Distinct Phosphotyrosine-dependent Functions of the ShcA Adaptor Protein Are Required for Transforming Growth Factor β (TGFβ)-induced Breast Cancer Cell Migration, Invasion, and Metastasis

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Background: ShcA integrates TGFβ and ErbB2 signaling in breast cancer.

Results: Distinct motifs within ShcA facilitate TGFβ-induced effects in ErbB2-expressing cells.

Conclusion: ShcA transduces distinct signals via Grb2 downstream of Tyr313 and Crk adaptors downstream of Tyr239/Tyr240, and all three residues are required for metastasis.

Significance: ShcA is essential for the tumorigenesis and metastasis of ErbB2-expressing breast tumors with active TGFβ signaling.

The ErbB2 and TGFβ signaling pathways cooperate to promote the migratory, invasive, and metastatic behavior of breast cancer cells. We previously demonstrated that ShcA is necessary for these synergistic interactions. Through a structure/function approach, we now show that the phosphotyrosine-binding, but not the Src homology 2, domain of ShcA is required for TGFβ-induced migration and invasion of ErbB2-expressing breast cancer cells. We further demonstrate that the tyrosine phosphorylation sites within ShcA (Tyr239/Tyr240 and Tyr313) transduce distinct and non-redundant signals that promote these TGFβ-mediated effects. We demonstrate that Grb2 is required specifically downstream of Tyr313, whereas the Tyr239/Tyr240 phosphorylation sites require the Crk adaptor proteins to augment TGFβ-induced migration and invasion. Furthermore, ShcA Tyr313 phosphorylation enhances tumor cell survival, and ShcA Tyr239/Tyr240 signaling promotes endothelial cell recruitment into ErbB2-expressing breast tumors in vivo, whereas all three ShcA tyrosine residues are required for efficient breast cancer metastasis to the lungs. Our data uncover a novel ShcA-dependent signaling axis downstream of TGFβ and ErbB2 that requires both the Grb2 and Crk adaptor proteins to increase the migratory and invasive properties of breast cancer cells. In addition, signaling downstream of specific ShcA tyrosine residues facilitates the survival, vascularization, and metastatic spread of breast tumors.

Collaboration between the ErbB2 and TGFβ signaling pathways promotes the invasive and metastatic behavior of breast cancer cells (1). The clinical significance of these findings is reflected by the fact that an active TGFβ signaling gene expression signature is not only enriched within the HER2+, basal, and luminal B subtypes but is also predictive of reduced relapse-free and overall survival times in breast cancer patients (2).

We previously identified the ShcA adaptor protein as an important mediator of the synergistic migratory and invasive effects observed downstream of the TGFβ and ErbB2 signaling pathways (3). ShcA functions as a mediator of protein-protein interactions through two conserved phosphotyrosine-binding motifs, which include the phosphotyrosine-binding (PTB)4 and Src homology 2 (SH2) domains (4, 5), and three tyrosine residues (Tyr239/Tyr240 and Tyr313 in mouse) that can be inducibly phosphorylated downstream of receptor and cytoplasmic tyrosine kinases (6–8). In particular, Tyr239 and Tyr313 have been characterized for their ability to initiate Ras/MEK/ERK signaling cascades by recruiting the Grb2 adaptor protein (6, 8, 9). The Crk adaptor proteins also bind ShcA in a phosphotyrosine-dependent manner in response to NGF stimulation (10). However, the requirement for these ShcA domains/motifs and the

4 The abbreviations used are: PTB, phosphotyrosine-binding; SH, Src homology; shRNAmir, microRNA-adapted shRNA; IHC, immunohistochemical; CH1, collagen homology 1; 2F, Y239F/Y240F; 3F, Y239F/Y240F/Y313F; TβRII, TGFβ type II receptor; TβRI, TGFβ type I receptor; NIC, Neu-IRES-CRE.
signaling molecules they engage in TGFβ-induced migration and invasion remain undefined. Accumulating evidence supports important roles for the ShcA, Grb2, and Crk signaling pathways during several aspects of breast cancer progression. Transgenic mouse models have revealed an important role for ShcA during various stages of the tumorigenic process, including breast cancer initiation, outgrowth, survival, angiogenesis, and the establishment of an immunosuppressive state (11–14). This is consistent with the observation that ShcA may serve as a prognostic indicator in breast cancer patients as elevated ShcA tyrosine phosphorylation and reduced p66 ShcA levels predict poor prognosis (15, 16). As with ShcA, Grb2-dependent signals play important roles downstream of oncogene-induced mammary tumorigenesis (11, 17, 18). Finally, elevated expression of Crk family adaptor proteins is observed in patients with breast adenocarcinoma, and Crk-dependent signaling is important for mammary tumorigenesis and breast cancer migration and invasion (19, 20).

Herein, we demonstrate that the ShcA adaptor requires the PTB, and not the SH2, domain for TGFβ-induced migration and invasion of ErbB2-expressing breast cancer cells. Moreover, the Tyr239/Tyr240 and Tyr313 phosphorylation sites transduce critical and non-redundant signals through the Crk and Grb2 adaptor proteins, respectively, to augment TGFβ-induced breast cancer cell migration and invasion. Signals emanating from these distinct ShcA tyrosine residues promote the vascularization (Tyr239/Tyr240) and survival (Tyr313) of tumor cells in vivo. Finally, all three tyrosine residues are required for efficient dissemination of breast cancer cells to the lungs.

EXPERIMENTAL PROCEDURES

**DNA Constructs**—ShcA-targeting shRNAs were expressed using the LTRmiR30-PIG vector system (21), and shRNA mirs were designed to target the 3'-untranslated region (UTR) of ShcA using the RNAi central website at Cold Spring Harbor Laboratories. The sequences (described in the supplemental Experimental Procedures) were PCR-amplified, digested, and cloned into the LTRmiR30-PIG vector as Xhol/EcoRI fragments following the manufacturer’s instructions. The NeuNT cDNA was inserted into MSCV-puro (Clontech) as described previously (3). This vector was subsequently modified by substituting the puromycin resistance cassette with a hygromycin resistance cassette derived from MSCV-hygro using the restriction enzyme sites AgeI and ClaI. All ShcA cDNAs were cloned into an MSCV-blunt expression vector as Xhol/EcoRI fragments.

**Cell Culture**—The NMuMG normal murine mammary cell line was obtained from the American Type Culture Collection (Manassas, VA) and cultured as described previously (3). The NIC cell line was derived from mammary tumors that formed in murine mammary tumor virus/NIC mice and were maintained as described (13). Retroviruses were generated in 293SVS cells according to the manufacturer’s instructions (Clontech).

**Immunoblotting/Immunoprecipitation**—Cells grown to 70–90% confluence were incubated for the indicated times in the absence or presence of TGFβ1 (2 ng/ml; catalog number HZ-1011, Humanzyme, Chicago, IL) prior to incubation in Tris-NaCl-EDTA lysis buffer as described previously (22). Total protein lysate (20–40 μg) was subjected to immunoblot analysis with the following primary antibodies: ShcA (1:1,000; catalog number 610081, BD Biosciences), FLAG (M2) (1:1,000 dilution; catalog number F1804, Sigma), α-tubulin (1:5,000 dilution; catalog number T9026, Sigma), ErbB2 (C-18) (1:1,000 dilution; catalog number sc-284-G, Santa Cruz Biotechnology, Santa Cruz, CA), Grb2 (C23) (1:500 dilution; catalog number sc-255, Santa Cruz Biotechnology), CrkII (1:1,000 dilution; catalog number 610035, BD Biosciences), and Crkl (C-20) (1:500 dilution; catalog number sc-319, Santa Cruz Biotechnology). Immunoblots were incubated with the appropriate hors eradish peroxidase-conjugated anti-IgG secondary antibodies and visualized with the Pierce enhanced chemiluminescence (ECL) system (catalog number 32106, Thermo Scientific, Rockford, IL). Co-immunoprecipitations were performed on cells treated with or without TGFβ for 24 h as described (3) using the following antibodies: ErbB2 (Ab-4) (catalog number OP16, Oncogene, San Diego, CA) and ErbB3 (C-17) (catalog number sc-285, Santa Cruz Biotechnology).

**Migration and Invasion Assays**—Migration and invasion assays were performed as described previously (20) with the following modifications. For invasion assays, cells were plated onto a 6% Matrigel layer coated over the inner surface of a Boyden chamber porous membrane (catalog number 35-3097, BD Biosciences). The number of cells plated in Figs. 1, 3, 4, 6, and 7 was 9 × 10^4 for migration assays and 1.5 × 10^5 for invasion assays. In Fig. 2, the number of cells plated was reduced to 7.5 × 10^4 and 1.25 × 10^5 for migration and invasion assays, respectively. The migration and invasion data are representative of the average pixel count from five independent images that were quantified using Scion Image software (Scion Corp., Houston, TX). Two independent inserts were quantified for each explant in the absence or presence of TGFβ1, and the data represent the average of two to three independent experiments.

Migration and invasion assays using NIC cells were performed largely as described above with the exception that NIC cells were plated in the upper chamber of the Transwell system with or without TGFβ (2 ng/ml). NIC tumor cells were plated at a density of 9 × 10^4 cells for migration assays and 1.5 × 10^5 cells for invasion assays. The migration and invasion data are representative of the average pixel count from five independent images that were quantified using Scion Image software (Scion Corp.). Two independent inserts were quantified for each explant in the absence or presence of TGFβ, and the data represent the average of two independent experiments.

**siRNA Transfections**—For Grb2 knockdowns, cells were transfected with a pool of two dicer substrate duplex siRNAs (duplexes 1 and 3) targeting mouse Grb2 (TriFECTa kit, catalog number NM_008163.10, Integrated DNA Technologies, Coralville, IA) or a scrambled dicer substrate duplex siRNA using Lipofectamine 2000 reagent (catalog number 11668-019, Invitrogen). Crk adaptor siRNAs were also obtained from Integrated DNA Technologies, and sequences are provided in the supplemental Experimental Procedures; however, they were not prepared with a dicer substrate design. All siRNA transfections were performed as described previously (3), ensuring that cells were transfected with equal concentrations of scrambled versus Crk and/or Grb2 pools of multiple siRNAs. A portion of the siRNA-transfected cells was maintained and lysed following the
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completion of migration and invasion assays to monitor the efficacy and duration of the transient adaptor knockdowns.

Mammary Tumor Growth, Quantification of Lung Metastases, and Generation of Tumor Explants—To assess primary tumor growth and spontaneous metastasis of the pooled ErbB2/ShcA high, and ErbB2/ShcA low-expressing cells, 1 × 10⁶ cells from each cell population were resuspended in a 50:50 solution of 1 × PBS and Matrigel (BD Biosciences) and injected into the number 4 mammary fat pad of female athymic mice. Tumor volumes were monitored by weekly caliper measurement and calculated using the following formula:

\[ V = \frac{4}{3} \pi L W^2 \]

where \( L \) is the length and \( W \) is the width of the mammary tumor. Mammary tumors were harvested at 5 and 7 weeks postinjection, and portions were 1) flash frozen in liquid nitrogen, 2) fixed and embedded in paraffin, and 3) used to generate explant populations in tissue culture. To generate explant populations, tumor tissue was first minced and incubated at 37 °C in 0.05% trypsin (Wisent, St. Bruno, Québec, Canada) for 20 min. Cells were plated in fresh medium containing purumycin and hygromycin to maintain expression of the shRNA mir and ErbB2, respectively. For spontaneous metastasis assays, mice were sacrificed at a 7-week common end point (Fig. 2), or tumors were resected at a common volume, and mice were sacrificed at a fixed time point following resection (Fig. 11). Lungs were harvested for histological analysis, fixed in 4% paraformaldehyde, and embedded in paraffin. Four step sections (40 μm/step) were collected from each set of lungs (all lobes) and stained with hematoxylin and eosin (H&E) to visualize metastatic lesions. Lung metastases were quantified from digital images of H&E-stained lung sections that were scanned using a Scanscope XT digital slide scanner (Aperio, Vista, CA). From the scanned sections, the number of metastases per lung section was counted across all step sections. Mice were housed in facilities managed by the McGill University Animal Resources Centre, and all animal experiments were conducted under a McGill University-approved Animal Use Protocol in accordance with guidelines established by the Canadian Council on Animal Care.

Immunohistochemical Staining—Tissue fixation and immunohistochemical (IHC) staining were carried out as described (23). The proliferative index in the mammary tumors was assessed with a Ki67 antibody (1:100 dilution; catalog number ab15580, Abcam, Cambridge, MA), and anti–CD31 staining (1:200 dilution; catalog number 550274, BD Biosciences) was performed to quantify endothelial cell recruitment within the tumors. Primary antibodies were used in conjunction with the appropriate Biotin–SP–conjugated anti-IgG secondary antibodies (The Jackson Laboratory, Bar Harbor, ME). Apoptotic cells were detected using an ApopTag kit following the manufacturer’s instructions (ApopTag Peroxidase In Situ Apoptosis Detection kit, catalog number S7100, EMD Millipore, Billerica, MA) or by staining with an antibody that recognizes cleaved caspase 3 (catalog number 9661, Cell Signaling Technology, Danvers, MA). Primary antibodies were also used to detect phospho-Smad2 (catalog number 3101, Cell Signaling Technology) and total levels of Smad2 (catalog number 3102, Cell Signaling Technology). Tissue sections were developed with 3,3-di-aminobenzidine tetrahydrochloride and counterstained with hematoxylin before being scanned using a Scanscope XT digital slide scanner (Aperio). Five 20× images from each tumor were analyzed with Imagescope software (Aperio) using positive pixel count for CD31 and cleaved caspase 3 staining and IHC nuclear algorithms for Ki67 and TUNEL staining. In all analyses, only moderate (+2) staining and strong (+3) staining were considered as positive pixels. For CD31 staining, CD31-positive pixels were expressed as a percentage of the total pixels per field. For quantification of Ki67 and TUNEL staining, positively stained nuclei were represented as a percentage of total nuclei per field.

Statistical Analysis—Statistical significance values (p values) for tumor growth, migration, and invasion assays were obtained by performing a two-sample unequal variance Student’s t test.

RESULTS

Diminished ShcA Expression Impairs Mammary Tumor Growth and Lung Metastasis—We have engineered immortalized mouse mammary epithelial cells (NMuMG) to express shRNAs that target the 3′-UTR of mouse ShcA mRNA (ShcA low) or luciferase-targeting shRNAs (ShcA high) as controls. NMuMG populations possessing reduced ShcA levels ( supplemental Fig. S1, shRNA#1) were transduced with an oncogenic Neu/ErbB2 allele, NeuNT, rendering them tumorigenic in vivo (3).

To assess the effect of diminished ShcA expression on tumorigenesis, ErbB2/ShcA high, and ErbB2/ShcA low-expressing cells were injected into the mammary fat pads of female athymic mice. Tumor growth curves began to diverge at 3 weeks postinjection, and by 7 weeks postinjection, the tumor volumes of ErbB2/ShcA low mammary tumors had only reached ~50% of those that developed in mice injected with ErbB2/ ShcA high control cells (Fig. 1A). Diminished ShcA levels were associated with a 2.3-fold decrease in proliferation (Fig. 1B), a 2.6-fold increase in apoptosis (Fig. 1C), and a 3-fold decrease in endothelial cell recruitment (Fig. 1D) in ErbB2/ShcA low versus ErbB2/ShcA high mammary tumors.

Analysis of lung tissues harvested from mice sacrificed at 7 weeks postinjection revealed a 5.7-fold decrease in the average number of lung metastases per section (Fig. 2) in mice injected with ErbB2/ShcA low versus ErbB2/ShcA high, expressing breast cancer cells. Moreover, the size of the lung metastases formed by ErbB2/ShcA low-expressing breast cancer cells was considerably smaller than those formed by ErbB2/ShcA high-expressing breast cancer cells. Collectively, these data demonstrate that ShcA is important for the growth and dissemination of ErbB2-expressing breast cancer cells.

Reduced ShcA Expression Ablates TGFβ-induced Motility and Invasion of ErbB2-expressing Breast Cancer Cells—Immunoblot analysis confirmed that ShcA levels were diminished in ErbB2/ShcA low-expressing mammary tumor explants harvested at both 5 and 7 weeks postinjection when compared with ErbB2/ShcA high-expressing explants (Fig. 3A). Importantly, no significant difference in ErbB2 expression was observed between ErbB2/ShcA high and ErbB2/ShcA low tumor explant populations (Fig. 3A).

Migration and invasion assays were performed on two independent sets of ErbB2/ShcA high and ErbB2/ShcA low mammary...
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Tumor explants. In agreement with our previous data (3), TGFβ/H9252 stimulation induced a 2-fold increase in both migration and invasion of ErbB2/ShcAhigh cells that was abolished in ErbB2/ShcAlow-expressing cells (Fig. 3, B and C). To determine whether ShcA is similarly required in other ErbB2-expressing breast cancer cells, we stably reduced ShcA expression in primary mammary tumor cells harvested from murine mammary tumor virus/NIC mice (13). These NIC mammary tumor cells undergo a TGFβ/H9252-induced epithelial-to-mesenchymal transition in a similar fashion to the NMuMG cell system as revealed by changes in cell morphology and reduced expression of known epithelial markers concomitant with elevated expression of mesenchymal markers (data not shown). Two clonal populations expressing either luciferase- (NIC/ShcAhigh) or ShcA-targeting (NIC/ShcAlow) shRNAs were generated (supplemental Fig. S2A). Further characterization revealed that NIC/ShcAhigh cells exhibit up to a 3-fold increase in their migratory and invasive responses to TGFβ/H9252 stimulation that was completely abolished by stable depletion of ShcA (NIC/ShcAlow; supplemental Fig. S2, B and C). These data confirm in two independent cell models that ShcA is required for TGFβ/H9252-induced migration and invasion of ErbB2-expressing breast cancer cells.

Previous reports describe a role for TGFβ in stimulating the shedding of EGF receptor family ligands from tumor cells, thereby leading to an enhanced association of EGF receptor and HER3 in HER2-expressing human breast cancer cells (2). Therefore, we investigated whether TGFβ signaling could alter

FIGURE 1. Reduced ShcA expression impairs primary tumor growth. A, ErbB2-expressing mammary epithelial cells (NMuMG) harboring either luciferase shRNAmirs (ErbB2/ShcAhigh) or ShcA shRNAmirs (ErbB2/ShcAlow) were injected into mammary fat pads of athymic mice. Tumor growth was followed by weekly caliper measurements, and the average tumor volumes from independent ErbB2/ShcAhigh (weeks 1–5, n = 8; weeks 6–7, n = 4) and ErbB2/ShcAlow (weeks 1–5, n = 10; weeks 6–7, n = 5) tumors were plotted (*, p < 0.03). Mice bearing ErbB2/ShcAhigh (n = 4) or ErbB2/ShcAlow (n = 5) tumors were sacrificed at 5 weeks postinjection to establish mammary tumor explants. The remaining animals were sacrificed at 7 weeks postinjection. Primary tumor tissue was harvested and subjected to IHC staining for proliferation (B), apoptosis (C), and endothelial cell recruitment (D). Data are quantified from ErbB2/ShcAhigh and ErbB2/ShcAlow mammary tumors (n = 8 per group) with averages calculated from five images (20×) from each tumor. ErbB2/ShcAlow mammary tumors show reduced proliferation (B; *, p < 0.001), diminished endothelial cell recruitment (D; *, p < 0.003), and elevated apoptosis (C; *, p < 0.02) compared with ErbB2/ShcAhigh mammary tumors. The scale bar in B (upper image) represents 50 μm and applies to all images in B–D. The error bars represent S.E.

FIGURE 2. Diminished lung metastases in mice bearing ErbB2/ShcAlow mammary tumors. The number of lung lesions per lung section was quantified at necropsy (7 weeks) from an average of four ErbB2/ShcAhigh and 5 ErbB2/ShcAlow mammary tumor-bearing mice. Representative images of the lungs from mice injected with ErbB2/ShcAhigh- and ErbB2/ShcAlow-expressing breast cancer cells are shown. Scale bars represent 2 mm for the images of the entire lung and 500 μm for the insets. *, p = 0.025. The error bars represent S.E.
activation of ErbB2 or ErbB3 and the subsequent recruitment of ShcA to these receptors. We observed no evidence for receptor activation in ErbB2/ShcA{}^{{\text{high}}}{} cells as measured by the degree of ErbB2 or ErbB3 phosphorylation in response to TGFβ/H9252 stimulation (supplemental Fig. S3). In addition, similar levels of ShcA were immunoprecipitated with either ErbB2 or ErbB3 in the presence or absence of TGFβ stimulation (supplemental Fig. S3). Similar observations were also made in NIC/ShcA{}^{{\text{high}}}{} cells (data not shown). Overall, these data indicate that TGFβ signaling does not augment ErbB receptor activation and ShcA recruitment, arguing that TGFβ-induced migration and invasion occur via distinct mechanisms in our cell model systems.

**Rescue of TGFβ-induced Migration and Invasion Requires an Intact ShcA Phosphotyrosine-binding Domain**—To define functional domains within ShcA that are required for the observed synergy between the TGFβ and ErbB2 signaling pathways, we performed rescue experiments with a panel of ShcA mutants. We began by determining whether the PTB or SH2 domain was important for ShcA function in this context. An ShcA cDNA encoding wild-type p46 and p52 ShcA isoforms as well as those encoding PTB (R175Q) or SH2 (R397K) mutants (24) were expressed in ErbB2/ShcA{}^{{\text{low}}}{} cells (Fig. 4A). These cDNAs are not targeted by the ShcA shRNA, which is directed to the 3'-UTR of endogenous ShcA. The p66 isoform of ShcA was not included in these analyses given that...

**FIGURE 3.** Reduced ShcA levels abrogate TGFβ-induced motility and invasion of ErbB2-expressing breast cancer cells. A, immunoblot analyses for ErbB2, ShcA, and α-tubulin were performed on ErbB2/ShcA{}^{{\text{high}}}{} and ErbB2/ShcA{}^{{\text{low}}}{}-expressing breast tumor explants. Ear tag numbers of animals designate individual mammary tumors. Arrowheads indicate ShcA isoforms. Migration (B) and invasion (C) assays were performed on two independent ErbB2/ShcA{}^{{\text{high}}}{} and ErbB2/ShcA{}^{{\text{low}}}{} breast tumor explants (designated by the last two digits of the ear tag number from A). Data represent three independent experiments performed in duplicate. Representative images are shown for migration (B) and invasion (C) assays. Significant differences between TGFβ-stimulated versus control cultures were observed for both migration (B; *, p < 0.04; **, p < 0.01) and invasion (C; *, p < 0.02; **, p < 0.01) in ErbB2/ShcA{}^{{\text{high}}}{} but not in ErbB2/ShcA{}^{{\text{low}}}{} cells. The error bars represent S.E.
current evidence suggests it mediates both antimitogenic and proapoptotic responses (25). In addition, reduced levels of p66 ShcA have been associated with poor prognosis in breast cancer patients (16).

Immunoblot analysis confirmed that equivalent levels of the indicated ShcA proteins were expressed in ErbB2/ShcA low cells (Fig. 4B, second panel). A FLAG epitope tag permitted an accurate assessment of exogenous ShcA expression (Fig. 4B, third panel) and accounts for the observed mobility shift (Fig. 4B, second panel, *). Immunoblot analysis also verified that ErbB2 levels were similar in all explant populations expressing the indicated ShcA mutants (Fig. 4B). We next analyzed the ability of these ShcA constructs to rescue TGFβ/H9252-induced migration and invasion of ErbB2/ShcA low breast cancer cells. Importantly, wild-type (WT) ShcA fully rescued TGFβ/H9252-induced migration and invasion (Fig. 4, C and D). The SH2 mutant (R397K) also restored TGFβ-induced migration and partially rescued TGFβ-induced invasion in ErbB2/ShcA low cells (Fig. 4, C and D). In contrast, TGFβ stimulation augmented neither the motility nor invasion of ShcA R175Q-expressing cells above baseline (Fig. 4, C and D). Together, these data conclusively demonstrate the requirement for an intact ShcA PTB domain for mediating the migratory and invasive phenotypes elicited by the TGFβ and ErbB2 signaling pathways.

Signaling Downstream of the Three Tyrosine Residues in ShcA

In order to investigate the requirement for signaling pathways engaged downstream of these ShcA tyrosine residues for TGFβ-induced migration and invasion, we transiently depleted Grb2 levels in ShcA-WT-, ShcA-313F-, and ShcA-2F-expressing cells using a pool of two independent
siRNAs. We also included scrambled control siRNAs as negative controls. Immunoblot analysis confirmed that Grb2 levels were efficiently diminished over the time frame needed to perform in vitro motility and invasion assays (Fig. 6A). We observed that Grb2 depletion selectively abrogated TGFβ-induced migration and invasion in ShcA-2F-expressing cells (Fig. 6, B and C). In contrast, these TGFβ-induced responses were unperturbed in Grb2-deficient ShcA-313F-expressing cells (Fig. 6, B and C). Therefore, TGFβ-initiated signaling downstream from Tyr313, but not Tyr239/Tyr240, of ShcA functions in a Grb2-dependent manner to augment TGFβ-induced migration and invasion of ErbB2-expressing cells.

Crk Adaptor Proteins Act Downstream of Tyrosine Residues 239/240 in ShcA to Mediate TGFβ-induced Migration and Invasion of ErbB2-expressing Cells—Previous reports have suggested that the Crk family of adaptor proteins can associate with ShcA (10). Given the known importance of Crk adaptor proteins for cell spreading and migration in breast cancer cells (20), we examined whether Crk adaptors could augment TGFβ-induced migration and invasion by performing transient

FIGURE 5. All three tyrosine residues within the ShcA CH1 domain are required for a complete migratory and invasive response to TGFβ in ErbB2-expressing cells. A, schematic representation of ShcA illustrating tyrosine (Y) to phenylalanine (F) mutations. B, immunoblot analyses of ErbB2/ShcA-low cells harboring an empty vector control (VC) or expressing WT ShcA or the indicated tyrosine mutants (313F, 2F, and 3F). Exogenous constructs were FLAG-tagged (*) as in Fig. 4. These cells were subjected to migration (C) and invasion (D) assays. Data are representative of three independent experiments performed in duplicate. Significant differences in migration between unstimulated and TGFβ-treated cells were observed in ErbB2/ShcA-low cells expressing WT, 313F, and 2F forms of the ShcA adaptor (C, * p < 0.002; ** p < 0.006; *** p < 0.0001). ErbB2/ShcA-low cells expressing ShcA-3F were refractory to TGFβ-induced migration and invasion. The error bars represent S.E.

FIGURE 6. ShcA elicits both Grb2-dependent and Grb2-independent signaling to mediate synergy between ErbB2/Neu and TGFβ pathways. A, immunoblot analysis of ErbB2/ShcA-low cells expressing WT ShcA, ShcA-313F, or ShcA-2F lines that were transfected with control (C) or Grb2-specific (G) siRNAs. Antibodies specific for Grb2, ShcA, and α-tubulin were used as indicated. Migration (B) and invasion (C) assays were performed, and the data represent three independent experiments performed in duplicate. TGFβ-induced motility and invasion downstream of Tyr239/Tyr240 in ShcA (313F) are independent of Grb2, whereas Grb2 is required downstream of Tyr313 in ShcA (2F) (B, * p < 0.001; C, * p < 0.01). The error bars represent S.E.
knockdowns of all Crk isoforms (Crk/I/II and Crk/L). A pool of siRNAs targeting the individual Crk isoforms was introduced into ErbB2/ShcAlow cells expressing ShcA-WT or the ShcA-313F and ShcA-2F mutants. The pooled Crk siRNAs reduced endogenous Crk levels by >50% when compared with cells transfected with scrambled siRNA control (Fig. 7A). Reduction of all three Crk adaptors resulted in the complete ablation of TGFβ-induced migration and invasion in cells expressing a mutant ShcA that only retained the Tyr239/Tyr240 phosphorylation sites (ShcA-313F). In contrast, reduced expression of the Crk adaptor proteins minimally perturbed the migratory or invasive characteristics of ErbB2/ShcAhigh cells reconstituted either with wild-type ShcA or the ShcA-2F mutant following TGFβ stimulation (Fig. 7B and C). These data strongly indicate that signals emanating from Tyr313 in ShcA are Grb2-dependent, whereas Tyr239/Tyr240 require the recruitment of Crk adaptor proteins.

Simultaneous Reduction of Grb2 and Crk Adaptor Proteins in ErbB2-Expressing Cells Renders Them Refractory to TGFβ-induced Migration and Invasion—We next examined whether Grb2 and Crk are the primary signaling complexes recruited to ShcA to increase TGFβ-induced breast cancer cell invasion. Therefore, we co-transfected siRNAs targeting Grb2 and the individual Crk isoforms in ShcA-proficient breast cancer cells (ErbB2/ShcAhigh). Scrambled siRNAs were included as negative controls (Fig. 8A). We demonstrate that simultaneous reduction of Grb2 and all three Crk isoforms completely abolished migration (Fig. 8B, upper graph) and invasion (Fig. 8B, lower graph) of ErbB2/ShcAhigh cells in response to TGFβ (Fig. 8B). These data completely mimicked the phenotype observed using ErbB2/ShcAhigh cells (Fig. 3). Importantly, simultaneous reduction of the Grb2 and Crk adaptor proteins in an independent ErbB2-expressing ShcA-proficient (NIC/ShcAhigh) breast cancer cell line also abrogated TGFβ-induced migration and invasion (supplemental Fig. S4). Thus, we have identified that the Grb2 and Crk signaling axes function downstream of ShcA for maximal TGFβ-induced migration and invasion of ErbB2-expressing breast cancer cells (Fig. 8C).

Distinct ShcA Tyrosine Residues Are Required for ErbB2- and TGFβ-mediated Tumor Growth and Progression—Prior to assessing the functional contribution of ShcA tyrosine residues, we first determined whether ErbB2/ShcAhigh-expressing mammary tumors were responding to TGFβ signals in vivo. To accomplish this, we performed phospho-Smad2 and total Smad2 immunohistochemical staining on tumor sections and lung metastases arising in mice injected with ErbB2/ShcAhigh cells. All primary tumors and metastases stained positively for nuclearly localized phospho-Smad2, clearly demonstrating that tumor cells are responding to TGFβ (Fig. 9). Thus, TGFβ signaling is active in both ErbB2-expressing mammary tumors and their derived lung metastases.

We next injected ErbB2/ShcAlow breast cancer cells harboring an empty vector or expressing our panel of ShcA phosphorylation site mutants into the mammary fat pads of mice. As expected, reduced ShcA expression in cells harboring the vector control exhibited diminished growth compared with cells in which wild-type p46 ShcA and p52 ShcA were re-expressed (Fig. 10A). ErbB2/ShcAhigh cells expressing the ShcA-313F mutant formed tumors that grew to the same extent as the ShcA-WT cell population. In contrast, ShcA-2F- and ShcA-3F-expressing cells grew at rates comparable with the ErbB2/ShcAhigh cells harboring an empty vector (Fig. 10A).

To understand which aspects of tumor progression depend on ShcA tyrosine signaling, immunohistochemical staining for markers of proliferation, apoptosis, and endothelial cell recruitment was performed on tumor sections prepared from tumors that were harvested at 3 weeks postinjection. Tumor cell proliferation was only decreased in the ErbB2/ShcAhigh cells containing the vector control or those expressing the ShcA-3F mutant (Fig. 10B). In contrast, expression of either the ShcA-2F or ShcA-313F mutants was capable of restoring proliferation rates to those observed in tumors expressing ShcA-WT (Fig. 10B). The presence of apoptotic cells was increased 2.6-fold in ErbB2/ShcAlow mammary tumors containing the empty vector or expressing the ShcA-313F and ShcA-3F mutants when compared with tumors expressing ShcA-WT or ShcA-2F (Fig. 10C). Finally, only the ShcA-313F-expressing tumors exhibited a partial restoration of endothelial cell recruitment when compared with ShcA-WT-expressing tumors at 3 weeks postinjection (Fig. 10D). Given the increased apoptosis in ShcA-313F-expressing tumors, it was surprising that these tumors grew at the same rate as those expressing ShcA-WT. However, an
assessments of the levels of proliferation and apoptosis by immunochemical methods in tumors at 5 weeks postinjection revealed that ShcA-313F-expressing tumors are able to overcome the defects in tumor cell survival that are present at the early 3-week time point, whereas proliferation rates remain unchanged (Fig. 10, E and F). Interestingly, the level of CD31 positivity was the highest in ShcA-

FIGURE 8. Simultaneous reduction of Crk family and Grb2 adaptor protein expression renders ShcA proficient cells refractory to TGFβ-induced migration and invasion. A, immunoblot analysis of ErbB2/ShcA high cells that were transfected with scrambled control siRNAs (C) or siRNAs targeting Grb2 and all three Crk adaptor isoforms (GK). Antibodies specific for CrkL, CrkII, Grb2, ShcA, and α-tubulin were used as indicated. β, migration and invasion assays were performed, and the data represent two independent experiments performed in duplicate. TGFβ-induced motility and invasion of ErbB2-expressing cells requires Grb2 and Crk adaptor protein signaling downstream of ShcA signaling (β, *p < 0.01). C, schematic model showing the requirement for ShcA signaling to mediate synergy between the TGFβ and ErbB2 pathways. Grb2 is required downstream of ShcA tyrosine 313, whereas Crk adaptors are recruited downstream of tyrosine residues 239/240 of ShcA for a complete migratory and invasive response to TGFβ in cells overexpressing ErbB2. The error bars represent S.E.

FIGURE 9. TGFβ signaling is uniformly engaged in ErbB2/ShcA high mammary tumors and their derived lung metastases. Five mammary tumor sections and five lung sections containing breast cancer metastases were immunohistochemically stained for phosphorylated Smad2 (P-Smad2) and total Smad2 protein. Positive staining of both markers was found to be uniformly distributed throughout all tumor samples and metastatic lesions, indicating that TGFβ signaling is active in ErbB2/ShcA high cells in vivo. Representative images of staining from one tumor and one lung section are shown. Scale bars represent 25 (large panels) or 750 μm (insets).

ShcA Integrates TGFβ and ErbB2 Signaling Pathways

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phosphorylation in the growth of ErbB2-expressing breast tumors. Taken together, these data support distinctive roles for ShcA control (Fig. 10).

**FIGURE 10.** Distinct ShcA tyrosine residues mediate tumor cell survival and endothelial cell recruitment. A, ErbB2/ShcAlow cells harboring a vector control (VC) or expressing ShcA-WT, ShcA-313F, ShcA-2F, and ShcA-3F were injected into the mammary fat pads of mice. Tumor growth was followed by weekly caliper measurements, and the average tumor volumes (n = 10 mice for weeks 1-3, n = 5 mice for weeks 3-5) were plotted (*, p < 0.02). Five mice from each group were sacrificed at 3 and 5 weeks postinjection, and mammary tumors were processed for immunohistochemical analyses. Tumor sections from mice sacrificed at 3 weeks were subjected to IHC staining for proliferation (B), apoptosis (C), and endothelial cell recruitment (D). IHC staining for proliferation (E), apoptosis (F), and endothelial cell recruitment (G) was again performed on tumor sections from mice sacrificed at 5 weeks. Data are representative of n = 5 mice for each group with averages calculated from at least five images (20×) from each tumor. ErbB2/ShcAlow mammary tumors expressing a vector control and ShcA-3F show reduced proliferation at 3 weeks (*, p < 0.05) and 5 weeks (*, p < 0.003) weeks postinjection. ErbB2/ShcAlow mammary tumors expressing a vector control, ShcA-313F, and ShcA-3F exhibit elevated apoptosis at 3 weeks (C, *, p < 0.001), whereas tumors expressing a vector control and ShcA-3F show elevated apoptosis at 5 weeks (E, *, p < 0.02). ErbB2/ShcAlow tumors expressing ShcA-WT or ShcA-313F exhibit increased endothelial cell recruitment at 3 and 5 weeks postinjection (D and G; *, p < 0.01). The error bars represent S.E.

**DISCUSSION**

We have established ErbB2-expressing breast cancer models that demonstrate the importance of ShcA in mediating tumor growth and highlight its role as a key integrator of the ErbB2 and TGFβ pathways in breast cancer cells. This is the first study to molecularly dissect the discrete phosphotyrosine-binding domains and motifs of ShcA in this process. Furthermore, we demonstrate that ShcA functions as a molecular scaffold by simultaneously recruiting Grb2 and Crk adaptor proteins into signaling complexes in a PTB-dependent manner.

The observed requirement of the ShcA PTB domain for TGFβ-induced responses in ErbB2-expressing breast cancer cells may depend on recruitment of ShcA to Tyr1221/1222 of ErbB2 that occurs via this domain (26, 27). ShcA may be recruited to other receptors in response to TGFβ stimulation. In this regard, an association between ShcA and the TGFβ type II (TβRII) or type I (TβRI) receptors has been described. One study suggests that the SH2 domain of ShcA binds directly to tyrosine residue 284 within TβRII (28) that is phosphorylated by Src in a TGFβ-dependent manner (29). Given our observations that the SH2 domain of ShcA is dispensable for TGFβ-induced motility and invasion, this mechanism is not likely to account for our observed phenotype. In contrast, another study described a PTB-dependent association between ShcA and the TGFβ receptor complex in response to TGFβ stimulation.

TGFβ signaling induces clustering of ErbB2 into integrin-containing complexes (31). TGFβ can also induce the expression of β3 integrin, which subsequently interacts with TβRII (28). This complex permits the Src-mediated phosphorylation of Tyr284 within TβRII and facilitates p38 MAPK activation, epithelial-to-mesenchymal transition, and increased migration and invasion (28, 29). Interestingly, ShcA is recruited to the tyrosine phosphorylation sites within the cytoplasmic tails of integrin β3 and integrin β4 in a PTB-dependent manner (27, 32, 33). Thus, it is conceivable that ShcA is recruited to ErbB2-integrin and/or TGFβ receptor-integrin complexes in response to TGFβ stimulation.

Grb2 is a central signaling intermediate downstream of ShcA that is capable of binding to both Tyr239 and Tyr143 sites (8). Our data, however, suggest that Grb2 function is required downstream of Tyr143, but not Tyr239/Tyr240, to mediate TGFβ-induced migration and invasion in ErbB2-expressing ShcA Integrates TGFβ and ErbB2 Signaling Pathways

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breast cancer cells. To our knowledge, this is one of the few examples in which Grb2 function is implicated downstream of a specific tyrosine residue in ShcA. Moreover, there is evidence that Grb2 can function independently of the Ras/MAPK pathway to promote the migration of breast cancer cells through its ability to bind neural Wiskott-Aldrich syndrome protein (34). Finally, inhibition of Grb2-mediated signaling with SH2 domain antagonists impairs the formation of melanoma metastases (35).

There are few examples that demonstrate unique signaling outputs emanating from Tyr239/Tyr240 when compared with Tyr313. Signaling downstream of Tyr239/Tyr240, but not Tyr313, is linked to c-Myc expression (36, 37). Grb2-independent ShcA signaling has also been inferred from experiments examining the relative role of Grb2 and ShcA signaling downstream of ErbB2-mediated epithelial dispersion. In this study, recruitment of Grb2 was insufficient to induce hepatocyte growth factor-mediated epithelial cell scattering and tubule formation, whereas recruitment of ShcA supported these responses (38). In addition, Grb2 recruitment to ErbB2 failed to induce VEGF expression and enhanced angiogenesis that were observed with an ErbB2 receptor capable of recruiting ShcA (39). Thus, ShcA must signal via molecules other than Grb2. In agreement with this idea, we observed that TGFβ promotes the association of ShcA with Crk adaptor proteins specifically through residues Tyr239/Tyr240. Importantly, we demonstrate that these interactions contribute to the migratory and invasive responses manifested by ErbB2-expressing breast cancer cells in response to TGFβ treatment. Our data strongly suggest that the Grb2 and Crk proteins are key players downstream of ShcA that mediate the synergistic responses to ErbB2 and TGFβ signaling.

The Crk adaptor proteins are key regulators of epithelial morphogenesis as well as breast cancer migration and invasion (20, 40, 41). p130Cas is known to recruit Crk adaptor proteins, which in turn bind DOCK180/ELMO to facilitate Rac1 GTPase activation (42–44). Rac1 and cdc42 activation are common responses during TGFβ-induced breast cancer cell migration and invasion (45, 46). Interestingly, p130Cas is a critical component downstream of both ErbB2 (47–49) and TGFβ signaling (50) for mammary tumor growth and metastasis. Our data suggest an alternate way in which the Crk adaptors can be engaged in a TGFβ-dependent fashion via their association with ShcA.

We were able to further determine that specific ShcA tyrosine residues have distinct roles in promoting mammary tumor growth downstream of TGFβ and ErbB2 signaling. We discovered that expression of the ShcA Tyr313 mutant promotes the survival of breast tumor cells, whereas the ShcA Tyr239/Tyr240 mutant is capable of supporting endothelial cell recruitment. These data are nearly identical to results previously described for the function of these tyrosines in breast cancer progression (13). The only distinguishable difference between these data was in the growth of ErbB2/ShcAlow cells expressing ShcA-313F. In our study, ShcA-313F expression could rescue tumor growth to the same degree as expression of ShcA-WT, whereas previous results demonstrated impaired growth in all of the ShcA tyrosine site mutants (13). This might be due in part to differences in experimental design. For instance, in the previous report, ShcA mutants were expressed as dominant negatives in the context of normal levels of endogenous ShcA (13). A further explanation for these divergent results may lie in the observation that ShcA-313F-expressing tumors were able to overcome an early wave of apoptosis (3 weeks postinjection). Indeed, this was verified by reassessing the proliferative and apoptotic markers in end stage mammary tumors (5 weeks postinjection). In addition, the ability of ShcA-313F-expressing tumors to recruit endothelial cells remained high in these late stage tumors. These in vivo results combined with the in vitro data demonstrating a requirement for ShcA tyrosine residues 313 and 239/240 for TGFβ-induced migration and invasion support our findings that mutation of any of these three residues rendered breast cancer cells less capable of forming spontaneous lung metastases in vivo.

FIGURE 11. ShcA tyrosine residues are required for the efficient lung metastasis of ErbB2-expressing breast cancer cells. Established ErbB2/ShcAlow tumors expressing a vector control (VC; n = 5) or expressing ShcA-WT (n = 6), ShcA-313F (n = 6), ShcA-2F (n = 7), and ShcA-3F (n = 5) were resected at a common volume (150–200 mm³), and mice were sacrificed at a fixed time following resection (3 weeks). The number of lung lesions per lung section was quantified at necropsy. Representative images of the lungs are shown. The loss of ShcA signaling through its tyrosine residues severely impaired metastasis (*, p < 0.1). Scale bars represent 2 mm for the images of the entire lung and 500 μm for the insets. The error bars represent S.E.
Further experiments will decipher the roles of Crk- and Grb2-initiated signaling downstream of ShcA tyrosine residues. However, this study clearly positions ShcA as a critical integrator of the TGFβ and ErbB2 signaling pathways, which synergize to promote the aggressive behaviors of breast cancer cells.

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