The G12 family of heterotrimeric G proteins promotes breast cancer invasion and metastasis

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Although the prognosis for patients with early-stage breast cancer has improved, the therapeutic options for patients with locally advanced and metastatic disease are limited. To improve the treatment of these patients, the molecular mechanisms underlying breast cancer invasion and metastasis must be understood. In this study, we report that signaling through the G12 family of heterotrimeric G proteins (G12 and G13) promotes breast cancer cell invasion. Moreover, we demonstrate that inhibition of G12 signaling reduces the metastatic dissemination of breast cancer cells in vivo. Finally, we demonstrate that the expression of Gα12 is significantly up-regulated in the earliest stages of breast cancer, implying that amplification of G12 signaling may be an early event in breast cancer progression. Taken together, these observations identify the G12 family proteins as important regulators of breast cancer invasion and suggest that these proteins may be targeted to limit invasion- and metastasis-induced patient morbidity and mortality.

G12 Signaling Promotes Breast Cancer Cell Invasion. Notably, in addition to promoting cell growth, many of the signaling pathways downstream of the Gα12 and Gα13 have been implicated in cancer cell invasion and metastasis (7, 14). Therefore, we next sought to determine whether G12 proteins affect breast cancer invasion and metastasis. First, we examined the impact of G12 signaling on breast cancer cell invasion by using the transwell invasion assay (15). Expression of activated Gα12 or activated Gα13 in T47D, BT549, MDA-MB-231, and 4T1 (Fig. 1B) breast cancer cells significantly increased cellular invasion. In contrast, expression of activated GαQ had no effect (Fig. 1B). Thrombin stimulates cancer cell invasion and metastasis through protease-activated receptor-1 (PAR-1) (16, 17), and stimulation of PAR-1 activates both G12 and Gq proteins (8). Thrombin-induced invasion of the BT549 and MDA-MB-231 cell lines was blocked by inhibition of G12 signaling (by expression of p115-RGS), whereas the inhibition of Gq signaling (by expression of RGS2) had no effect (Fig. 1D). As an additional control, an adenovirus was engineered to express a disabled form of p115-RGS, p115-RGS(E29K). This point mutant of the p115-RGS

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Abbreviation: RGS, regulator of G protein signaling.

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Fig. 1. G12 signaling promotes breast cancer cell invasion. (A) Expression of the activated forms of Ga12 (Ga12QL) and Ga13 (Ga13QL) but not Gq (GqQL) induces breast cancer cell invasion in vitro. Cells were transduced with the indicated adenovirus, starved for 18 h, and then allowed to invade growth-factor-reduced Matrigel (T47D, MDA-MB-231, and BT549) or collagen-coated (4T1) transwell filters for 30 h. (B) Immunoblot analysis showing expression levels of Ga12, Ga13, and Gq in the T47D cell line after infection with the indicated adenovirus. Similar expression was observed in the other cell types. (C and D) Expression of p115-RGS blocks thrombin (C) and thromboxane A2-induced (U46619 (D) invasion of breast cancer cells, whereas expression of RGS2 or expression of an inactive form of the p115-RGS, p115-RGS(E29K), has no effect. Cells were transduced with the indicated adenovirus, starved for 18 h, then allowed to invade growth-factor-reduced Matrigel-coated transwell filters for 30 h in the presence of 1 unit/ml thrombin (A), 100 nM U46619 (B), or vehicle control. (E) Immunoblot analysis showing expression levels of p115-RGS, p115-RGS(E29K), and RGS2 in the MDA-MB-231 cell line after infection with the indicated adenovirus. Similar expression was observed in the BT549 cells. (A, C, and D) Experiments were performed in triplicate, and the results are presented as the fold increase over vehicle-treated GFP control. All results are presented as mean ± SE from a single experiment. All experiments were performed at least three times. *P < 0.05 as determined by paired Student t test.

The Role of Rho G Proteins in G12-Promoted Breast Cancer Cell Invasion. In most cell types, stimulation of the G12 proteins results in the activation of the RhoA/B/C family of monomeric G proteins (7). Many studies have demonstrated that the Rho family of proteins plays a significant role in breast cancer invasion (19). Thus, we examined the role of Rho signaling in G12-induced breast cancer invasion. First, we confirmed that the G12 proteins are able to activate Rho in breast cancer cell lines. Expression of the activated forms of Ga12 and Ga13 in the MDA-MB-231 (Fig. 2A), T47D (Fig. 6A), which is published as supporting information on the PNAS web site, and 4T1 (Fig. 6A) cell lines induced a significant increase in the levels of GTP-bound Rho. Interestingly and consistent with recent reports (20), the activated form of Gq also induced a small increase in the level of GTP-bound Rho (Fig. 2A). In addition, stimulation of the MDA-MB-231 cell line with thrombin resulted in ~5-fold increase in the levels of GTP-bound Rho (Fig. 2B). This stimulation was inhibited by expression of the p115-RGS but not by expression of the p115-RGS(E29K) or RGS2 (Fig. 2B). Taken together these data confirm that G12 signaling is able to activate the Rho proteins in breast cancer cells.

Next we sought to determine the contribution of signaling through the Rho proteins in G12-induced breast cancer cell invasion. Treatment of the MDA-MB-231, BT549, T47D (Fig. 2C), and 4T1 (Fig. 6C) cell lines with C3 toxin, a specific and irreversible inhibitor of RhoA/B/C, blocked Ga12(QL)-induced invasion. These data demonstrate that Rho signaling is required for G12-stimulated invasion. However, given that C3 toxin treatment of these breast cancer cells also reduced basal invasion, it was not clear from these data whether G12-stimulated invasion simply requires Rho signaling or whether G12 must activate Rho to induce invasion. To address these possibilities, we used a mutant form of Ga12 [Ga12 (Δ244–249)] that is unable to activate Rho but is still able to interact with other putative G12 effectors (21). Expression of the constitutively active form of this protein [Ga12QL (Δ244–249)] in the MDA-MB-231, BT549 (Fig. 2D), or 4T1 (Fig. 6D) cell lines did not promote invasion. These results suggest that that G12 must activate the Rho pathway to induce invasion in these cell types. Interestingly, however, when Ga12QL (Δ244–249) was expressed in T47D cell line, although it did not activate Rho (Fig. 6A), it did induce a small but significant increase in invasion (Fig. 2D). This finding suggests that, although maximal G12-stimulated breast
Fig. 2. G12 signaling is able to promote breast cancer cell invasion through the activation of the Rho family of monomeric G proteins. (A) Expression of G12QL and G13QL induces RhoA activation in the MDA-MB-231 cell line. Cells were transduced with the indicated adenovirus and then starved for 18 h. Cells were lysed, and the lysates were subjected to pull-down assays using a GST fusion of the activated RhoA-binding domain of rhotekin. Levels of precipitated RhoA were determined by immunoblot analysis using anti-RhoA antibody. Levels of total RhoA, G12, G13, and Gq also were determined. All lanes are representative of two or more separate experiments. (B) Thrombin-stimulated RhoA activation in MDA-MB-231 cells is inhibited by expression of p115-RGS but not by expression of RGS2 or p115-RGS(E29K). Cells were transduced with the indicated adenovirus, starved for 18 h, and then stimulated with thrombin (1 unit/ml) or a vehicle control for 5 min. Levels of activated RhoA were determined as in A. Levels of total RhoA, myc-p115-RGS, and RGS2 also were determined. All lanes are representative of two or more separate experiments. (C) G12-mediated breast cancer cell invasion requires the activity of the Rho family of G proteins. Cells were transduced with the indicated adenovirus, starved for 18 h in the presence and absence of C3 toxin as indicated, and then allowed to invade growth-factor-reduced Matrigel-coated transwell filters for 30 h. Experiments were performed in duplicate, and the results are presented as the fold increase over that observed with GFP control. All experiments were performed at least three times. All results are presented as mean ± SE. *, P < 0.05 as determined by paired Student t test. (D) The effect of the expression of G12QL (L244–249), a mutant of G12QL that is functionally uncoupled from the Rho axis but is still able to interact with other putative G12 effectors, on breast cancer cell invasion. Experiments were performed as in C. Experiments were performed in duplicate, and the results are presented as the fold increase over that observed with GFP control. All experiments were performed at least three times. All results are presented as mean ± SE. *, P < 0.05 as determined by paired Student t test.
G12 proteins in tumor invasion and metastasis by assessing its expression in breast biopsies obtained from patients with breast cancer. Immunohistochemical detection of Gα12 in sections of both ductal carcinoma of the breast and lobular carcinoma of the breast revealed that breast cancer cells consistently expressed higher levels of Gα12 compared with benign breast epithelial cells within the same tissue section (Fig. 4). Gα12 staining could be completely blocked by preincubation of the antibody with its blocking peptide, demonstrating antibody specificity (Fig. 9, which is published as supporting information on the PNAS web site). Interestingly, no signal above background away from the primary site. Mice that received p115-RGS-expressing 4T1 cells had significantly longer metastasis-free survival times than did mice that received control 4T1 cells (P < 0.0001), RGS2-expressing cells (P < 0.001), or p115-RGS(E29K)-expressing cells (P < 0.0013). Statistical analyses were performed by using the log-rank test. (B) Overall mouse survival. Mice that received p115-RGS-expressing 4T1 cells survived significantly longer than did mice that received control 4T1 cells (P < 0.00001), RGS2-expressing cells (P < 0.001), or p115-RGS(E29K)-expressing cells (P < 0.002). Statistical analyses were performed by using the log-rank test.

Discussion

In this report, we demonstrate that G12 is markedly up-regulated in adenocarcinoma of the breast and identify G12 signaling as an important regulator of breast cancer invasion and metastasis. Previous studies have shown that G12 signaling is able to promote cell growth in some cell lines (5, 6, 12, 13). Thus, our results that G12 signaling did not promote tumor cell growth were surprising. However, it is interesting to note that much of the previous work on the mitogenic effects of G12 signaling was done in nontransformed, fibroblast-derived cell lines (5, 6, 12, 13). Because our studies used transformed cells of epithelial origin, it is possible that the discrepancy results from fundamental differences between normal and transformed cells and/or between cells of mesenchymal and epithelial origin. Thus, although it is clear that in some cell types Gα12 and Gα13 may be important promoters of cell growth, they do not appear to promote breast cancer cell growth.

In contrast to the data on proliferation, this study provides compelling evidence for a role for G12 signaling in breast cancer cell invasion. Activation of the pathway by expression of the activated forms of the G12 proteins or by receptor stimulation of the endogenous G12 proteins resulted in a significant increase in the invitro invasiveness of several breast cancer cell lines. Although this finding is novel in the field of cancer biology, studies in other systems have implicated G12 signaling in the related biologies of cell migration and extracellular matrix adhesion. In particular, studies in neutrophils have demonstrated that the G12 proteins are critically
important in establishing cell polarity (28). Developmental studies in Drosophila (29) and in zebrafish (30) have demonstrated that G12 signaling is critical for cell migration during gastrulation. Furthermore, genetic ablation of G13 in mice impairs the organization of the vascular system (31). Embryonic fibroblasts cultured from these mice display a reduced chemokinetic response to several G protein-coupled receptor ligands, and Offermanns et al. speculated that this defect in cell migration may underlie the failed angiogenesis (31).

Although the results from the in vitro assays used in this study strongly suggest a role for G12 in tumor invasion, the most compelling data supporting this hypothesis comes from the in vivo studies with the 4T1 mammary carcinoma line. In these experiments, inhibition of G12 signaling resulted in a significant decrease in metastasis from the mammary fat pad but did not affect either primary tumor growth or seeding of the lungs when cancer cells were introduced via the tail vein. Recent evidence suggests that 4T1 cells metastasize to the lung and, possibly, other organs by embolizing to the capillary bed and then growing in the intravascular space (32). This phenomenon of metastasis without extravasation does not appear to be limited to the 4T1 cells (32). However, exiting the vascular bed does appear to be a requirement for the metastasis of other cell types (23). Therefore, it is possible that, for other tumor types and at other sites of distant metastasis, G12 signaling also plays a role in these later stages of metastasis. Nevertheless, given that we saw no difference in metastatic potential of the 4T1 cells expressing p115-RGS and control cells when they were seeded into the blood stream directly, the difference in the metastatic dissemination of these cells from the mammary fat pad must reflect a disparity in the rate that cells are shed from the primary tumors. Thus, G12 signaling appears to promote cancer metastasis by stimulating tumor cell invasion and entry into the blood stream.

The findings that blockade of G12 signaling dramatically impacts metastatic behavior of cancer cells prompted us to examine the expression of the G12 proteins in tissue samples from patients with adenocarcinoma of the breast. We found that Gα12 expression was significantly higher in situ and in invasive carcinomas compared with normal epithelium. This finding is consistent with a previous report suggesting that Gα12 and Gα13 expression is elevated in breast cancer cells with metastatic human Gaq (Q209L), Gaq12(Q231L), Gaq13(Q226L), hemagglutinin-RGS2 (University of Missouri cDNA Resource, Rolla, Mo), myc-p115 (gift of Tohru Kosaza, University of Illinois, Chicago) and myc-p115(E29K) (generated by site-directed mutagenesis of the myc-p115) into the Adtrack-CMV vector (gift of Robert Weinberg, The Johns Hopkins University Medical Center) then recombinating these with pAdEasy-1 in BJ5183 Escherichia coli (Strategen). The resulting DNA was transfected into HEK 293 cells with Lipofectamine (Invitrogen), then the viruses were amplified and purified with Adeno-X Virus Purification kits (BD Biosciences). Cell lines were infected at a multiplicity of infection of 5–50, for 6–24 h at 37°C. Infection efficiencies ranged from 80–100%. For experiments using C3 toxin, 1 mg of purified C3 and 20 hemolytic units (as defined by Biomol) of tetanolysin were added to the cells for 1 h after the infection media was removed. The cells were then washed twice with PBS and then were incubated for 18 h in DMEM with 0.1% fatty-acid-free BSA.

### Table 1. Distribution of breast specimen subtypes by Gα12 staining

<table>
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<th>Specimen</th>
<th>N</th>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>Avg. ± SEM</th>
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<td>19</td>
<td>9</td>
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<td>1.2 ± 0.1</td>
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<td>Ductal carcinoma in situ</td>
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<td>0</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>2.3 ± 0.4*</td>
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<tr>
<td>Invasive lobular carcinoma</td>
<td>40</td>
<td>1</td>
<td>10</td>
<td>15</td>
<td>15</td>
<td>2.1 ± 0.1**</td>
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* *P < 0.05 for ductal carcinoma in situ vs. benign breast epithelium. **P < 0.0001 for invasive lobular carcinoma vs. benign breast epithelium. Avg., average.

**Adenoviral Infections.** Recombinant adenoviruses were constructed by subcloning human Gaq (Q209L), Gaq12(Q231L), Gaq13(Q226L), hemagglutinin-RGS2 (University of Missouri cDNA Resource, Rolla, Mo), myc-p115 (gift of Tohru Kosaza, University of Illinois, Chicago) and myc-p115(E29K) (generated by site-directed mutagenesis of the myc-p115) into the Adtrack-CMV vector (gift of Robert Weinberg, The Johns Hopkins University Medical Center) then recombinating these with pAdEasy-1 in BJ5183 Escherichia coli (Strategen). The resulting DNA was transfected into HEK 293 cells with Lipofectamine (Invitrogen), then the viruses were amplified and purified with Adeno-X Virus Purification kits (BD Biosciences). Cell lines were infected at a multiplicity of infection of 5–50, for 6–24 h at 37°C. Infection efficiencies ranged from 80–100%. For experiments using C3 toxin, 1 mg of purified C3 and 20 hemolytic units (as defined by Biomol) of tetanolysin were added to the cells for 1 h after the infection media was removed. The cells were then washed twice with PBS and then were incubated for 18 h in DMEM with 0.1% fatty-acid-free BSA.

**Retrovirus Production.** Recombinant retroviruses were constructed by subcloning human Gaq (Q209L), Gaq12(Q231L), Gaq13(Q226L), hemagglutinin-RGS2 (University of Missouri cDNA Resource), myc-p115, and myc-p115RGS2(E29K) into the pLXRN vector (Clontech) and pG-L2 firefly luciferase into pLPCX (Clontech). Constructs were then cotransfected into the GP2–293 packaging line (Clontech) with FuGene (Roche Applied Science). Viral supernatants were collected 48 h later, clarified by filtration, and concentrated by ultracentrifugation. The concentrated virus was used to infect 1 × 10^5 cells in a 60-mm dish with 8 μg/ml polybrene. 4T1 cells were selected with 2 μg/ml puromycin (Sigma) and 1.2 mg/ml Genetricin (GIBCO). MDA-MB-231 and PC3 cells were selected with 2 μg/ml puromycin (Sigma) and 800 mg/ml Genetricin (GIBCO).
Cell Invasion Assay. For invasion assays, transwell chamber filters (8-μm pore size, polycarbonate filter, 6.5-mm diameter; Costar) were coated with 50 μg of growth-factor-reduced Matrigel (T47D, MDA-MB-231, and BT549) or 20 μg of collagen (4T1). After infection with adenovirus, cells were starved for 18 h in DMEM containing 0.1% BSA and detached, and 3 × 10^5 cells in 100 μl were placed to the upper chamber of the transwell with and without agonists. The insert was then transferred to a well containing 600 μl of 5 μg/ml of fibronectin in conditioned medium from NIH 3T3. Cells were incubated for 30 h at 37°C in a humidified incubator. Cells in the top well were removed by swiping the top of the membrane with cotton swabs. The membranes were then stained (Hema3 staining kit, Fisher Scientific), and the remaining cells were counted. Five high-powered fields were counted for each membrane.

Rho Activity Assays. The levels of activated Rho were determined by using pull-down assays with a GST fusion of the RhoA-binding domain of rhoetkin as described in ref. 21.

In Vivo Metastasis. Animal handling and procedures were approved by the Duke University Medical Center Institutional Animal Care and Use Committee. 4T1 cells expressing firefly luciferase (4T1-luc) were injected into the lateral tail vein of 6-week-old female BALB/c mice. For invasion assays, transwell chamber filters were placed to the upper chamber of the transwell with 10^4 4T1-Luc cells. The volume of the primary tumors was quantified with caliper measurements in three dimensions. At day 21, the primary tumors were measured as described in ref. 26. Bioluminescence imaging was performed on all of the mice at 3-day intervals.

For direct metastasis to the lung, 10^5 4T1-Luc cells were injected into the lateral tail vein of 6-week-old female BALB/c mice. Bioluminescence imaging was performed on all of the mice at 3-day intervals.

Bioluminescence Imaging. Mice were anesthetized and given 150 μg/g D-luciferin in PBS by i.p. injection. Fifteen minutes after injection, bioluminescence was imaged with a charge-coupled device camera (IVIS, Xenogen, Alameda, CA). Bioluminescence images were obtained with a 15-cm field of view, binning (resolution) factor of 8, 1/f stop, open filter, and with an imaging time of 30 sec. Bioluminescence from relative optical intensity was defined manually, and data were expressed as photon flux (photons/s per cm²/steradian). Background photon flux was defined from a relative optical intensity drawn over a mouse that was not given an injection of luciferin.

Immunohistochemistry. Institutional Review Board–approved breast samples were from Ardais (Lexington, MA). Tissue microarray (#BR801) was from U.S. Biomax (Rockville, MD). After paraffin removal and quenching of endogenous peroxidase, 5-μm sections were steamed in 10 mM citrate, pH 6.0, for 15 min in a steamer (model no. HS900, Black & Decker), then incubated with Background Buster (Innovex Biosci, Richmond, CA) for 30 min. Sections were then incubated with G12a antisera (Santa Cruz Biotechnology) diluted 1:100 in PBS for 1 h, followed by biotinylated goat anti–rabbit antiserum (Vector Laboratories, Burlingame, CA) diluted 1:200 in PBS for 30 min, followed by horseradish peroxidase–labeled streptavidin (Jackson ImmunoResearch) for 30 min, all at room temperature. Bound immune complex was visualized with DAB (Innovex Biosci, Richmond, CA); hematoxylin counterstain (Fisher, Pittsburgh, PA) was used. The G12 staining was graded 0–3 based on intensity, and data were analyzed by using one-way ANOVA and Dunn’s multiple comparison test in PRISM, Version 4.0c (GraphPad, San Diego).

Supporting Information. Additional details can be found in Supporting Materials and Methods, which is published as supporting information on the PNAS web site.

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Supplementary Fig. 1. G12 signaling inhibits anchorage dependent and anchorage-independent breast cancer cell growth. (A) Cells were plated in triplicate and transduced with adenovirus encoding the indicated protein. The cells were starved for 24 h, labeled with [3H] thymidine and stimulated with serum 24 h. DNA was precipitated and thymidine incorporation was determined by liquid scintillation detection. (B) MDA-MB-231 cells stably expressing the p115-RGS, RGS2 and Gα13(Q226L) and 4T1 cells stably expressing the p115-RGS and RGS2 were grown suspended in soft agar for 21 days and 14 days respectively. Colonies greater than 1 mm in size were counted. For both panels, the data represent triplicate determinations and were performed at least three times. All results are presented as mean ± S.E. * P < 0.05 as determined by student t-test.
Supplementary Fig. 2. G12 signaling is able to promote breast cancer cell invasion through the activation of the Rho family of monomeric G proteins. Expression of Gα12QL and Gα13QL induces RhoA activation in the (A) T47D and (B) 4T1 lines. Cells were transduced with the indicated adenovirus and then starved for 18 h. Cells were lysed and lysates were subjected to pull-down assays using a GST fusion of the activated RhoA-binding domain of rhotekin. Levels of precipitated RhoA were determined by immunoblot analysis using anti-RhoA antibody. Levels of total RhoA, Gα12, Gα13 and Gαq were also determined. All panels are representative of two or more separate experiments. (C) Gα12 mediated 4T1 cancer cell invasion requires the activity of the Rho family of G proteins. Cells were transduced with the indicated adenovirus, starved for 18 h in the presence and absence of C3 toxin as indicated, and then allowed to invade GFR matrigel coated transwell filters for 30 h. Experiments were performed in duplicate and results are presented as fold increase over that observed with GFP control. All experiments were performed at least three times. All results are presented as mean ± S.E. * P < 0.05 as determined by paired student t-test. (D) The effect of the expression of Gα12QL (Δ244-249), a mutant of Gα12QL that is functionally uncoupled from the Rho axis but is still able to interact with other putative G12 effectors, on breast cancer cell invasion. Experiments were performed as in (C). Experiments were preformed in duplicate and results are presented as fold increase over that observed with GFP control. All experiments were performed at least three times. All results are presented as mean ± S.E. * P < 0.05 as determined by paired student t-test.
**Supplementary Fig. 3.** Inhibition of G12 signaling has no effect on the growth of 4T1 cells orthotopically implanted in the mouse mammary fat pad. 4T1-Luc cells (5 X 10^5) stably expressing either p115-RGS (n=16, blue), RGS2 (n=12, yellow), p115-RGS(E29K) (n=5, green) or retroviral vector control (n=17, red) were implanted into the mammary fat pads of six-week-old female Balb/c mice. The volume of the primary tumors was quantified as the product of caliper measurements in three dimensions. All results are presented as mean ± S.E.
**Supplementary Fig 4.** Inhibition of G12 signaling has no effect on 4T1 cell metastatic spread following tail vein injection. *(A)* 4T1-Luc cells (1 X 10^5) stably expressing either p115-RGS (n=6) or retroviral vector control (n=6) were injected into the lateral tail vein of six-week-old female Balb/c mice. The mice were then imaged using the IVIS® Imaging System (Xenogen) at 3 day intervals at maximal sensitivity. Average metastatic burden for mice injected with control 4T1 cells (red) or p115-RGS expressing 4T1 cells (blue) is reported in photons/sec/cm^2^. Average metastatic burden per mouse was determined by total luciferase signal from each cohort averaged over number of mice remaining in the cohort. *(B)* Kaplan-Meier curve showing the overall survival of the mice receiving control 4T1 cells (red) or p115-RGS expressing 4T1 cells (blue).
Supplementary Fig 5. Pre-incubation of the anti-Gα12 antibody with its blocking peptide abrogates staining of invasive breast cancer. Serial sections of formalin fixed paraffin embedded tissue were stained with either (A) anti-Gα12 antibody alone or (B) anti-Gα12 antibody pre-incubated with its blocking peptide as described in Methods and Materials. All sections were counterstained with hematoxylin. Original images were taken with the 40X objective
**Supplementary Fig. 6.** Gαq protein is expressed at comparable levels in normal breast and adenocarcinoma of the breast. Sections of formalin-fixed paraffin-embedded breast cancer breast tissue were stained for Gαq with anti-Gq anti-sera (Santa Cruz Biotechnology) as described in Methods. Original images were taken with the 40X objective. *(A)* Benign breast tissue. *(B)* Ductal carcinoma *in situ* of the breast. *(C)* Invasive ductal carcinoma of the breast.