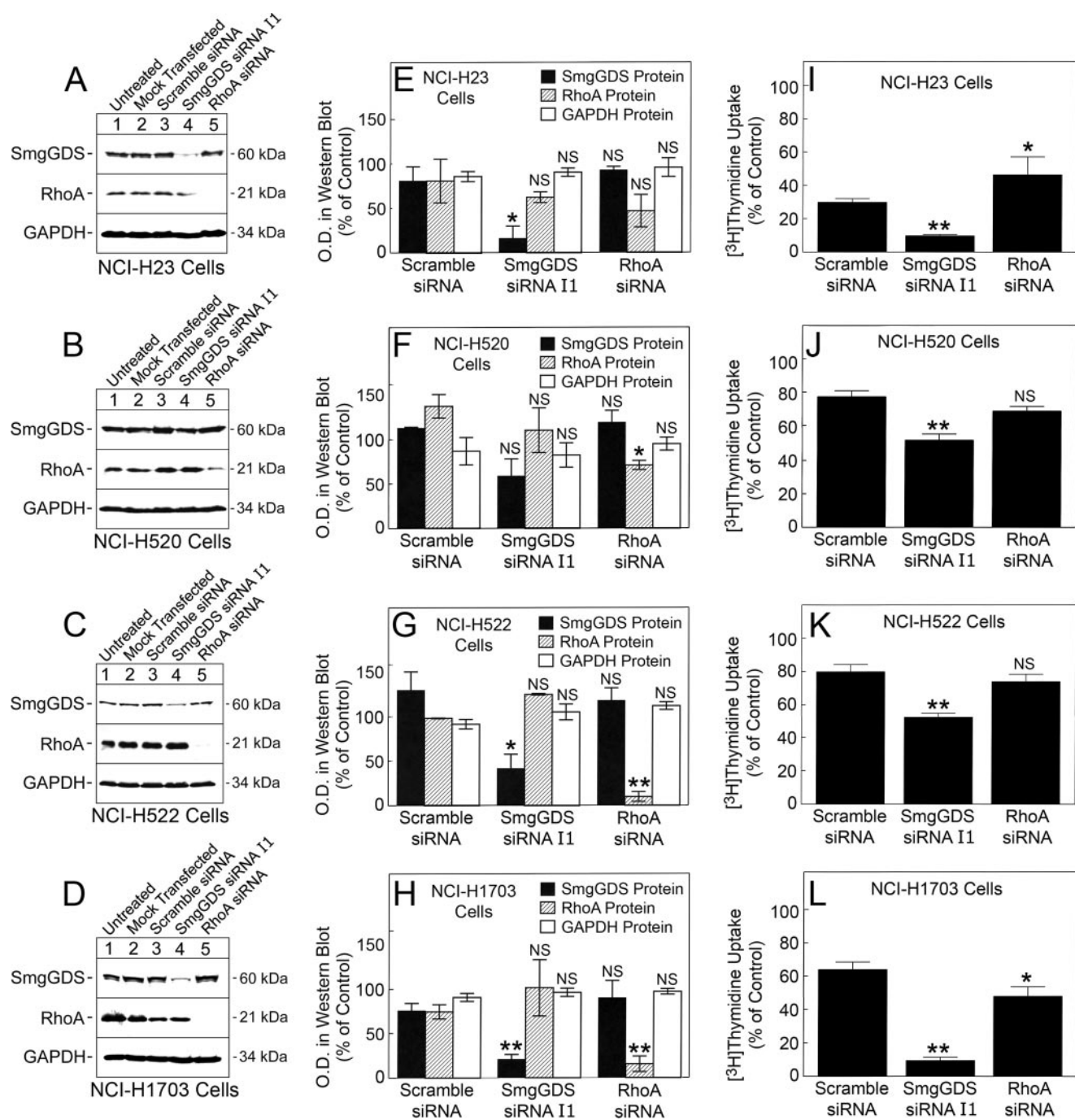


FIGURE 3. Cell proliferation is significantly diminished by silencing SmgGDS expression, and more modestly diminished by silencing RhoA expression, in multiple NSCLC cell lines. A–D, to characterize silencing of SmgGDS or RhoA expression, the indicated cell lines were untransfected (lane 1), subjected to mock transfection without siRNA (lane 2), or transfected with 100 nM Scramble siRNA (lane 3), 100 nM SmgGDS siRNA I1 (lane 4), or 100 nM RhoA siRNA (lane 5). Cell lysates were prepared 72 h post-transfection, and subjected to ECL-Western blotting using antibodies to SmgGDS, RhoA, and GAPDH. Results are representative of three to five independent experiments. E–H, quantitative densitometry was conducted to determine the protein levels of SmgGDS (solid bars), RhoA (striped bars), and GAPDH (open bars) in the ECL-Western blots described in A–D. The values are normalized to densitometric values obtained from ECL-Western blots of cells subjected to mock transfection without siRNA. Results are the mean \pm S.E. from three to five independent experiments. Symbols above a column indicate a statistical comparison between the indicated protein expressed by experimental cells and the same protein expressed by control cells transfected with Scramble siRNA (NS, not significant; *, $p < 0.05$; **, $p < 0.001$). I–L, cell proliferation was assayed by measuring [3 H]thymidine uptake 72 h after transfecting the cells with 100 nM of the indicated siRNAs. The values are normalized to [3 H]thymidine uptake by cells subjected to mock transfection without siRNA. Results are the mean \pm S.E. from three independent experiments conducted with quadruplicate samples in each experiment. Symbols above a column indicate a statistical comparison between the indicated sample and the control sample of cells transfected with Scramble siRNA (NS, not significant; *, $p < 0.05$; **, $p < 0.001$).

reduction in the proliferation of NCI-H1703 cells with silenced SmgGDS expression (supplemental Fig. 1). However, this comparison should be interpreted with caution, because the proliferation of NHBE cells was extremely low in culture (supplemental Fig. 1), making it difficult to assess the anti-proliferative effects of silencing SmgGDS expression in NHBE cells.

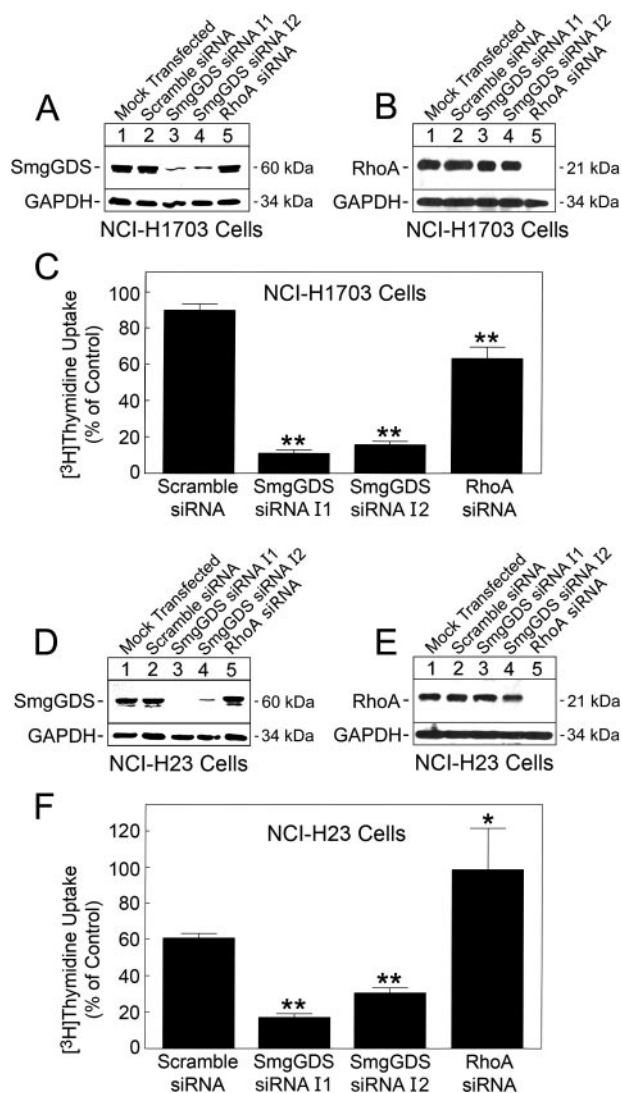


FIGURE 4. Cell proliferation is significantly diminished by silencing SmgGDS expression using SmgGDS siRNA I1 or SmgGDS siRNA I2. NCI-H1703 cells (A and B) and NCI-H23 cells (D and E) were transfected without siRNA (lane 1), or transfected with 25 nM Scramble siRNA (lane 2), 25 nM SmgGDS siRNA I1 (lane 3), 25 nM SmgGDS siRNA I2 (lane 4), or 25 nM RhoA siRNA (lane 5). Cell lysates were prepared 72 h post-transfection, and subjected to ECL-Western blotting using antibodies to SmgGDS (A and D), RhoA (B and E), and GAPDH (A, B, D, and E). Results are representative of three independent experiments. The proliferation of NCI-H1703 cells (C) and NCI-H23 cells (F) was assayed by measuring [³H]thymidine uptake 72 h after transfecting the cells with 25 nM of the indicated siRNAs. The values are normalized to [³H]thymidine uptake by cells subjected to mock transfection without siRNA. Results are the mean \pm S.E. from three independent experiments conducted with quadruplicate samples in each experiment. Symbols above a column indicate a statistical comparison between the indicated sample and the control sample of cells transfected with Scramble siRNA (NS, not significant; *, $p < 0.05$; **, $p < 0.001$).

We next tested the time-dependent changes in cell cycle progression after transfection with SmgGDS siRNA or RhoA siRNA. We used NCI-H1703 cells for these assays because these cells express high levels of both SmgGDS and RhoA (Fig. 2, D and I) and exhibit high siRNA transfection efficiency (Figs. 3, D and H, and 4, A and B). SmgGDS expression in NCI-H1703 cells declines within 24 h of transfection with 100 nM SmgGDS siRNA I1, and remains suppressed for 96 h after transfection (Fig. 5A). Similarly, RhoA expression declines within 24 h of

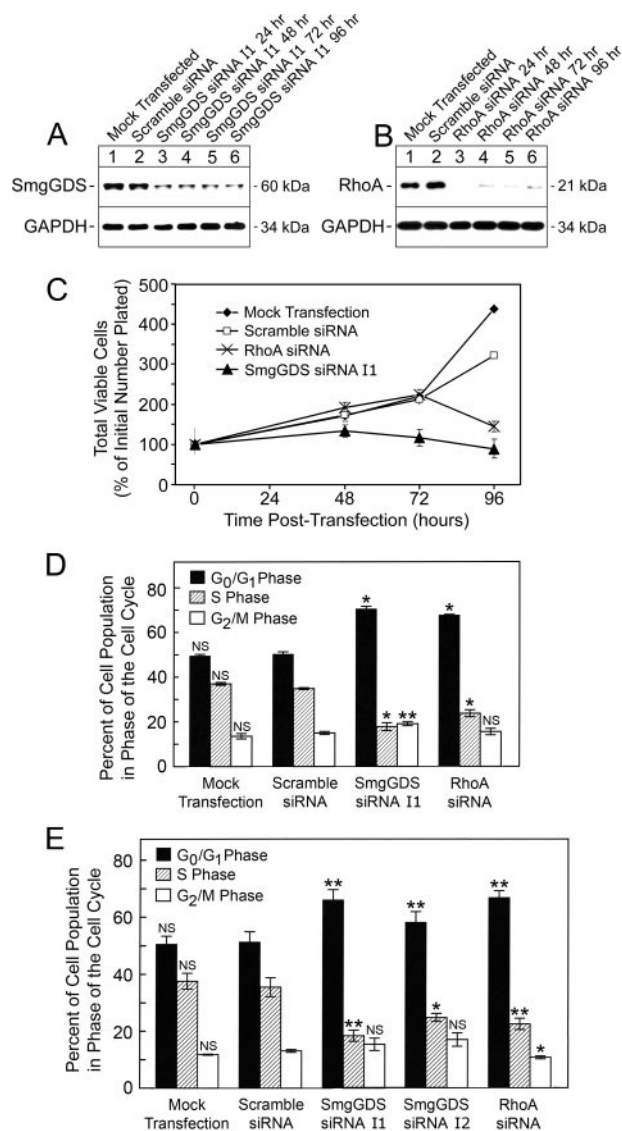


FIGURE 5. Diminished cell proliferation and G₁ arrest in NCI-H1703 cells occurs more rapidly after transfection with SmgGDS siRNA than with RhoA siRNA. Silencing of SmgGDS expression (A) or RhoA expression (B) in NCI-H1703 cells was characterized by transfecting the cells in the absence (lane 1) or presence (lanes 2–6) of 100 nM of the indicated siRNAs, and lysing the cells 24 (lane 3), 48 (lane 4), 72 (lane 5), or 96 h (lanes 1, 2, and 6) later. The lysates were subjected to ECL-Western blotting using the indicated antibodies. Changes in cell proliferation (C) were determined by transfecting NCI-H1703 cells without siRNA (mock transfection) or with 100 nM of the indicated siRNAs, and culturing the cells in complete medium. The total number of cells was counted and viability was determined by trypan blue exclusion at the indicated times. Changes in cell cycle progression were determined by transfecting NCI-H1703 cells in the absence of siRNA (mock transfection) or in the presence of 100 nM of the indicated siRNAs (D) or 25 nM of the indicated siRNAs (E), and staining the cells with propidium iodide 72 h post-transfection, followed by fluorescence-activated cell sorter analysis. Results are the mean \pm S.E. from three independent experiments. Symbols above a column indicate a statistical comparison of progression through each phase of the cell cycle by the indicated cells versus the control cells transfected with Scramble siRNA (NS, not significant; *, $p < 0.05$; **, $p < 0.001$).

transfection with 100 nM RhoA siRNA, and remains suppressed for 96 h after transfection (Fig. 5B). Cells transfected with 100 nM SmgGDS siRNA I1 do not proliferate in culture (Fig. 5C), whereas cells transfected with 100 nM RhoA siRNA appear to divide normally for the first 72 h but then exhibit a decrease in the number of viable cells 96 h after transfection (Fig. 5C). Cell

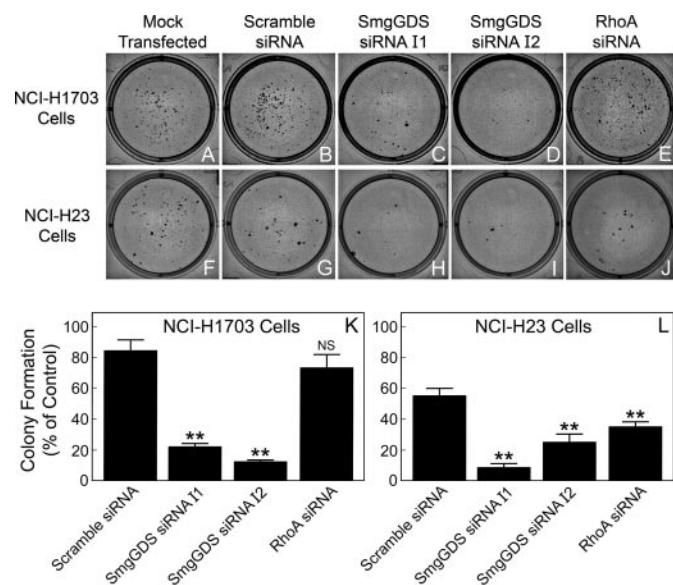


FIGURE 6. Silencing of SmgGDS expression in NSCLC cells diminishes colony formation in soft agar. NCI-H1703 (A–E and K) and NCI-H23 cells (F–J and L) were transfected in the absence of siRNA (mock transfected) or in the presence of 25 nM of the indicated siRNAs and plated 24 h later in soft agar. Digital images of the cells were collected 4 (A–E) or 5 weeks (F–J) later, and the numbers of visible colonies in the agar were counted (K and L). The results in K and L are the mean \pm S.E. from three independent experiments conducted with triplicate samples. Symbols above a column indicate a statistical comparison between the indicated sample and the control sample of cells transfected with Scramble siRNA (NS, not significant; **, $p < 0.01$).

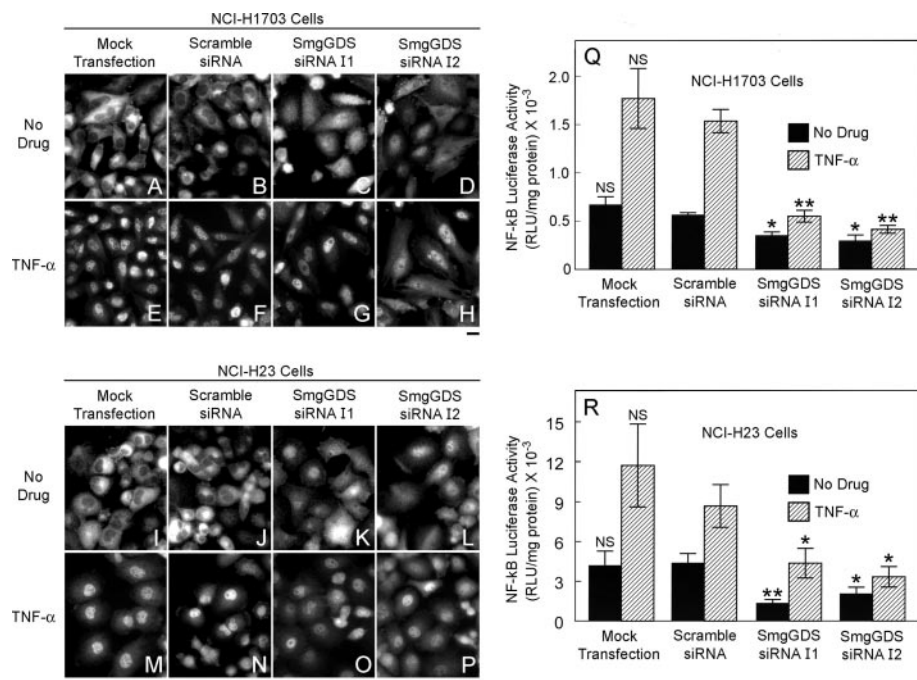


FIGURE 7. Silencing of SmgGDS expression promotes nuclear translocation of the NF- κ B RelA subunit, but inhibits NF- κ B transcriptional activity. To examine RelA nuclear translocation, NCI-H1703 (A–H) or NCI-H23 cells (I–P) were transfected without siRNA (mock transfection) or transfected with 25 nM of the indicated siRNAs and cultured for 72 h. The cells were incubated in the absence (A–D and I–L) or presence (E–H and M–P) of TNF- α (10 ng/ml) for 30 min, and immunofluorescently labeled with antibody to the NF- κ B RelA subunit. Results are representative of three independent experiments. The bar represents 50 μ m. To examine NF- κ B activity, NCI-H1703 (Q) or NCI-H23 cells (R) were transfected without siRNA (mock transfection) or with 25 nM of the indicated siRNAs, cultured for 48 h, and then transfected with the pNifty-Luc NF- κ B reporter plasmid. After 24 h, the cells were incubated in the absence or presence of TNF- α (100 ng/ml) for 3 h, and luciferase activity was measured. The results are the mean \pm S.E. from three independent experiments, with triplicate samples measured in each experiment. Symbols above a column indicate a statistical comparison of luciferase activity in the indicated treatment groups compared with control cells transfected with Scramble siRNA (NS, not significant; *, $p < 0.05$; **, $p < 0.001$).

cycle analysis 72 h after siRNA transfection indicates that 100 nM SmgGDS siRNA I1 causes cells to accumulate in G₁ phase with exclusion from S phase (Fig. 5D), whereas 100 nM RhoA siRNA causes a more moderate accumulation in G₁ phase with less exclusion from S phase (Fig. 5D). Similar results were obtained when the cells were transfected with 25 nM siRNA instead of 100 nM siRNA and when SmgGDS siRNA I2 was included in the experiments (Fig. 5E).

We also examined the effects of silencing the expression of SmgGDS or RhoA on the anchorage-independent proliferation of the cells in soft agar (Fig. 6). NCI-H1703 or NCI-H23 cells were transfected without siRNA (mock transfected) or transfected with 25 nM of the indicated siRNAs 24 h before plating in agar, and the numbers of colonies in the agar were counted 4 (for NCI-H1703 cells) or 5 weeks (for NCI-H23 cells) later. We found that NCI-H1703 cells form fewer colonies when the cells are transfected with either SmgGDS siRNA I1 or SmgGDS siRNA I2, compared with mock transfected cells or cells transfected with Scramble siRNA or RhoA siRNA (Fig. 6, A–E and K). Similar results were obtained using NCI-H23 cells, except that transfection with RhoA siRNA significantly diminished the number of colonies formed by NCI-H23 cells (Fig. 6, F–J and L).

Several small GTPases that are activated by SmgGDS promote the nuclear translocation and transcriptional activity of the RelA subunit of NF- κ B (22–26). Thus, we expected that silencing SmgGDS expression would diminish both the nuclear

accumulation and transcriptional activity of RelA. Unexpectedly, we observed increased nuclear accumulation of RelA in both NCI-H1703 and NCI-H23 cells transfected with either SmgGDS siRNA I1 or SmgGDS siRNA I2, compared with mock-transfected cells or cells transfected with Scramble siRNA (Fig. 7, A–D and I–L). Despite this high level of nuclear RelA, NF- κ B activity is significantly diminished in NCI-H1703 and NCI-H23 cells with silenced SmgGDS expression, as indicated by luciferase production from the pNifty-Luc NF- κ B reporter plasmid (Fig. 7, Q and R). These findings indicate that silencing SmgGDS expression promotes the nuclear accumulation of RelA, but this nuclear RelA is transcriptionally inactive and may repress NF- κ B-dependent transcription. In contrast to the effects of silencing SmgGDS expression, we observed that silencing RhoA expression does not inhibit NF- κ B transcriptional activity (data not shown).

To determine whether TNF- α -dependent pathways that stimulate NF- κ B activity are disrupted by silencing SmgGDS, we tested the

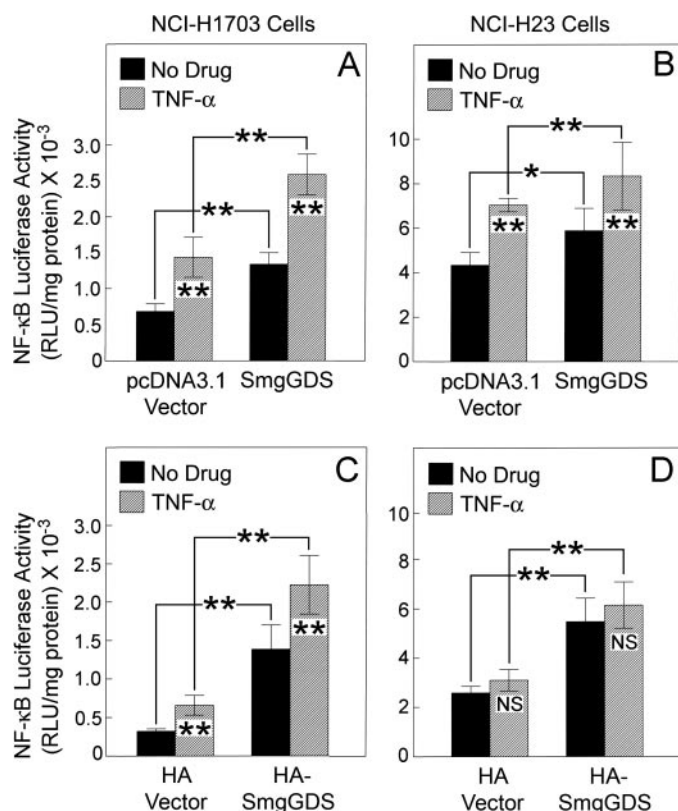


FIGURE 8. Increased expression of SmgGDS increases NF-κB transcriptional activity. NCI-H1703 (A and C) and NCI-H23 cells (B and D) were co-transfected with the pNifty-Luc NF-κB reporter plasmid plus a cDNA encoding SmgGDS (A and B) or a cDNA encoding hemagglutinin (HA)-tagged SmgGDS (C and D). Control cells were co-transfected with the pNifty-Luc NF-κB reporter plasmid plus the pcDNA3.1 vector (A and B) or the hemagglutinin vector (C and D). Luciferin was added to the cultures 24 h after transfection, and luciferase activity was measured. The results are the mean \pm S.E. from three independent experiments, with triplicate samples measured in each experiment. Symbols in the brackets indicate a statistical comparison between the bracketed samples (NS, not significant; *, $p < 0.05$; **, $p < 0.01$).

effects of TNF- α on NF- κ B activity in the cells. Treatment with TNF- α promotes RelA nuclear accumulation in all treatment groups (Fig. 7, E–H and M–P), and increases NF- κ B transcriptional activity in the mock-transfected cells and in cells transfected with Scramble siRNA (Fig. 7, Q and R). In contrast, cells transfected with SmgGDS siRNA continue to have significantly reduced NF- κ B activity after treatment with TNF- α (Fig. 7, Q and R).

The role of SmgGDS in promoting NF- κ B activity was further tested by co-transfecting the cells with the pNifty-Luc NF- κ B reporter plasmid in the presence of a cDNA encoding SmgGDS or in the presence of the pcDNA3.1 vector (control cells). NF- κ B activity was determined 24 h after transfection of the cells. We found that increased expression of SmgGDS increases NF- κ B activity in the cells (Fig. 8, A and B), regardless of whether the cells are treated with or without TNF- α (Fig. 8, A and B). Similar results were obtained when the cells were transfected with a cDNA encoding hemagglutinin-tagged SmgGDS (Fig. 8, C and D). These results indicate that SmgGDS actively promotes NF- κ B activity in the cells.

To determine the effects of SmgGDS siRNA on the organization of the actin/myosin cytoskeleton, we immunofluorescently stained NCI-H1703 cells with an antibody to the myosin

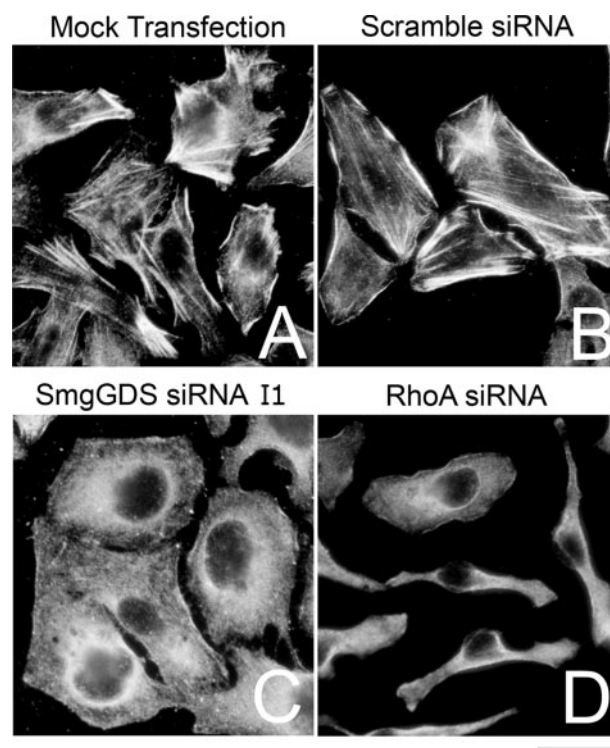


FIGURE 9. Silencing of SmgGDS or RhoA expression disrupts myosin organization and cell morphology. NCI-H1703 cells were transfected in the absence (A) or presence (B–D) of 100 nm of the indicated siRNAs, cultured for 72 h, and immunofluorescently labeled with the SA-2 antibody to the myosin heavy chain. Results are representative of three independent experiments. The bar represents 50 μ m.

heavy chain 72 h after siRNA transfection (Fig. 9). Myosin-containing stress fibers are present in mock-transfected cells (Fig. 9A) and in cells transfected with Scramble siRNA I1 (Fig. 9B), indicating that myosin associates with actin in these control cells. In contrast, myosin-containing stress fibers are absent in cells transfected with SmgGDS siRNA I1 (Fig. 9C) or RhoA siRNA (Fig. 9D), indicating an inability of myosin to associate with actin in these cells. Silencing of SmgGDS or RhoA expression also induces unique morphological changes in the cells. Loss of SmgGDS induces cell spreading, resulting in large elliptical cells (Fig. 9C), whereas loss of RhoA results in thin, elongated cells (Fig. 9D). Transfection with SmgGDS siRNA I2 disrupted myosin organization and caused morphological changes that resembled the responses induced by transfection with SmgGDS siRNA I1 (data not shown).

The cytoskeletal changes induced by silencing SmgGDS or RhoA expression may affect the ability of the cells to migrate. We first tested this possibility by examining the ability of NCI-H1703 cells to migrate over a 24-h period from a confluent monolayer into an area devoid of cells (Fig. 10). NCI-H1703 cells transfected with SmgGDS siRNA I1 (Fig. 10C) re-populate the cell-free area much slower than cells transfected without siRNA (Fig. 10A) or cells transfected with Scramble siRNA (Fig. 10B) or RhoA siRNA (Fig. 10D). Transfection with SmgGDS siRNA I2 inhibited cell migration to the same extent as transfection with SmgGDS siRNA I1 (data not shown). These findings suggest that migration is diminished in cells transfected with SmgGDS siRNA. However, reduced proliferation by these

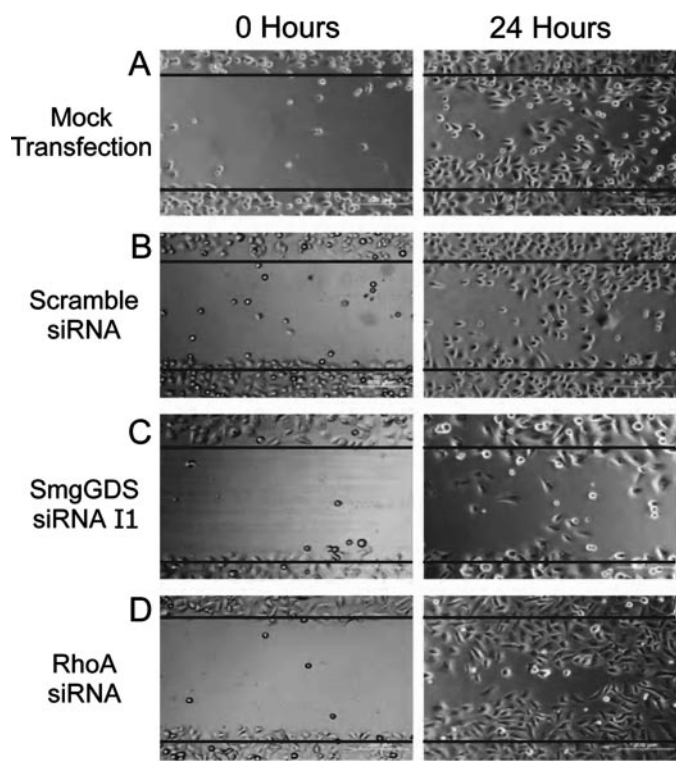


FIGURE 10. Silencing of SmgGDS expression slows the migration of cells in confluent monolayers. NCI-H1703 cells were transfected in the absence (A) or presence (B–D) of 100 nM of the indicated siRNAs and cultured for 72 h. The confluent monolayers were scratched to remove cells, and images of the cells were collected immediately (left panels) and 24 h later (right panels). The results are representative of three independent experiments. The bar represents 200 μ m.

cells may also contribute to their slower re-population of the cell-free area. To eliminate this possibility, we also measured the area of the tracks left by single NCI-H1703 cells as they migrated over colloidal gold (Fig. 11). These measurements indicated that migration of single NCI-H1703 cells is diminished by SmgGDS siRNA (Fig. 11, C and E), but not by RhoA siRNA (Fig. 11, D and E). This finding indicates that SmgGDS promotes cell migration.

DISCUSSION

The results of this study are the first to demonstrate that SmgGDS promotes the malignant characteristics of carcinoma cells. SmgGDS differs from all other known GEFs because it activates multiple members of the Ras and Rho families of small GTPases (reviewed in Refs. 38 and 39). These small GTPases promote malignancy by multiple mechanisms, including enhancing the proliferation and migration of carcinoma cells (4–20). The ability of SmgGDS to act as a master regulator of these multiple small GTPases makes SmgGDS a very unique candidate to regulate multiple forms of cancer. Our observation that SmgGDS protein levels are elevated in a significant number of NSCLC tumors supports a role for SmgGDS in promoting the development or progression of NSCLC. This role is further supported by our finding that silencing SmgGDS expression diminishes the proliferation and migration of cultured NSCLC cells. These results identify SmgGDS as an important regulator of the malignant phenotype in NSCLC.

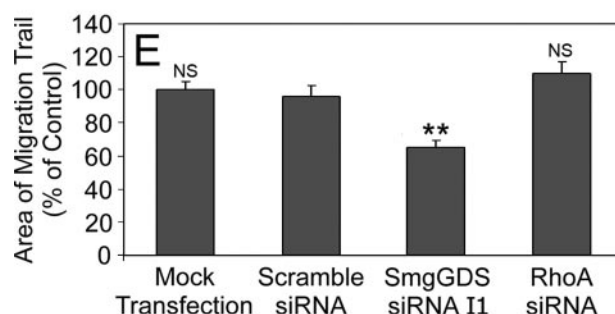
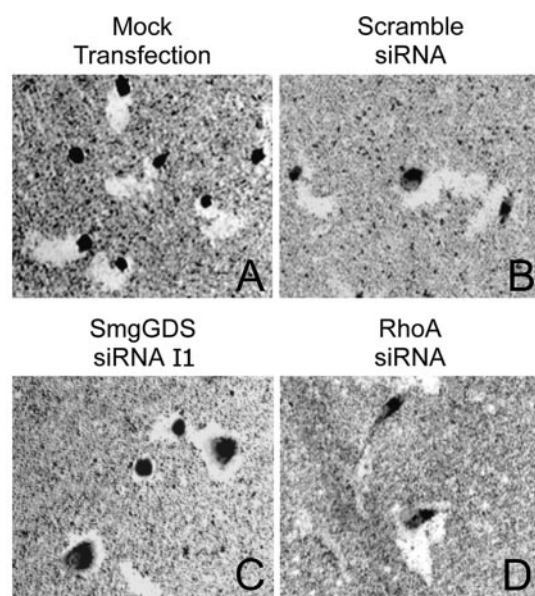


FIGURE 11. Silencing of SmgGDS expression slows the migration of individual cells. NCI-H1703 cells were transfected in the absence (A) or presence (B–D) of 100 nM of the indicated siRNAs, cultured for 72 h, and allowed to migrate on colloidal gold coated with collagen Type I. Digital images of the cells were collected 24 h later. The bar represents 50 μ m. E, the areas of the migration trails were measured after the cells migrated for 24 h, and the values were normalized to migration by the mock-transfected cells. Results are the mean \pm S.E. from three independent experiments. Symbols above a column indicate a statistical comparison of migration by the indicated cells compared with control cells transfected with Scramble siRNA (NS, not significant; **, $p < 0.001$).

The molecular mechanisms responsible for the increased expression of SmgGDS in NSCLC tumors are currently unknown. A recent study examining mRNA transcripts in squamous cell lung carcinoma detected increased SmgGDS mRNA expression in 69% of the 16 squamous cell lung tumors that were examined (7). We detected increased SmgGDS protein expression in 58% of the 117 squamous cell lung tumors that we examined. The finding that similar percentages of squamous cell lung tumors have elevated levels of SmgGDS mRNA (7) and SmgGDS protein (this study) suggests that increased transcription of SmgGDS mRNA is responsible for the elevated SmgGDS protein levels in squamous cell lung tumors. Changes in DNA methylation and histone acetylation, as well as other epigenetic events occurring in NSCLC (reviewed in Ref. 54) may increase SmgGDS mRNA transcription in the tumor cells.

We expected SmgGDS expression to be significantly higher in cultured NSCLC cell lines than in cultured NHBE cells, based on our finding that 22 of 28 patients (78.5%) had higher Smg-

GDS expression in their NSCLC tumor than in their matched normal lung tissue (Fig. 1, *I* and *J*). Unexpectedly, we observed that SmgGDS expression was higher in the cultured NHBE cells than in the NSCLC cell lines, which exhibited variable expression of SmgGDS. The small sample size of NHBE and NSCLC cell lines that we tested makes it difficult to assign significance to this unexpected finding. However, one potential explanation for this result is that the NHBE cells (obtained from a commercial source) may have been derived from an individual with high levels of SmgGDS expression in their lung tissue. We detected such individuals at a low frequency when we examined SmgGDS expression in a group of healthy individuals (Fig. 1*A*) and when we examined SmgGDS expression in normal lung tissue from patients with matched squamous cell lung carcinoma tumors (Fig. 1*J*). Interestingly, a patient in this latter group had healthy lung tissue with SmgGDS expression at a high enough level to be scored +1 in the immunohistochemical analysis, which was equal to or greater than the level of SmgGDS in the majority of the squamous cell lung tumors from the other individuals in this group (Fig. 1*J*).

Although the NHBE cells that we examined exhibited elevated SmgGDS expression, silencing of SmgGDS expression had less of an inhibitory effect on the proliferation of these NHBE cells than on the NCI-H1703 cells (supplemental Fig. 1). This result suggests that SmgGDS is needed more for the proliferation of NSCLC cells than for NHBE cells. This result is consistent with the model of "oncogene addiction" (55), in which the NCI-H1703 cells have become dependent on the functions of SmgGDS as an oncogene that is required for cell proliferation. However, caution should be used when comparing the anti-proliferative effects of silenced SmgGDS expression in NHBE cells *versus* NSCLC cells, because the low proliferation of the cultured NHBE cells may complicate the measurement of these anti-proliferative effects.

The variable level of SmgGDS expression among the NSCLC cell lines was also somewhat unexpected. The original tumors that generated these NSCLC cell lines and the matched healthy lung tissues from the patients with these tumors are not available. Thus, we could not determine whether these NSCLC cell lines express SmgGDS levels that are similar to those expressed by the original tumors. Similarly, we could not determine whether the tumors that gave rise to these cell lines expressed SmgGDS levels that were higher than those expressed by the matched healthy lung tissue from the patients with these tumors.

Although we observed variable expression of SmgGDS in the different NSCLC cell lines, all four NSCLC cell lines exhibited significantly reduced cell proliferation when SmgGDS expression was silenced. The NSCLC cell lines with the greatest loss of SmgGDS expression exhibited the greatest loss of cell proliferation. These findings provide evidence that SmgGDS promotes NSCLC cell proliferation.

SmgGDS may promote NSCLC cell proliferation by enhancing NSCLC cell cycle progression or by suppressing NSCLC cell death. We could not detect significant changes in necrotic or apoptotic cell death in cells transfected with SmgGDS siRNA, as indicated by the number of cells in the sub-G₀/G₁ fraction determined by fluorescence-activated cell sorter analysis, or by

cleavage of caspase-3 determined by Western blotting (data not shown). However, we found that NSCLC cells transfected with SmgGDS siRNA arrest in the G₁ phase of the cell cycle, indicating that SmgGDS is required for the progression of NSCLC cells from G₁ phase to S phase. Thus, SmgGDS most likely promotes NSCLC cell proliferation by enhancing cell cycle progression, rather than by suppressing cell death.

SmgGDS may regulate NSCLC cell proliferation by regulating NF- κ B activity. We found that silencing SmgGDS expression diminishes NF- κ B activity while increasing SmgGDS expression enhances NF- κ B activity, indicating that SmgGDS regulates NF- κ B transcriptional activity in NSCLC cells. NF- κ B promotes the transcription of gene products that are required for cell cycle progression from G₁ phase through S phase (reviewed in Ref. 56). The loss of NF- κ B-mediated transcription in NSCLC cells with silenced SmgGDS expression may account for the reduced proliferation of these cells and their inability to progress through G₁ phase. However, it is also possible that other uncharacterized SmgGDS-dependent signaling events, which do not involve NF- κ B, also contribute to the SmgGDS-dependent regulation of NSCLC cell proliferation.

Several lines of evidence suggest that the SmgGDS-dependent regulation of NF- κ B activity may confer a greater selective advantage to NSCLC cells in tumors than to NSCLC cells maintained in tissue culture. NF- κ B is thought to be one of the most important signaling molecules regulated by cytokines (reviewed in Refs. 57–62) and oxidative stress (reviewed in Ref. 63–68) during the inflammatory process that leads to cancer development. Activation of NF- κ B in pulmonary epithelial cells induces transcription of a variety of proteins that protect the cells from the inflammatory process and promote their neoplastic transformation. For example, NF- κ B induces transcription of antioxidant enzymes such as manganese superoxide dismutase (69, 70), which may protect the cells from oxidative stress induced by inflammation or inhaled particulate matter (reviewed in Refs. 71 and 72). In addition to inducing expression of antioxidants, NF- κ B also induces the transcription of many other proteins that promote lung cancer development and progression, including proteins that promote immortalization, invasion, and metastasis, such as cyclin D1, cyclooxygenase-2, and matrix metalloproteases (reviewed in Ref. 57). These features make NF- κ B an important therapeutic target for chemoprevention and chemotherapy of lung cancer (reviewed in Refs. 30, 57, 60, and 61). Previous studies indicate that NF- κ B activity is elevated in 70% of pre-neoplastic bronchial biopsies exhibiting dysplasia (73) and in 67% of NSCLC tumors (31), providing additional evidence of the importance of this transcription factor in NSCLC tumors. Elevated SmgGDS levels in NSCLC tumors may contribute to elevated NF- κ B activity in these tumors, protecting the tumor cells from the inflammatory process and promoting tumor progression and metastasis.

SmgGDS most likely regulates the activity of NF- κ B by activating the small GTPases that promote NF- κ B transcriptional activity. The SmgGDS-dependent activation of many different small GTPases (40–45), several of which can activate NF- κ B through different pathways (22–26), may stimulate several signaling pathways that ultimately converge to increase NF- κ B activity, resulting in a cumulative enhancement of NF- κ B tran-

scriptional activity when SmgGDS is active or overexpressed. Diminished signaling by these small GTPases when SmgGDS is inactive or underexpressed (as in cells with silenced SmgGDS expression) may reduce NF- κ B activity in cells with inactive or underexpressed SmgGDS.

Silencing SmgGDS causes the nuclear accumulation of RelA, but surprisingly this nuclear RelA is apparently unable to induce transcription. Previous studies identified other agents that enhance the nuclear accumulation of RelA or the binding of RelA to DNA, but paradoxically inhibit the transcriptional activity of RelA (35–37). These agents may incompletely or only partially activate the signaling pathways that stimulate RelA transcriptional activity, resulting in the generation of some but not all of the post-translational modifications that are needed for RelA transcriptional activity, such as RelA phosphorylation and acetylation. The generation of only a few of the modifications that are needed for RelA transcriptional activity may lead to the accumulation of nuclear RelA that binds to its promoters but is unable to activate these promoters, resulting in transcriptional repression (reviewed in Ref. 33). A similar model may explain why silencing SmgGDS expression represses NF- κ B activity in NSCLC cells. SmgGDS may be needed to coordinate the activities of multiple small GTPases that synergistically regulate RelA nuclear translocation, phosphorylation, acetylation, and interactions with co-activators and DNA-binding partners. Loss of SmgGDS may disrupt the activities of these small GTPases, causing the uncoordinated generation of signals that regulate RelA, ultimately resulting in the nuclear accumulation of transcriptionally inactive RelA.

Our observation that silencing SmgGDS expression diminishes colony formation by NSCLC cells in soft agar provides further evidence that SmgGDS promotes the malignant phenotype. The anchorage-independent proliferation of cells in soft agar is a characteristic of malignant transformation (reviewed in Ref. 74). The diminished colony formation of NCI-H1703 and NCI-H23 cells transfected with either SmgGDS siRNA I1 or SmgGDS siRNA I2 is consistent with the diminished cell proliferation (Fig. 4) and decreased NF- κ B activity (Fig. 7) in these cells that have reduced SmgGDS expression.

RhoA is considered to be a preferred substrate for SmgGDS (42, 43). Due to the potentially important role for RhoA in SmgGDS signaling pathways, we investigated whether silencing RhoA induces the same effects as silencing SmgGDS in the cells. Silencing RhoA should mimic the effects of silencing SmgGDS, if loss of RhoA activity is the major cause for the observed effects of silencing SmgGDS. We found that silencing RhoA expression induces different effects in different cell lines and is less deleterious to the cells than silencing SmgGDS expression. Silencing RhoA expression in NCI-H1703 cells modestly diminishes thymidine uptake (Fig. 4C) but does not diminish colony formation in soft agar (Fig. 6K). In contrast, silencing RhoA expression in NCI-H23 cells does not inhibit thymidine uptake (Fig. 4F) but diminishes colony formation in soft agar (Fig. 6L). We also observed that silencing RhoA does not inhibit NF- κ B activity (data not shown) or cell migration, in contrast to the diminished NF- κ B activity and migration of cells that have silenced SmgGDS expression. These findings support the conclusion that reduced RhoA signaling is not the major

cause of the reduced cell proliferation or other changes induced by silencing SmgGDS expression.

We also observed different time-dependent changes in the proliferation of the cells following transfection with SmgGDS siRNA *versus* RhoA siRNA. Cells transfected with SmgGDS siRNA exhibit reduced cell proliferation within 48 h of siRNA transfection (Fig. 5C). In contrast, cells transfected with RhoA siRNA proliferate normally for several days after RhoA expression is silenced, but after the cells have doubled in number, they apparently undergo a crisis and exhibit reduced proliferation by the third or fourth day after transfection (Fig. 5C). The reasons for this crisis are not clear, but it may involve abnormalities in cytokinesis, which is a process that is known to be dependent on active RhoA (reviewed in Ref. 75). This reduction in the proliferation of RhoA siRNA-transfected cells is apparently transient, because NCI-H1703 cells transfected with RhoA siRNA do not exhibit abnormalities in colony formation in soft agar 4 weeks after siRNA transfection (Fig. 6K).

Silencing the expression of SmgGDS or RhoA induces unique changes in cell morphology and migration, which probably result from changes in the actin/myosin cytoskeleton. It is not surprising that silencing SmgGDS or RhoA expression disrupts the actin/myosin cytoskeleton, because RhoA and other small GTPases that are activated by SmgGDS are known to regulate actin/myosin interactions (reviewed in Ref. 19–21). Silencing SmgGDS induces cell spreading and diminishes cell migration, whereas silencing RhoA induces cell narrowing and does not diminish cell migration. These differences in the effects induced by silencing SmgGDS compared with silencing RhoA probably occur because silencing SmgGDS diminishes the activities of multiple small GTPases in addition to RhoA. Cell migration depends on coordinated changes in the actin/myosin cytoskeleton that are regulated by multiple small GTPases, including RhoA, Rac1, and the Rap1 proteins (reviewed in Ref. 19). When SmgGDS expression is silenced, this coordination may be lost due to the reduced activities of these small GTPases, resulting in cytoskeletal changes that cannot support migration.

In all experiments using siRNAs, we included control samples consisting of cells transfected without siRNA (mock transfection) and cells transfected with Scramble siRNA. Responses induced by the Scramble siRNA did not significantly differ from those induced by mock transfection in many assays (Figs. 5, 7, and 9–11). However, transfection with the Scramble siRNA unexpectedly reduced the proliferation of NCI-H23 cells, compared with the proliferation of the mock-transfected cells (Figs. 3I, 4F, and 6L). Transfection of these cells with 100 nM Scramble siRNA diminished thymidine uptake by 70% (Fig. 3I), whereas transfection with 25 nM Scramble siRNA reduced thymidine uptake by 40% (Fig. 4F) and colony formation by 40% (Fig. 6L), compared with the mock-transfected cells. The reasons for these effects of the Scramble siRNA on NCI-H23 cells are unknown. The Scramble siRNA may nonspecifically silence the expression of a protein needed for the proliferation of NCI-H23 cells, even though this siRNA was designed to not target any human mRNAs. Alternatively, the Scramble siRNA may competitively inhibit the functions of endogenous microRNAs in NCI-H23. The competition between synthetic siRNAs and

endogenous microRNAs has only recently been recognized to play an important role in siRNA and microRNA functions (76). The roles of different microRNAs in NSCLC cells are just now being characterized (reviewed in Ref. 77), and it is possible that the Scramble siRNA may competitively interfere with the functions of some of these microRNAs in NCI-H23 cells, resulting in diminished proliferation. Despite this nonspecific anti-proliferative effect of the Scramble siRNA, NCI-H23 cell proliferation was diminished significantly more by transfection with SmgGDS siRNAs I1 or I2 than by transfection with the Scramble siRNA (Figs. 3I, 4F, and 6L).

In conclusion, the results of this study identify SmgGDS as an important regulator of the malignant phenotype of NSCLC cells. Our finding that SmgGDS protein levels are increased in a significant number of NSCLC tumors supports a role for SmgGDS in NSCLC tumorigenesis. SmgGDS may promote NSCLC tumorigenesis by enhancing NF- κ B activity and stimulating the proliferation of NSCLC cells. SmgGDS may promote metastasis by regulating the organization of the cytoskeleton and promoting the migration of NSCLC cells. Our demonstration that the loss of SmgGDS expression is more deleterious than the loss of RhoA in NSCLC cells is consistent with our model that SmgGDS promotes the malignant NSCLC phenotype by regulating the activities of multiple small GTPases in addition to RhoA. Studies are currently underway in our laboratory to determine how SmgGDS regulates the activities of multiple small GTPases to control the malignant characteristics of NSCLC cells.

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