The SmgGDS Splice Variant SmgGDS-558 Is a Key Promoter of Tumor Growth and RhoA Signaling in Breast Cancer

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Abstract

Breast cancer malignancy is promoted by the small GTPases RhoA and RhoC. SmgGDS is a guanine nucleotide exchange factor that activates RhoA and RhoC in vitro. We previously reported that two splice variants of SmgGDS, SmgGDS-607, and SmgGDS-558, have different characteristics in binding and transport of small GTPases. To define the role of SmgGDS in breast cancer, we tested the expression of SmgGDS in breast tumors, and the role of each splice variant in proliferation, tumor growth, Rho activation, and NF-κB transcriptional activity in breast cancer cells. We show upregulated SmgGDS protein expression in breast cancer samples compared with normal breast tissue. In addition, Kaplan–Meier survival curves indicated that patients with high SmgGDS expression in their tumors had worse clinical outcomes. Knockdown of SmgGDS-558, but not SmgGDS-607, in breast cancer cells decreased proliferation, in vivo tumor growth, and RhoA activity. Furthermore, we found that SmgGDS promoted a Rho-dependent activation of the transcription factor NF-κB, which provides a potential mechanism to define how SmgGDS-mediated activation of RhoA promotes breast cancer. This study demonstrates that elevated SmgGDS expression in breast tumors correlates with poor survival, and that SmgGDS-558 plays a functional role in breast cancer malignancy. Taken together, these findings define SmgGDS-558 as a unique promoter of RhoA and NF-κB activity and a novel therapeutic target in breast cancer.

Implications: This study defines a new mechanism to regulate the activities of RhoA and NF-κB in breast cancer cells, and identifies SmgGDS-558 as a novel promoter of breast cancer malignancy. Mol Cancer Res; 12(1); 130–42. © 2013 AACR.

Introduction

Members of the Rho family of small GTPases, especially RhoA and RhoC, have significant roles in breast cancer progression and metastasis. RhoA expression is often increased in primary breast tumors, and its expression correlates with more aggressive breast cancers and poorer survival (1). Knockdown of RhoA expression in aggressive breast cancer cell lines significantly decreases proliferation and tumor formation in vivo (2, 3). Taken together, these findings indicate that therapeutically targeting the activation of RhoA has potential to treat aggressive breast cancers (4).

RhoA and RhoC have been shown to regulate the malignancy of breast cancer cells through the prosurvival transcription factor, NF-κB. Rho increases NF-κB transcriptional activity by activating ROCK (5, 6), which is associated with increased breast cancer malignancy (7). Clinical studies have shown that about 13% of patients with breast cancer have highly active NF-κB in their tumors and that these tumors are resistant to chemotherapy (8, 9). Targeting Rho activity in breast cancer may inhibit NF-κB transcriptional activity, which may diminish malignancy.

One way to suppress Rho activity is to target Rho regulators such as guanine nucleotide exchange factors (GEF). SmgGDS (gene name RAP1GDS1) is a noncanonical GEF that promotes the activity of RhoA and RhoC (10–15). SmgGDS interacts with many small GTPases that have a polybasic region at their C-termini (16, 17). There have been reports that SmgGDS can activate K-Ras, Rap1, Rac1, and RhoA due to these interactions (10–14). A recent report has shown that SmgGDS is most likely a true GEF, specifically for RhoA and Rac, in cells (15). These properties indicate that SmgGDS is a novel target to regulate Rho activity in breast cancer.

The present study compares the roles of SmgGDS-558 with SmgGDS-607, which are two SmgGDS splice variants whose expression and function in breast cancer have not been previously characterized. Our results show the
importance of SmgGDS in the development and progression of breast cancer. We find that both SmgGDS-558 and SmgGDS-607 are expressed in all breast cancer cell lines studied, and SmgGDS expression is elevated in breast tumors compared with normal mammary tissue. By comparing the effects of selectively diminishing SmgGDS-558 or SmgGDS-607 expression, we found that SmgGDS-558 plays a greater role in activating RhoA and promoting breast cancer progression than does SmgGDS-607. To investigate a mechanism to explain how the SmgGDS-mediated activation of Rho promotes breast cancer, we tested the ability of SmgGDS to promote Rho-dependent activation of the transcription factor, NF-kB. We found that SmgGDS-558 overexpression promotes NF-kB transcriptional activity in breast cancer cells and that this response is diminished by the expression of dominant-negative Rho. These results indicate that SmgGDS promotes Rho-dependent NF-kB transcriptional activity in breast cancer cells. Taken together, these findings demonstrate that SmgGDS-558 plays an important role in breast cancer, making it a novel therapeutic breast cancer target.

Materials and Methods

Tumor tissues and tissue microarrays

Nine human tumor tissue microarrays from archival samples were analyzed. The number of tumor tissue cores for each case ranged from two to three, and included 16 benign breast (including both normal and benign proliferative lesions), 11 ductal carcinoma in situ (DCIS), 96 invasive ductal adenocarcinoma (IDC), and 52 metastatic lymph node specimens. Histologically normal breast tissue controls were present in each array. Tissue was fixed in 10% neutral-buffered formalin. All protocols were approved by the Medical College of Wisconsin (Milwaukee, WI) Institutional Review Board.

Antibodies and immunohistochemistry

Immunohistochemical (IHC) staining was performed using an automated DAKO Autostainer Plus stainer as we previously described (18), utilizing the following antibodies: calponin (DAKO clone CALP), CK5/6 (DAKO clone D5/16 B4), ER (DAKO clones 1D5 and SP1), Her2 (DAKO HercepTest), ki-67 (DAKO clone MIB-1), p63 (Biocare Medical clone BC4A4), PR (DAKO clone PgR636), and SmgGDS (BD Transduction Laboratories, 612511). Cytoplasmic and membranous expression of SmgGDS was quantified manually in each core using an Automated Cellular Imaging System III (DAKO) as previously described (19). For all measurements, only benign or malignant ductal epithelia were analyzed; stroma, inflammatory cells, vessels, and other nonepithelial elements were excluded. There were between four to 20 regions per case sampled. The automated cellular imaging system (ACIS) system measures the intensity of the staining based on three related parameters: hue (color), luminosity (brightness), and saturation (density of color). ACIS software was programmed by setting the color-specific thresholds to determine and calculate SmgGDS staining intensity and the ratio of SmgGDS-positive breast epithelial cells to total breast epithelial cells. The percentage of SmgGDS-positive ductal epithelial cells was then determined. The breast cancer cases were categorized as luminal A, luminal B, basal-like, Her2+, and other as described (20).

Cell lines and reagents

All cell lines were obtained from the American Type Culture Collection (ATCC). T47D and MDA-MB-231 cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM), high glucose with 1-glutamine media supplemented with sodium pyruvate, and 10% FBS. BT-474 and MDA-MB-468 cells were maintained in RPMI-1640 with 1-glutamine supplemented with 10% FBS. MCF-7, HCC-1954, MCF-10A, and MCF-12A cells were maintained as indicated by the ATCC. Culture media were supplemented with penicillin and streptomycin.

Immunoblotting

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 the following antibodies: SmgGDS (BD Transduction Laboratories, 612511), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, sc-32233), HA (Covance, MMS-101P), RhoA (Cytoskeleton, ARH03), and RhoC (Abcam, ab64659). Bound antibodies were visualized using horseradish peroxidase-linked secondary antibodies (Amersham Biosciences) as previously described (17, 21). Densitometry of blots was determined using ImageJ software (22).

siRNA transfection

Previously optimized siRNA duplexes for SmgGDS knockdown were used (17). Cells were transfected with 25 nmol/L siRNA using DharmaFECT-1 transfection reagent (Dharmacon) according to the manufacturer’s instruction.

[3H]-thymidine uptake

Cell proliferation was assessed by measuring [3H]-thymidine uptake by the cells, as we previously described (23).

Coimmunoprecipitation assays

The cDNAs encoding SmgGDS, or HA-tagged wild-type (WT-) or dominant negative (T19N mutation, DN-) Rho, were generated as described previously (17, 21, 24). Lipofectamine 2000 (Invitrogen) was used to transfect cells with a cDNA construct encoding either HA-tagged, WT- or DN- Rho, or the HA vector, in the presence or absence of a cDNA encoding SmgGDS-607 or SmgGDS-558. After 24 hours, the cells were lysed and immunoprecipitated with HA-conjugated agarose beads, and the immunoprecipitates were immunoblotted for SmgGDS and HA as previously described (17).

Rho activation assay

Rho activation was measured using a pulldown assay conducted according to the manufacturer’s instructions.
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NF-κB transcriptional activation assay

In SmgGDS knockdown assays, cells were transfected with the indicated siRNAs. After 48 hours, pNifty-Luc NF-κB luciferase reporter and β-galactosidase reporter plasmids were transfected using Lipofectamine 2000 (Invitrogen) into the cells. After an additional 24 hours, luminescence was quantified by addition of luciferin (0.15 mg/mL) to each well followed by measurement of luminescence using a FLUOstar Omega plate reader. Transfection efficiency was normalized by measuring β-galactosidase activity after luminescence measurements by washing cells with PBS and incubating the wells with a β-galactosidase reagent (Pierce) for 30 minutes or after detection of color. β-galactosidase activity was measured using a FLUOstar Omega plate reader at 405 nm absorbance. NF-κB activity was calculated using the luminescence divided by the optical density.

In overexpression assays, the indicated Rho and SmgGDS plasmids were transfected with the pNifty-Luc NF-κB luciferase reporter and β-galactosidase reporter plasmids using Lipofectamine 2000 (Invitrogen) into the cells. After 24 hours, NF-κB activity was determined similarly to the SmgGDS knockdown assays.

MDA-MB-231 cells stably expressing tet-inducible shRNA

MDA-MB-231 cells were transduced with lentiviral vectors encoding the luciferase protein (GeneCopoeia) and the tetracycline repressor protein (Invitrogen) and selected for stable expression. Lentiviral vectors expressing inducible short hairpin RNA (shRNA) for SmgGDS-558 (shRNA BD), SmgGDS-607 (shRNA C2), or a nontargeting control (shRNA Scr4) were generated using the “Block-iT”-inducible BD, SmgGDS-607 (shRNA C2), or a nontargeting control (shRNA Scr4) were generated using the “Block-iT”-inducible H1 lentiviral RNAi system (Invitrogen, K4925-00) as previously described (17). Cells were maintained in DMEM triple selection media (100 μg/mL Zeocin, 14 μg/mL blasticidin, 500 ng/mL puromycin). Expression of shRNA was induced using 625 mg/kg doxycycline in feed for in vivo studies.

Orthotopic xenograft study in SCID hairless outbred mice

Animal studies were conducted according to protocols approved by the MCW Institutional Animal Care and Use Committee. Xenografts were established in the mammary fat pad of 6-week-old female SCID hairless outbred (SHO) mice (Charles River) by injecting 4 × 10⁶ cells in a 1:1 PBS: Matrigel solution. Mice were separated into two groups after 2 weeks; one group remained on normal feed, whereas the other group was given doxycycline feed (Tekland). Tumor size was measured weekly by bioluminescence imaging using a biophotonic imager (Xenogen). Mice were injected with 200 μL of 15 mg/mL luciferin 5 minutes before bioluminescent imaging.

Computational studies

RNA microarray data mining was done by using the Oncomine database (www.oncomine.com). SmgGDS (RAP1GDS1) overexpression was determined using these Oncomine filters: RAP1GDS1, cancer versus normal analysis, and breast cancer. Analyses were chosen by having a P value of 0.05 or lower and RAP1GDS1 gene expression being expressed in the top 10% of genes. Kaplan–Meier plotter data were obtained using the 2012 version of the database (http://kmplot.com/analysis/; ref. 25). The Affymetrix ID 209444_at for SmgGDS was used with the follow-up threshold set at 15 years. The auto select best cutoff and array quality control to exclude biased array were also used in the computation of the plots. Statistics obtained were generated by the Kaplan–Meier plotter software.

Statistical analysis

Results are medians or mean ± SEM. Symbols above a column indicate a statistical comparison between the control and experimental group by Student t test, by one-way ANOVA, or by two-way ANOVA with secondary Bonferroni multiple comparisons test as indicated in the figure legends. P values less than 0.05 were considered significant.

Results

SmgGDS protein and RNA levels are upregulated in breast cancer biopsies

The percentage of SmgGDS-positive breast epithelial cells in benign, DCIS, IDC, and metastatic lymph node specimens were quantified using a digital imaging system (Fig. 1A–C). Nearly all cases of invasive and metastatic breast cancer demonstrated moderate to strong cytoplasmic SmgGDS staining; nuclear staining was absent to weak. Because the percentage of SmgGDS-positive cells demonstrated a much wider dynamic range, varying from less than 10% to 100% of the total malignant cells, we compared cases based on the percentage of SmgGDS-positive cells (Fig. 1B). The relative number of benign epithelial cells that stained SmgGDS positive was significantly lower with less intense staining compared with malignant breast cancer cells (Fig. 1B). A subset of benign breast epithelial cells demonstrated strong cytoplasmic SmgGDS staining (Fig. 1A, ii). On the basis of the similar distribution and localization as p63-positive cells (Fig. 1A, iv), we interpret the strong SmgGDS-positive cells as myoepithelial. The primary tumors were also grouped into different subtypes: luminal A, luminal B, basal like, Her2⁺, and others. SmgGDS expression was high in all subtypes with no significant difference in expression between the different subtypes (Fig. 1C). These results indicate that SmgGDS expression is upregulated at tumor initiation and stays high through tumor progression.

We used Oncomine’s online database to determine whether SmgGDS (RAP1GDS1) RNA levels are upregulated in breast cancer. SmgGDS/RNA levels were in the top 10% of genes upregulated in eight different breast cancer microarrays compared with normal breast tissue (Fig. 1D). These data, along with our IHC results, indicate that SmgGDS
RNA and protein expression are upregulated in breast cancer compared with normal breast tissue.

High SmgGDS expression correlates with poor clinical outcome

To determine whether SmgGDS expression is prognostic in breast cancer, we examined whether SmgGDS expression in breast cancer patients’ tumors correlated with clinical outcomes. We used software from Kaplan–Meier plotter to examine the relapse-free survival of patients with different subtypes of breast cancer. We found that patients with high expression of SmgGDS in luminal A (Fig. 2A), luminal B (Fig. 2B), Her2+ (Fig. 2C), and basal (Fig. 2D) subtypes of breast cancer had a significantly lower relapse-free survival.
When we examined Kaplan–Meier survival curves for survival data combining all breast cancer subtypes, we found that high SmgGDS expression correlated with significantly lower relapse-free survival and overall survival (Fig. 2E and F). The results obtained from these Kaplan–Meier survival curves correlate with our IHC and Oncomine data, indicating that high SmgGDS expression leads to worse prognosis for patients with breast cancer.

**SmgGDS is overexpressed in breast cell lines**

We next determined the expression of SmgGDS in cell lines representing different breast cancer subtypes. SmgGDS
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Figure 3. Knockdown of SmgGDS decreases proliferation in multiple breast cell lines. A, armadillo repeats of SmgGDS-607 and SmgGDS-558 are shown with each armadillo repeat designated by the letters A–M. SmgGDS siRNAs targeting the indicated regions specifically knockdown SmgGDS-607 (siRNA C2), SmgGDS-558 (siRNA BD), or both splice variants (siRNA I1). B, lysates from untransfected breast cell lines were immunoblotted for SmgGDS and GAPDH. C and D, breast cells were transfected with the indicated siRNAs. After 72 hours, cell lysates were immunoblotted for SmgGDS and GAPDH. C and D, breast cells were incubated with [3H]-thymidine for 3 hours, followed by scintillation counting (D). Values are normalized to [3H]-thymidine uptake in cells transfected with scramble siRNA (Scr3). V, P < 0.05; **, P < 0.01; and ***, P < 0.001.

SmgGDS-558 knockdown decreases cell proliferation

We previously reported that simultaneous knockdown of both SmgGDS splice variants in lung and prostate cancer cell lines significantly decreases cell proliferation (18, 26). To test the specific functions of SmgGDS-607 and SmgGDS-558 in breast cancer cell proliferation, we transfected cell lines with siRNAs that specifically target either one or both forms of SmgGDS. siRNA I1 targets both SmgGDS-607 and -558 by recognizing the mRNA region encoding armadillo domain I (Fig. 3A). siRNA BD targets SmgGDS-558 because the siRNA overlaps armadillo domain B and D, which only occurs when SmgGDS mRNA is spliced into SmgGDS-558 (Fig. 3A). siRNA C2 targets SmgGDS-607 specifically because SmgGDS-607 is the only splice variant that encodes armadillo domain C (Fig. 3A). Figure 3C shows the expression of SmgGDS 72 hours after transfecting the eight different breast cell lines with our siRNA duplexes.

All breast cell lines exhibited a 30% to 50% decrease in proliferation when both SmgGDS forms were knocked down with siRNA I1 (Fig. 3D). Knocking down SmgGDS-558 with siRNA BD decreases proliferation by 50% to 65%, whereas knocking down SmgGDS-607 with siRNA C2 did not significantly decrease proliferation (Fig. 3D). These results suggest that in highly proliferative breast cells, SmgGDS-558 is the more important splice variant for promoting proliferation.

Inducible knockdown of SmgGDS-558 decreases tumor formation of orthotopic xenografts

To examine the effects of SmgGDS knockdown on murine xenografts, we developed MDA-MB-231 cell lines stably expressing luciferase (Luc), tet-repressor (TR), and doxycycline-inducible shRNA that specifically targets different SmgGDS splice variants (Fig. 4). We observed that doxycycline-inducible knockdown of both SmgGDS splice variants significantly decreased Luc-231-TR-BD2 tumor size by week 9 (Fig. 4D). Doxycycline-inducible knockdown of SmgGDS-558 also significantly decreased growth of the Luc-231-TR-BD tumors by week 8 (Fig. 4B). In contrast, doxycycline-inducible knockdown of SmgGDS-607 did not
significantly decrease the growth of Luc-231-TR-C2 tumors (Fig. 4C). There was no difference in the growth rate of Luc-231-TR-Scr4 tumors after doxycycline-inducible expression of nontargeting shRNA (Fig. 4A).

To confirm that doxycycline induced the expected loss of the SmgGDS splice variants, we conducted immunoblotting of lysates from tumors formed by each inducible cell line. SmgGDS expression was similar in all tumors expressing scramble shRNA, and in tumors from mice treated without doxycycline (Fig. 4E, lanes 1–12, 17–20, and 25–28). In contrast, SmgGDS-558 expression was specifically diminished by the doxycycline induction of BD shRNA in the Luc-231-TR-BD tumors (Fig. 4E, lanes 13–16). SmgGDS-607 expression was specifically diminished by the doxycycline induction of C2 shRNA in the Luc-231-TR-C2 tumors (Fig. 4E, lanes 21–24). Both SmgGDS splice variants were diminished by inducing both BD and C2 shRNAs with doxycycline in the Luc-231-TR-BDC2 tumors (Fig. 4E, lanes 29–32).

Taken together, our results indicate that SmgGDS-558 is more important than SmgGDS-607 in promoting the proliferation of breast cancer cells and tumor growth in vivo.

Figure 4. Decreased SmgGDS-558 expression slows tumor growth in the mammary fat pad of mice. Luc-231-TR-Scr4 (A), Luc-231-TR-BD (B), Luc-231-TR-C2 (C), and Luc-231-TR-BDC2 (D) cells were injected into the mammary fat pad of SHO mice. At week 2, doxycycline (dox) was administered in the feed of half of each group of mice. In each panel, the top image shows representative raw luminescence images at 2, 5, and 8 weeks after injection of the indicated cells into mice receiving feed with or without doxycycline. The graphs in each panel show relative weekly growth of tumors derived from Luc-231-TR-Scr4 cells (A, n = 7 mice without doxycycline and 8 mice with doxycycline), Luc-231-TR-BD cells (B, n = 9 mice without doxycycline and 9 mice with doxycycline), Luc-231-TR-C2 cells (C, n = 10 mice without doxycycline and 10 mice with doxycycline), and Luc-231-TR-BDC2 cells (D, n = 9 mice without doxycycline and 8 mice with doxycycline). Values are normalized to luminescence obtained at week 2 for each tumor and are the mean ± SEM assessed by two-way ANOVA with secondary Bonferroni multiple comparisons test (*, P < 0.05; **, P < 0.001). E, tumors excised from 32 mice used in the experiments depicted in A–D were examined for SmgGDS expression by immunoblotting.
define a potential mechanism for this critical role of SmgGDS-558, we next compared the abilities of SmgGDS-558 with SmgGDS-607 to interact with Rho proteins because Rho activity is needed for breast cancer progression (1, 27).

SmgGDS splice variants associate with both RhoA and RhoC in breast cancer cells

We first examined with endogenous and overexpressed SmgGDS-607 and SmgGDS-558 associate with overexpressed HA-WT-Rho and HA-DN-Rho in breast cancer cells. In MDA-MB-231 (Fig. 5A and C) and MCF-7 (Fig. 5B and D) cells, both SmgGDS splice variants communoprecipitated (CoIP) with HA-DN-RhoA (Fig. 5, lanes 5–7 and 19–21) and HA-DN-RhoC (Fig. 5, lanes 12–14 and 26–28) consistent with previous reports that SmgGDS is a GEF for RhoA and RhoC (10–12, 14, 15). In MDA-MB-231 cells, but not MCF-7 cells, both SmgGDS splice variants detectably CoIP with HA-WT-RhoA (Fig. 5, lanes 2–4 and 16–18) and HA-WT-RhoC (Fig. 5, lanes 9–11 and 23–25). These results indicate that SmgGDS splice variants stably associate better with the dominant negative form of Rho than with the WT form, consistent with SmgGDS activity as a GEF. These results also indicate that SmgGDS associates more with RhoA than with RhoC, indicating that SmgGDS may be a better activator of RhoA than RhoC.

SmgGDS-558 knockdown decreases the activity of endogenous RhoA in MDA-MB-231 cells

We tested whether knockdown of the different SmgGDS splice variants affected the activity of RhoA and RhoC in MDA-MB-231 cells. Knockdown of SmgGDS-558 significantly decreased RhoA activity at 1 minute after serum addition, whereas knockdown of both splice variants or only SmgGDS-607 modestly, but not significantly, decreased RhoA activity (Fig. 6A, B, and E). Knockdown of SmgGDS-558, SmgGDS-607, or both splice variants did not induce a significant change in RhoC activity (Fig. 6C–E). These results indicate that SmgGDS-558 has a greater ability than SmgGDS-607 to activate RhoA in MDA-MB-231 cells, supporting the model that SmgGDS-558 is the form of SmgGDS that is most important to activate RhoA in breast cancer.

Figure 5. SmgGDS associates with both RhoA and RhoC in breast cancer cells. MDA-MB-231 cells (A and C) or MCF-7 cells (B and D) were transfected with a cDNA encoding the WT or dominant negative form of HA-tagged RhoA (A and B) or RhoC (C and D) in the absence or presence of a cDNA encoding SmgGDS-607 or SmgGDS-558. After 24 hours, each sample was immunoprecipitated using HA antibody and immunoblotted for SmgGDS and HA. Multiple exposures of the immunoblot analyses are shown (indicated by times at the left of each figure) to allow assessment of the relative amounts of coprecipitated proteins. Results represent two independent experiments.
SmgGDS promotes Rho-dependent activation of NF-κB in MDA-MB-231 cells

We next tested whether SmgGDS could regulate Rho-dependent activation of NF-κB in MDA-MB-231 cells. We found that NF-κB activity was significantly diminished by knockdown of SmgGDS-558 alone using siRNA BD, but not by knockdown of SmgGDS-607 alone using siRNA C2 (Fig. 7A). NF-κB activity was inhibited by knocking down both splice variants simultaneously using siRNAs BD and C2, almost to the same level as knocking down only SmgGDS-558 (Fig. 7A).

We hypothesized that RhoA activation contributes to the unique ability of SmgGDS-558 to activate NF-κB in the cells. In support of this hypothesis, we found that NF-κB activity is increased by overexpressing SmgGDS-558 but not by overexpressing SmgGDS-607 (Fig. 7B). These results support the model that activation of endogenous RhoA by overexpressed SmgGDS-558 promotes NF-κB activity.

To further define the role of RhoA in SmgGDS-mediated activation of NF-κB, we examined NF-κB activity in MDA-MB-231 cells coexpressing SmgGDS-558 with either WT- or DN-RhoA (Fig. 7C). As expected, we found that NF-κB activity is significantly higher in cells coexpressing SmgGDS-558 and WT-RhoA, compared with cells expressing WT-RhoA alone (Fig. 7C). These results are consistent with the model that SmgGDS-558 activates RhoA to promote RhoA-dependent activation of NF-κB. Most importantly, the coexpression of DN-RhoA with SmgGDS-558 does not significantly increase NF-κB activity, compared with cells coexpressing WT-RhoA with SmgGDS-558 (Fig. 7C). Intriguingly, we observed a similar pattern of changes in NF-κB activity when RhoC was used instead of RhoA in these experiments (Fig. 7D). Taken together, these results suggest that SmgGDS-558 promotes breast cancer in part by inducing a Rho-dependent activation of NF-κB.

Discussion

This study identifies SmgGDS as an important promoter of breast cancer malignancy, and indicates that malignancy is promoted more by SmgGDS-558 than by SmgGDS-607. This conclusion is based on our finding that silencing the expression of SmgGDS-558, but not SmgGDS-607, significantly diminishes cell proliferation in multiple breast cancer cell lines, and suppresses tumorigenesis by MDA-MB-231 xenografts in mice. Intriguingly, we found that RhoA activity depends more on the presence of SmgGDS-558 than SmgGDS-607 in breast cancer cells. This specific ability of SmgGDS-558 to promote RhoA activity may explain why SmgGDS-558 is the unique form of SmgGDS that promotes malignancy because RhoA activity is associated with the malignant phenotype (1–3). In most cases, we found that the malignant characteristics of the cells were inhibited to the same extent by knocking down either SmgGDS-558 alone, or knocking down both SmgGDS-558 and SmgGDS-607.
splice variants simultaneously. These results indicate that therapeutics targeting only SmgGDS-607 will not be particularly effective. Instead, therapeutics should be designed to target either SmgGDS-558 alone, or both SmgGDS splice variants simultaneously, for the treatment of breast cancer.

We observed that elevated SmgGDS expression occurs very early in breast cancer development. SmgGDS expression is elevated in precancerous lesions and in all stages of invasive breast cancer, including metastatic breast cancer. Furthermore, SmgGDS expression is elevated in all subtypes of breast cancer. This expression pattern indicates that elevated SmgGDS expression is an early event in breast cancer development, and is required for breast cancer progression.

The high expression of SmgGDS was shown to correlate clinically to poorer survival rates of patients with breast cancer. These data obtained from the Kaplan–Meier plots are supported by the results of a previous study that correlated the expression of certain genes, including SmgGDS (RAP1GDS1), with worse response to neoadjuvant chemotherapy in patients with breast cancer (28). These results indicate that high SmgGDS expression may be a negative prognostic indicator in patients with breast cancer.

Our immunohistochemical analyses did not define the expression levels of SmgGDS-607 versus SmgGDS-558 in the mammary tissues. Thus, we cannot yet determine whether one or both SmgGDS splice variants contribute to elevated SmgGDS expression in breast tumors. Additional techniques, including the immunohistochemical use of antibodies that differentiate between SmgGDS-558 and SmgGDS-607, will be needed to determine the expression of SmgGDS splice variants in the breast tumor arrays.

In addition to observing SmgGDS expression in breast tumors, we also observed significant SmgGDS expression in established breast cancer cell lines. Interestingly, there is also significant SmgGDS expression in the breast epithelial cell lines, MCF-10A and MCF-12A. Although these cell lines are not considered cancerous, they are highly proliferative with a doubling time of approximately 20 hours (29, 30). Normally, the breast ducts are nonproliferating, except during the menstrual cycle when the ducts proliferate rapidly and resind quickly if pregnancy does not occur (31). MCF-10A and MCF-12A cells may be mimicking this highly proliferative state of the breast ducts. It is intriguing to
speculate that SmgGDS is needed for this highly proliferative state, and the dysregulation of SmgGDS in normally non-proliferating ducts might contribute to the initiation of breast tumors.

Only a few GEFs that activate RhoA in breast cancer cells have been characterized. SmgGDS is the first GEF for RhoA that has been found to be upregulated in many types of breast cancers. Two other characterized RhoGEFs, GEF-H1 and MyoGEF, were previously found to be upregulated in only a subset of breast cancer cells with invasive characteristics (32, 33). For example, GEF-H1 and MyoGEF were found to be detectably upregulated in invasive MDA-MB-231 cells, but not in noninvasive MCF-7 cells (32, 33). In stark contrast, we show that SmgGDS is highly expressed in all examined breast cancer cell lines and in all stages of breast tumors. In addition to its upregulation, SmgGDS also utilizes a unique mechanism to activate Rho (15). SmgGDS activates Rho by a currently uncharacterized mechanism because it lacks the DH-PH domain that is used by typical RhoGEFs such as GEF-H1 and MyoGEF (15). The upregulation of SmgGDS in all types of breast cancer, and its atypical mechanism of Rho activation, make SmgGDS, a particularly unique target in breast cancer.

We found that RhoA activity is promoted more by SmgGDS-558 than by SmgGDS-607 in breast cancer cells. One explanation for this finding is that SmgGDS-558 specifically interacts with prenylated RhoA, whereas SmgGDS-607 specifically interacts with nonprenylated RhoA (17). We found that the majority of RhoA in MDA-MB-231 cells is prenylated, as indicated by the greater solubilization of RhoA in a TX-114 detergent phase compared with the aqueous phase (Supplementary Fig. S1). Thus, the large proportion of RhoA that is prenylated in breast cancer cells will be regulated by SmgGDS-558, whereas the smaller proportion of RhoA that is nonprenylated in the cells will be regulated by SmgGDS-607. In contrast with RhoA, we found that the majority of RhoC fractionates into the TX-114 aqueous phase (Supplementary Fig. S1). This result could occur because either RhoC is nonprenylated in the cells, or endogenous RhoGDI is binding the prenylated RhoC and solubilizing it in the aqueous phase. Both of these events could diminish SmgGDS-558-mediated activation of RhoC, consistent with our finding that SmgGDS-558 does not significantly promote RhoC activity in MDA-MB-231 cells.

SmgGDS-558 may promote breast cancer malignancy through its ability to activate RhoA, which in turn activates NF-kB. It is known that RhoA promotes the proliferation of MDA-MB-231 breast cancer cells and is needed for tumorigenesis of MDA-MB-231 xenografts (2, 3). RhoA is known to activate NF-kB, passingage into S phase of the cell cycle and increasing the proliferation of breast cancer cells (34–36). The model that SmgGDS-558 promotes Rho-dependent activation of NF-kB is supported by our findings that (i) silencing SmgGDS-558 expression diminishes both RhoA activity and NF-kB activity, and (ii) overexpressing SmgGDS-558 increases the ability of WT-RhoA to promote NF-kB activity, and (iii) expressing DN-RhoA diminishes NF-kB activity in the presence of overexpressed SmgGDS-558. Although the results are consistent with the possibility that SmgGDS-mediated RhoA activity contributes to NF-kB activation, it is likely that SmgGDS also promotes NF-kB activity through additional small GTPases, such as Rac1 and K-Ras. Both of these small GTPases interact with SmgGDS (10–13) and are known to promote NF-kB activity (5, 37–39).

RhoA and RhoC are known to be associated with breast cancer malignancy. However, RhoC is associated more with tumor invasion and metastasis than with cell proliferation (27, 40). We observed greater serum-induced activation of RhoA than RhoC in the MDA-MB-231 cells. Furthermore, we observed that silencing SmgGDS expression reduced RhoA activity more than RhoC activity in the cells. These results suggest that in our assay conditions, RhoA may be more active than RhoC, and therefore more sensitive to perturbations in SmgGDS levels. It is possible that SmgGDS regulates RhoC activity in breast cancer cells, even though we did not detect it in the RhoC activation assays. This possibility is supported by our observation that SmgGDS-558 and RhoC synergize together to activate NF-kB in MDA-MB-231 cells.

In summary, this study demonstrates that SmgGDS is an important promoter of breast cancer, as indicated by our observation that SmgGDS expression is elevated in breast tumors, and by Kaplan–Meier plots indicating that patients with high SmgGDS expression have clinically poorer outcomes. Our findings identify SmgGDS-558 as an activator of RhoA and NF-kB in breast cancer, and demonstrate a functional role for SmgGDS-558 in the proliferation of breast cancer cells and tumorogenesis. These results define a new mechanism to regulate the activities of RhoA and NF-kB in breast cancer cells, and validate SmgGDS-558 as a better therapeutic target than SmgGDS-607 in breast cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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