The ShcA PTB Domain Functions as a Biological Sensor of Phospho-tyrosine Signaling During Breast Cancer Progression

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Summary

ShcA (SHC1) is an adapter protein that possesses an SH2 and a PTB phosphotyrosine binding motif. ShcA generally utilizes its PTB domain to engage activated receptor tyrosine kinases (RTKs), but there has not been a definitive determination of the role of this domain in tumorigenesis. To address this question, we employed a ShcA mutant (R175Q) that no longer binds phospho-tyrosine residues via its PTB domain. Here we report that transgenic expression of this mutant delays mammary tumor onset in the MMTV-PyMT mouse model of breast cancer. Paradoxically, we observed a robust increase in the growth and angiogenesis of mammary tumors expressing ShcR175Q, which displayed increased secretion of fibronectin and expression of integrin α5/β1, the principle fibronectin receptor. Sustained integrin engagement activated Src, which in turn phosphorylated pro-angiogenic RTKs, including PDGFR, FGFR and Met, leading to increased VEGF secretion from ShcR175Q-expressing breast cancer cells. We defined a ShcR175Q-dependent gene signature that could stratify breast cancer patients with a high microvessel density. This study offers the first in vivo evidence of a critical role for intracellular signaling pathways downstream of the ShcA PTB domain, which both positively and negatively regulate tumorigenesis during various stages of breast cancer progression.
Introduction

The ShcA adaptor employs distinct domains and motifs to transduce phosphotyrosine dependent signals downstream of receptor and non-receptor tyrosine kinases (1). These interactions regulate signaling pathways in breast cancer cells that govern numerous biological processes including cell proliferation, survival, invasion, metastasis, immune suppression and induction of an angiogenic response (2-4). The ability of a tumor to reach a clinically-detectable mass requires neo-vascularization through a process called angiogenesis. Numerous studies suggest an important role for ShcA during normal and pathological angiogenesis. In the cardiovascular system, ShcA is required for proper heart development through the angiogenic remodeling of pre-existing vessels (5). Moreover, ShcA signaling downstream of receptor tyrosine kinases (RTK), such as ErbB2, increases VEGF production and promote an in vivo angiogenic response associated with tumor outgrowth (4).

Following RTK activation, ShcA utilizes its PTB or SH2 domains to bind phosphotyrosine residues in the cytoplasmic tails of growth factor or integrin receptors. It has been established that ShcA interacts with many receptor tyrosine kinases, including ErbB2, specifically through its PTB domain (6). Moreover, the MT antigen also binds the ShcA PTB domain via Y250, which resides within a consensus NPXpY motif (7-8). Activated RTKs, or associated cytoplasmic kinases, subsequently phosphorylate three tyrosine residues (Y239/Y240 and Y317) within the central collagen homology 1(CH1) domain of ShcA (9-10). Once phosphorylated, these tyrosines serve as docking sites for other PTB- and SH2-containing proteins. Transgenic and orthotopic mouse models of ErbB2- and Polyoma Virus middle T (MT) antigen-driven breast cancer demonstrate
that these tyrosine residues transduce critical and non-overlapping signals to promote breast cancer progression (4). Transgenic studies in MMTV/MT mice further demonstrate an important role for the ShcA SH2 domain in promoting breast cancer cell survival by engaging a 14-3-3/PI3'K signaling complex at the plasma membrane (11).

We sought to establish the requirement for the ShcA PTB domain during MT- and ErbB2-induced breast cancer progression. Using these in vivo mouse model systems, we demonstrate that the ShcA PTB domain functions as a biological sensor to regulate not only breast cancer initiation but to control the rate of tumor growth and angiogenesis, depending on the strength of the transforming oncogene.
Materials and Methods

Cells. NMuMG-NT2197 cells were generated and cultured as previously described (4) and transfected with a pMSCV/hygro expression vector (Clontech) expressing a wild-type ShcA cDNA or a ShcA mutant harboring an arginine to glutamine substitution at amino acid 175 (R175Q). Stable cell lines were generated by selection with 500 μg/ml hygromycin. The stable cell lines used here represent pools of 3-4 clonal populations expressing similar levels of FLAG-tagged ShcA.

Mice. MMTV/MT transgenic mice have been described (12). Mice expressing a FLAG-tagged mutant ShcR175Q allele under the control of the endogenous shcA promoter have also been described (13) and were interbred for ten generations onto an FVB background prior to their utilization in bigenic crosses. NT2197/ShcA and NT2197/ShcR175Q breast cancer cells (5 x 10^4) were injected into the fourth mammary fat pad of nude mice (Taconic) and tumor growth was determined bi-weekly by caliper measurements (4). Tumors were fixed in 10% buffered formalin, embedded in OCT or frozen in liquid nitrogen. For the Matrigel plug assays, 1 x 10^5 cells were injected as previously described (4). The plugs were excised 8 days later and embedded in OCT. All animal studies were approved by the Animal Resources Centre (ARC) at McGill University and comply with guidelines set by the Canadian Council of Animal Care.

SiRNA/Inhibitor Studies. For the siRNA studies, cells were transfected with a mixture of three dicer substrate duplex siRNAs targeting mouse Src (Origene-SR416929) or with a universal scrambled control (Origene-SR30004) using Interferin transfection reagents (Cat# 409-10; Polyplus). siRNAs were used at a final concentration of 60 nM per transfection (20 nM for each individual ShcA siRNA). Cells were serially transfected
a total of three times at 24, 48 and 72h post plating. Four hours after the third transfection, the cells were cultured in media in 0.5% FBS and condition media and lysates were harvested 20 hours later. For the PP2 inhibitor studies, 300,000 cells were seeded on 6 well dishes (n=6). The following day, cells were cultured in media containing either DMSO or 20 μM PP2 (Sigma). Whole cell lysates and media were collected 12hrs and 24hrs post-treatment, prior to which the cells were imaged on an Olympus Infinity 1 at 4X magnification using Infinity Capture software (Lemenera). Cell viability was determined by trypan blue exclusion.

**Enzyme-Linked Immunosorbent Assay (ELISA).** ELISAs were performed on the conditioned media using commercially available kits directed against mouse PDGF-AA (R&D), PDGF-AB (R&D), PDGF-BB (R&D), VEGF-A (R&D), and Fibronectin (Abcam). Cells were transferred to low serum (0.5%) at 70% confluency and allowed to condition the media for 24 hours. For the tumor cell lysates, 50 μg were loaded per well. Level of secreted protein in the conditioned media was normalized by the concentration of total protein within the corresponding whole cell lysates for each well.

**Immunohistochemistry.** Ki67 staining was performed on paraffin-embedded sections as described (4) (1:1000; AB15580, Abcam). Paraffin-embedded sections were also subjected to TUNEL staining (Apoptag Detection Kit, Chemicon) according to manufacturer’s instructions. CD31 (1:250; 550274, BD Biosciences) and F4/80 (1:250; MF48004-3, Invitrogen) staining was performed on OCT-embedded sections as previously described (11). Slides were scanned using a ScanScope XT Digital Slide Scanner (Aperio) and data was analyzed with positive pixel count or nuclear algorithms. A minimum of 15 fields (20x magnification) were quantified for each section.
Immunoprecipitation/Immunoblotting. Whole cell lysates were generated from cell lines or flash frozen tumor tissue as previously described (4). Lysates were separated by SDS-PAGE, transferred onto PVDF membranes and probed with the antibodies listed in Table S1. Densitometric analysis was performed using Image J software. Lysates were immunoprecipitated with FLAG-specific antibodies (1:100; Cat# F1804 – Sigma Aldrich). For the MT co-immunoprecipitation reactions, cell lines derived from MMTV/MT transgenic tumors were transfected with FLAG-tagged ShcA and ShcR175Q constructs. The mouse monoclonal MT antibody was a gift from Dr. Stephen Dilworth.

Quantitative real-time reverse transcription-PCR. Total RNA was isolated from cell lines using RNeasy midi-kits (Qiagen) according to manufacturer’s instruction and cDNA was generated using Superscript Reverse Transcriptase II (Invitrogen). Mouse Fibronectin mRNA levels were determined by quantitative using the QuantiTect SYBR Green RT-PCR kit (Qiagen) and the following primers: Forward: ACAGAAATGACCATTGAAGG and Reverse: TGTCTGGAGAAAGGTTGATT. Mouse GPNMB (Mm01328587_m1), NCF4 (Mm00476300_m1), CSF2RB2 (Mm00655763_m1), MMP2 (Mm00439498_m1) and GAPDH (4352339E) levels were determined using commercially available Taqman probes (Invitrogen) and a Taqman Fast Universal PCR Mastermix kit (Cat# 4352042 - Invitrogen). A constant annealing temperature of 60°C was used for all reactions. Expression levels of the FN-EDA and FN-EDB alternatively spliced variants were determined as previously described (14).

Adhesion Assay. Adhesion to fibronectin was determined using the xCELLigence RTCA system (Roche Applied Science). Prior to seeding of cells, wells of an E-plate (Roche Diagnostics; 05 469 830 001) were coated with fibronectin diluted in PBS (final
concentration 50 μg/mL) for 1 hour and rinsed twice with PBS. 80000 cells suspended in 100 μL of serum-free media were plated and measurements were taken every minute for 2 hours. The slope of the curve (rate of adhesion) was calculated according to the manufacturer’s protocol using the xCELLigence software. The graph represents data from 3 independent experiments performed in duplicate.

**Microarray hybridization, normalization and analysis.** Total RNA was isolated from NT/ShcA and NT/ShcR175Q cells (4 wells each). RNA extraction, linear amplification, aRNA and hybridization onto Agilent Whole Mouse Genome Oligo Microarrays (4x44K) were performed as described (3). Each sample was hybridized against the Universal Mouse Reference RNA (Agilent) to normalize the data. Raw probe intensities were background-corrected, within and between each array normalized using the normexp, loess and quantile methods respectively in Bioconductor. Differential expression was evaluated using LIMMA. A ShcR175Q signature was derived by selecting the top 100 significantly differentially expressed genes between NT/ShcA and NT/ShcR175Q breast cancer cells (FDR corrected p values < 2.6x10-6). Expression of this signature was examined in six breast cancer datasets encompassing 3100 breast cancer patients (15-20). In each dataset, the patients were ordered based on the correlation of expression of the genes in the ShcR175Q signature with the observed fold change between NT/ShcA and NT/ShcR175Q breast cancer cells. All expression analysis was conducted using Bioconductor. The microarray data are deposited on GEO (GSE41718).

**Statistical Analysis.** All statistical analysis was performed using a two tailed, two-sample equal variance student’s t test.
Results

Attenuated PTB-driven ShcA signaling leads to delayed breast tumor onset but enhanced outgrowth. We previously demonstrated that the ShcA adaptor protein transduces signals in mammary epithelial cells, which are essential for breast cancer initiation and progression (3-4). Many clinically-relevant RTKs that contribute to mammary tumorigenesis, bind to the PTB domain of ShcA. Thus, we sought to determine the functional importance of PTB-driven ShcA signaling during breast cancer progression. To achieve this, we first employed the well established polyoma virus middle T model (MMTV/MT) transgenic breast cancer mouse model (12). Importantly, the MT oncogene recruits ShcA via the PTB domain (7-8) and mutation of the ShcA binding site in MT severely delays the incidence and impairs the severity of mammary tumor formation (21). MMTV/MT transgenic mice were bred with animals in which the endogenous ShcA allele was replaced with a mutated allele harboring a point mutation in the PTB domain (R175Q), which ablates binding to phospho-tyrosine residues (13). We were unable to generate ShcAR175Q/R175Q homozygous mice due to embryonic lethality (13). We confirmed that the ability of the ShcR175Q mutant to bind MT is significantly impaired (Fig. 1A). We further demonstrate that reduced PTB-driven ShcA signaling significantly delays mammary tumor onset in MT/ShcA+/R175Q mice (herein referred to as MT/ShcR175Q) (Fig. 1B). This delay is associated with a reduced tumor burden observed in MT/ShcR175Q females four weeks post first palpation. However, the number of tumor bearing glands are identical between MT and MT/ShcR175Q mice at the experimental endpoint (7-8 weeks post-palpation), re-enforcing the idea that tumor initiation is delayed in MT/ShcR175Q animals (Fig. 1C, upper panel). Strikingly,
the average tumor volume is significantly enhanced in MT/ShcR175Q mice at the experimental end-point, suggesting that attenuated PTB-driven ShcA signaling paradoxically increases mammary tumor growth (Fig. 1C, lower panel). We next characterized MT and MT/ShcR175Q mammary tumors with respect to the engagement of signaling pathways known to be downstream of ShcA. We demonstrate that the ERK, p38MAPK, JNK and AKT signaling pathways were similarly active in both MT and MT/ShcR175Q mammary tumors (Fig. S1).

To better understand the mechanisms that enhance tumor growth in MT/ShcR175Q animals, we performed IHC staining of early and late-stage MT and MT/ShcR175Q mammary tumors for markers of proliferation, apoptosis and endothelial cell recruitment. These analyses indicate that tumor cell proliferation is marginally different between both genotypes (Fig. 1D, top panel). However, increased tumor volume correlates with a statistically significant reduction in apoptosis and enhanced neo-vascularization in MT/ShcR175Q tumors compared to MT control tumors (Fig. 1D, middle panels). Moreover, end-stage MT/ShcR175Q mammary tumors express moderately elevated VEGF levels relative to MT control tumors (Fig. S2).

Macrophages are essential for the angiogenic switch in MT-driven mammary tumors (22). However, the observed increase in vascular density at the 7-8 week time point is not reflective of enhanced macrophage recruitment. Moreover, early stage MT/ShcR175Q mammary tumors display a statistically significant decrease in macrophage recruitment compared to age-matched MT controls (Fig. 1D, lower panel). These results suggest that attenuated PTB-driven ShcA signaling in breast cancer cells
accelerates mammary tumor growth by increasing tumor angiogenesis through a mechanism that is unlikely to rely on enhanced macrophage infiltration.

**The ShcA PTB mutant enhances tumor angiogenesis.** It is important to recognize that the ShcR175Q mutant allele is expressed from the endogenous ShcA promoter (13). Therefore, PTB-driven ShcA signalling is attenuated both in the tumor epithelium and in cell types within the adjacent stroma. To confirm that the ShcR175Q mutant is acting in a cell autonomous fashion, we ectopically expressed FLAG-tagged wild-type ShcA or a ShcR175Q mutant in an immortalized mammary epithelial cell line (NMuMG), which we subsequently transformed with activated Neu/ErbB2 (NT2197). Pooled ShcA-expressing populations were generated by combining 4 clones, each expressing similar levels of FLAG-tagged ShcA or the ShcR175Q mutant (Fig. 2A). We chose Neu/ErbB2 as the transforming oncogene given that it recruits ShcA via its PTB domain (23) and is overexpressed in 20-30% of human breast cancer (24). Indeed, the ShcR175Q mutant is reduced in its ability to bind ErbB2 (Fig. 2A). In contrast, Grb2 binding to ShcA and the ShcR175Q mutant is comparable (Fig. 2B). Given that the SH2 domain of Grb2 binds phospho-tyrosine residues within the CH1 domain of ShcA (9-10), these data demonstrate the specificity of the R175Q mutation within the phospho-tyrosine binding pocket of the PTB domain.

Consistent with our transgenic studies, mammary tumor growth of NT/ShcR175Q cells is greatly accelerated relative to that observed with NT/ShcA breast cancer cells (Fig. 2C). Despite this fact, we do not observe appreciable differences in ERK, AKT, p38MAPK or JNK signaling between NT/ShcA and NT/ShcR175Q breast cancer cells (Fig. S3A). We further analyzed NT/ShcA and NT/ShcR175Q mammary tumors at an
early stage (<500mm\(^3\)) and at the experimental endpoint (1500mm\(^3\)) for their degree of proliferation, survival and endothelial cell recruitment as assessed by Ki67, TUNEL and CD31 IHC staining, respectively (Fig. 2D, upper panel). The degree of tumor cell proliferation and survival is unaltered at either time point between NT/ShcA and NT/ShcR175Q tumors (Fig. S3B, C). However, we observe a statistically significant increase in microvessel density in NT/ShcR175Q tumors during the early stages of their outgrowth, which normalize at end-stage (Fig. 2D, middle panel; Fig. 2E). This is coincident with diminished macrophage infiltration in early- and late-stage NT/ShcR175Q mammary tumors relative to NT/ShcA controls (Fig. 2D, lower panel; Fig. 2E). Taken together, these data suggest that expression of the ShcA PTB mutant functions in a cell autonomous fashion to accelerate tumor growth by virtue of its ability to stimulate neo-vascularization.

Enhanced microvessel density without a parallel increase in macrophage recruitment suggests that the ShcR175Q mutant may augment the expression of angiogenic factors from the breast cancer cells themselves. Indeed, it has previously been demonstrated that ShcA signaling promotes tumor angiogenesis by regulating VEGF production (4). We demonstrate that VEGF levels are significantly elevated in both and NT/ShcR175Q cells in vitro (Fig. 2F) and in early stage NT/ShcR175Q mammary tumors in vivo (Fig. 2G), which co-incides with the time point in which we observe a robust increase in tumor microvessel density. We further show that NT/ShcR175Q breast cancer cells are significantly more angiogenic in an in vivo Matrigel plug assay (Fig. S4). These data suggest that attenuated PTB-driven ShcA signaling increases VEGF production and promotes tumor angiogenesis.
Expression of a ShcA PTB mutant increases Src-dependent receptor tyrosine kinase signaling and VEGF production. To understand how the ShcR175Q mutant functions to increase tumor angiogenesis, we examined intracellular signaling pathways that are known to be pro-angiogenic. In this regard, PDGFR, FGFR and Met are three receptor tyrosine kinases that engage signaling pathways which promote tumor angiogenesis (25-26). Interestingly, we demonstrate that PDGFR, FGFR and Met signaling is elevated in NT/ShcR175Q breast cancer cells compared to NT/ShcA controls (Fig. 3A). We further demonstrate that Met and PDGFR ligands are comparably expressed (PDGF-AA) or reduced (PDGF-AB, HGF) from NT/ShcR175Q cells, relative to NT/ShcA cells (Fig. 3B). PDGF-BB and FGF2 were undetectable in the conditioned media of NT/ShcA and NT/ShcR175Q breast cancer cells by ELISA. These data suggest that increased PDGFR, FGFR and Met activation in ShcR175Q-expressing breast cancer cells is not the result of an autocrine loop. In contrast, we observe variable levels of Src, PDGFR, MET and FGFR phosphorylation in late stage MT/ShcA and MT/ShcR175Q tumors (Fig. S5) and in early and late stage NT/ShcA and NT/ShcR175Q tumors (Fig. S6). This is not surprising given that these signaling pathways are also engaged in numerous cell types within the tumor stroma.

Another mechanism to activate receptor tyrosine kinases, independently of ligand, is via lateral integrin-dependent signaling, which leads to Src-mediated phosphorylation of tyrosine residues in the cytoplasmic tails of RTKs. We observe increased Src activation, both in NT/ShcR175Q breast cancer cells and MT/ShcR175Q mammary tumors, relative to their respective controls (Fig. 4A). This coincides with a
A robust increase in the phosphorylation of Src-dependent phosphorylation sites within FAK (Y576/577) in NT/ShcR175Q breast cancer cells (Fig. 4B).

Integrin signaling proceeds primarily via the recruitment and activation of FAK and Src family kinases (27). Moreover, β1-containing integrins promote tumor angiogenesis in breast cancer transgenic mouse models (28). Thus, we examined whether increased Src activation in ShcR175Q-expressing breast cancer cells correlated with elevated β1 integrin expression. Indeed, we demonstrate a robust increase in both α5 and β1 subunit expression in NT/ShcR175Q cells, which together constitute the principal fibronectin receptor (Fig. 4B). This is coincident with elevated fibronectin mRNA and protein levels in NT/ShcR175Q cells compared to NT/ShcA controls (Fig. 4C, D). Published studies have shown that two fibronectin alternatively spliced variants (EDA, EDB) contribute to tumor neo-vascularization and are also highly expressed in stromal cell types, including the tumor endothelium (29-31). However, we do not observe differences in processing of the FN-EDA and FN-EDB alternatively spliced variants between NT/ShcA and NT/ShcR175Q cells in vitro or in mammary tumors in vivo (Fig. S7). Thus, fibronectin alternative splicing does not account for the increased VEGF production and tumor vascularization of NT/ShcR175Q mammary tumors relative to NT/ShcA controls. However, we demonstrate that the rate of NT/ShcR175Q breast cancer cell adhesion to fibronectin is significantly increased compared to that observed with NT/ShcA cells (Fig. 4E). These data suggest that NT/ShcR175Q cells establish an autocrine loop, which involves α5/β1 integrin engagement and sustained downstream Src and FAK activation.
In order to test whether enhanced Src activation contributes to increased VEGF production and RTK signaling, we employed small interfering RNA (siRNA) approaches to attenuate Src expression in NT/ShcA and NT/ShcR175Q cells. Relative to scrambled controls, NT/ShcA cells treated with the Src-specific siRNA exhibit a modest (1.4 fold) reduction in VEGF levels. In contrast, attenuated Src expression markedly (4.4 fold) reduces VEGF levels in NT/ShcR175Q cells (Fig. 5A). Immunoblot analysis confirms that both pSrc and Src levels are significantly reduced in NT/ShcA and NT/ShcR175Q cells transfected with the Src siRNA compared to the scrambled control (Fig. 5B). Moreover, the profound reduction in VEGF production observed in Src-deficient, NT/ShcR175Q cells is specifically associated with significantly reduced levels of PDGFR and MET phosphorylation. In contrast, Src inhibition does not impact PDGFR or MET phosphorylation in NT/ShcA cells (Fig. 5B).

We extended these observations by employing PP2, a highly specific Src family kinase inhibitor. We demonstrate that NT/ShcA, but not NT/ShcR175Q cells, display reduced cell viability following a 24 hour incubation with PP2 (Fig. S8A), which is reflective of morphological differences between both cell types (Fig. S8B). These data suggest that NT/ShcR175Q cells are refractory to the growth inhibitory effects of Src inhibition. We examined whether Src inactivation via PP2 was sufficient to attenuate VEGF synthesis in these cells. We demonstrate a dramatic reduction in VEGF levels from PP2-treated NT/ShcR175Q cells both at 12 and 24 hours following inhibitor treatment compared to controls (Fig. 5C). We were unable to ascertain VEGF levels in PP2-treated NT/ShcA cells at the 24 hour time point due to the cytostatic effects of the...
inhibitor on these cells (Fig. S8B, C). The amount of VEGF secreted by NT/ShcA cells at the 12 hour time point was below the detection limit of the assay.

We next assessed the consequence of impaired Src activity on phosphorylation of the PDGFR, FGFR and Met receptor tyrosine kinases. We demonstrate that treatment of both NT/ShcA and NT/ShcR175Q cells with PP2 for either 12 or 24 hours ablated Src activation, as assessed by reduced phosphorylation of the Y416 autophosphorylation site (Fig. 5D). Despite the reduced Src activity in NT/ShcA cells, PDGFR, FGFR and Met phosphorylation levels were comparable to DMSO-treated controls (Fig. 5D). In contrast, we show a robust diminishment of PDGFR and Met phosphorylation in PP2-treated NT/ShcR175Q cells as early as 12 hours, which was completely attenuated following 24 hours of PP2 treatment (Fig. 5D). FGFR phosphorylation levels were also greatly diminished following 24 hours of PP2 treatment (Fig. S8D). Taken together, these data suggest that elevated integrin engagement leads to lateral Src-dependent activation of pro-angiogenic receptor tyrosine kinases, which stimulates VEGF production in breast cancer cells expressing the ShcR175Q mutant.

The acquisition of a ShcR175Q signature is associated with increased microvessel density in primary human breast cancers. We next performed gene expression profiling to generate a ShcR175Q signature, representing the top 100 most differentially expressed genes between NT/ShcA and NT/ShcR175Q cells (Fig. S9A, Table S2). We confirmed that four of the most top differentially expressed genes are regulated by ShcA signaling in ErbB2-driven breast cancer cells. Relative to NT/ShcA cells, their expression levels are significantly increased in NT/ShcR175Q cells but strongly repressed in NT/Shc3F cells, a loss of function ShcA mutant which exhibits
reduced tumor growth and angiogenesis (4) (Fig. S9B). We used this signature to stratify 3100 primary breast cancers from six publically available data sets. We demonstrate that acquisition of the ShcR175Q signature is strongly associated with enhanced breast tumor CD31 positivity (Fig. 6). We further show a robust correlation between the attainment of the ShcR175Q signature and elevated PDGFRα and PDGFRβ mRNA expression (Fig. 6). In contrast, the ShcR175Q signature cannot segregate tumors based on FGFR1, FGFR2 or Met mRNA expression levels (Fig. S10). These results demonstrate the attenuated PTB-driven ShcA signaling regulates a transcriptional program that is strongly associated with enhanced PDGFR signaling and increased microvessel density in primary breast cancers.
Discussion

We demonstrate a novel role for the ShcA PTB domain in modulating tyrosine kinase signaling to promote breast cancer initiation but paradoxically impair the growth of established tumors. We have mapped this function to the phospho-tyrosine binding properties of the ShcA PTB domain as the ShcR175Q mutant used herein lies within the phospho-tyrosine binding pocket but retains the ability to bind to phospholipids, the latter of which requires R112, K116 and K139 residues with this domain (32). Moreover, we demonstrate that ShcA/Grb2 binding is unaffected by the PTB mutant (Fig. 2).

Previous studies have demonstrated that ShcA signaling is critical for breast cancer initiation, either by deletion of the ShcA binding site on transforming oncogenes such as MT or via deletion of the ShcA gene within the mammary epithelial compartment (3-4, 21). Given that ShcA binds MT via its PTB domain (7-8), it is not surprising that germline replacement of one ShcA allele with the ShcR175Q mutant delays mammary tumor onset. This suggests that perturbation of PTB-driven ShcA signaling impairs breast cancer initiation downstream of oncogenes that recruit ShcA via this domain. However, the observation that expression of a dominant negative ShcR175Q mutant enhances the growth of MT- and ErbB2-driven mammary tumors suggests that the strength of PTB-driven ShcA signaling is dynamically, and perhaps spatially, controlled in cancer cells to regulate activation of downstream pro-tumorigenic responses. Indeed, enhanced mammary tumor growth in ShcR175Q expressing cells is associated with a robust increase in tumor angiogenesis. Despite this fact, we observe a significant reduction in intra-tumoral macrophage recruitment in both MT/ShcR175Q and NT/ShcR175Q mammary tumors. This suggests that ShcR175Q-expressing tumor...
cells are less dependent on macrophages to undergo the angiogenic switch, in contrast to what is observed in parental MMTV/MT transgenic animals (22). Instead, we demonstrate that expression of a dominant negative PTB mutant increases tumor angiogenesis by stimulating VEGF production from the breast cancer cells themselves.

Relative to breast cancer cells only expressing wild-type ShcA (MT/ShcA+/+, NT/ShcA), we demonstrate enhanced tumor growth and angiogenesis in breast cancer cells expressing both wild-type ShcA and the ShcA PTB mutant (MT/ShcA+/R175Q, NT/ShcR175Q). Moreover, the ShcR175Q-dependent increase in tumor growth and angiogenesis is observed in breast cancer cells that were transformed by oncogenes (MT, ErbB2) that bind the PTB domain of ShcA. Therefore, the wild-type ShcA allele is available to signal downstream of the transforming oncogene. While the ShcA PTB mutant no longer binds MT or ErbB2, it is fully competent to participate in SH2-driven interactions. Indeed, the majority of cytoplasmic tyrosine kinases, including Src, bind the SH2 domain of ShcA (33). Therefore, we suggest a novel role for the ShcA PTB domain in attenuating SH2-driven, phospho-tyrosine-dependent ShcA signaling from intracellular pools, other than the transforming oncogene, through its ability to recruit one or more growth inhibitory molecules. Thus, overexpression of the ShcR175Q PTB mutant would create a ShcA pool that is refractory to these inhibitory proteins, thereby potentiating SH2-driven ShcA signaling from complexes other than the transforming oncogene (Figure 7). We propose a unique mechanism whereby ShcA utilizes its PTB domain as a biological sensor to integrate the total amount of tyrosine kinase signaling in breast cancer cells.
Members of the ShcA PTB domain interactome include RTKs such as insulin receptor, IGF1R, TrkA, TrkB, Ret and all members of the EGFR family (6). The ShcA PTB domain also participates in phospho-tyrosine independent interactions with proteins such as the cytoskeletal regulator IQ-GAP1 (34). However, the ShcA PTB domain also binds phospho-tyrosine containing proteins that may attenuate RTK signaling. For example, ErbB2 and the protein tyrosine phosphatase PTPε compete for binding to the PTB domain of ShcA. In this manner, PTB-mediated ShcA recruitment to ErbB2 protects ShcA from PTPε-mediated dephosphorylation and attenuation of signaling. However, PTPε does not contain an NPXY motif and mutations in the ShcA PTB domain that ablate phospho-tyrosine binding do not impair PTPε recruitment (35). Given that the R175Q mutant employed herein specifically ablates phospho-tyrosine binding to the PTB domain (32), it is unlikely that PTPε contributes to the observed increase in mammary tumor outgrowth and angiogenesis in ShcR175Q-expressing tumors. Further mechanistic studies will define the growth inhibitory molecule(s) that attenuate PTB-driven breast tumor growth and angiogenesis.

We show that breast cancer cells expressing the ShcR175Q mutant display increased Src activation. This is demonstrated by increased phosphorylation of the Src autophosphorylation site (Y416) and Src-dependent phosphorylation sites on FAK (Y576/577) in ShcR175Q-expressing cells. We also observe increased expression of fibronectin and both integrin subunits that comprise a high affinity fibronectin receptor (α5/β1) in ShcR175Q expressing cells. The observation that ShcR175Q expressing cells display enhanced adhesion to fibronectin and increased Src phosphorylation suggests that the ShcA PTB mutant establishes an autocrine loop to constitutively
activate integrin signaling. We further show that enhanced Src activity potentiates phosphorylation of several RTKs, including PDGFR, Met and FGFR in ShcR175Q-expressing breast cancer cells. Indeed, fibronectin induces β1 integrin and Src-dependent FGFR1 phosphorylation in endothelial cells (36). Fibronectin also induces HGF-independent activation of Met in ovarian cancer cells via the α5/β1 receptor (37). Finally, type I collagen increases PDGFRβ phosphorylation in smooth muscle cells in a Src and α2/β1-driven manner (38). Thus, these RTKs are sensitive to lateral signaling via integrin-mediated Src activation.

Studies have established cross-talk between ShcA, Src and integrin signaling. The ShcA PTB domain binds directly to β3 and β4 integrin subunits (39-40). In contrast, a subset of β1-containing integrins indirectly binds ShcA via the α-integrin subunit through a tri-molecular Caveolin-1, Shc- and Fyn- containing complex (41). In this instance, a poly-proline rich stretch in ShcA constitutively binds the SH3 domain of Fyn. ShcA also directly interacts with Src. Some studies suggest that the ShcA/Src complex is constitutive (42) while others show that Src binds inducibly to the SH2 domain of ShcA (33). Moreover, ShcA recruitment increases Src activity (42) and the Y239/Y240 residues within the CH1 domain of ShcA are Src phosphorylation sites (43). Thus ShcA and Src complexes potentiate signal transduction in a reciprocal manner. Given that the ShcR175Q mutant only impairs PTB-dependent phospho-tyrosine binding, it retains the ability to bind Src. This raises the intriguing possibility that the ShcR175Q mutant engages signaling pathways to activate Src, both directly and indirectly by increasing integrin signaling.
We demonstrate that the ShcR175Q mutant potentiates tumor angiogenesis and that increased VEGF production by ShcR175Q-expressing cells is Src dependent. Given that β1 integrin-containing complexes activate Src, it is interesting that loss of β1 expression within the mammary epithelial compartment attenuates breast tumor angiogenesis in mouse models (28). Moreover, ShcR175Q-expressing breast cancer cells display increased PDGFR, FGFR and Met phosphorylation in a Src-dependent manner. Overexpression of the ShcR175Q PTB mutant creates an intracellular ShcA pool that retains the ability to activate ShcA through the SH2 domain. Interestingly, Met, Src and Fak promote mammary tumorigenesis, induce tumor angiogenesis (44-46) and bind the ShcA SH2 domain (47-49). This reinforces our model that both PTB-driven (via endogenous ShcA) and SH2-driven (via ShcR175Q) ShcA signaling is elevated in breast cancer cells expressing this dominant negative ShcA mutant, which enhances tumor growth and angiogenesis (Figure 7).

There is no evidence for point mutations in ShcA. However, we demonstrate that a ShcR175Q gene signature is strongly associated with increased microvessel density and PDGFR overexpression. Therefore, we have identified a clinically relevant gene signature that is predictive of neo-vascularization. We suggest that primary breast cancers can acquire such an ShcR175Q signature, either by overexpressing receptor tyrosine kinases that engage the ShcA SH2 domain or via loss of an unidentified inhibitory molecule that attenuates PTB-driven ShcA signaling from intracellular ShcA pools that are not engaged to RTKs.
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Figure Legends

Figure 1: Attenuated PTB-driven ShcA signalling delays mammary tumor onset but accelerates tumor outgrowth in MMTV/MT transgenic mice. (A) FLAG immunoprecipitates from MT cell lines expressing ShcA and ShcR175Q were immunoblotted using ShcA and MT specific antibodies. (B) Kaplan Meier curve demonstrating the percentage of tumor free mice over time for the indicated genotypes is shown (n=20 each). T_{50} is the time at which 50% of the mice harbor a first palpable mammary tumor. (C) (Upper graph) The total number of tumor bearing mammary glands were quantified at 4-5 weeks and 7-8 weeks post first palpation for MT/ShcA^{+/+} or MT/ShcA^{+/R175Q} mice. (Lower graph) The rate of mammary tumor outgrowth was assessed by quantifying the average tumor volume (mm^3) either at 4-5 weeks or 7-8 weeks post palpation via caliper measurements. (D) IHC staining of MT/ShcA^{+/+} or MT/ShcA^{+/R175Q} mammary tumors at 4-5 (n=6) and 7-8 (n=10) weeks post palpation using Ki67-, CD31- and F4/80-specific antibodies. The degree of apoptosis was also evaluated via a TUNEL staining. For each tumor section, a minimum of 30,000 and 100,000 nuclei were counted for the 4-5 week and 7-8 week time points, respectively. The data is presented as the percentage of Ki67, TUNEL or F4/80 positive cells or as the percentage of CD31 positive pixels ±SEM. Representative images of IHC-stained sections of mammary tumors harvested at 7-8 weeks post palpation. All statistical analyses were performed using a two-tailed student’s t test.

Figure 2: The ShcA R175Q PTB mutant confers tumorigenic and angiogenic properties within breast cancer cells. (A) NeuNT-transformed NMuMG cells (NT2197) along with
populations stably expressing FLAG-tagged wild-type ShcA or the ShcR175Q mutant were established. Immunoblot analysis of the indicated cell lysates using FLAG- and ShcA-specific antibodies. The shift in molecular weight observed with the ShcA immunoblot reflects the presence of the FLAG epitope. Extracts were immunoprecipitated with an ErbB2-specific antibody followed by immunoblotting with ErbB2 or FLAG-specific antibodies. (B) FLAG immunoprecipitates from NT/ShcA and NT/ShcR175Q lysates were immunoblotted using ShcA and Grb2 specific antibodies. Control IPs using beads alone are shown. (C) Breast cancer cells ($5 \times 10^4$) were injected into the fourth mammary fat pad of nude mice (n=7). Tumor growth was monitored by bi-weekly caliper measurements. The data are represented as tumor volumes (mm$^3$) ± SD. (D) (Upper graph) NT/ShcA and NT/ShcR175Q breast tumor tissue (n=7 each) was harvested early during tumor outgrowth (average volume: 350mm$^3$) or at the experimental endpoint of the assay (average volume: 1500mm$^3$). Tumor sections were subjected to CD31 (middle graph) and F4/80 (lower graph) IHC staining. The data is shown as the percentage of F4/80+ cells and CD31+ pixels ± SEM. A minimum of 20,000 cells were counted per condition. (E) Representative images of CD31 and F4/80 IHC staining quantified in panel D. (F) VEGF levels secreted from NT/ShcA and NT/ShcR175Q cell lines in vitro (n=4) were quantified by ELISA (n=4). (G) VEGF levels present in NT/ShcA and NT/ShcR175Q breast tumor cell extracts harvested either early or late during their growth phase was quantified by ELISA (n=6 each). All statistical analyses were performed using a two-tailed student’s t test.
Figure 3: Enhanced activation of pro-angiogenic receptor tyrosine kinases in NT/ShcR175Q breast cancer cells. (A) Immunoblot analysis of whole cell lysates derived from NT/ShcA and NT/ShcR175Q cells using the indicated antibodies. (B) The amount of PDGF-AA, PDGF-AB and HGF secreted from NT/ShcA and NT/ShcR175Q cells was quantified by ELISA (n=6 wells each). All statistical analyses were performed using a two-tailed student’s t test.

Figure 4: The ShcA R175Q PTB mutant increases Src activity and establishes an autocrine loop involving integrin engagement. (A) Immunoblot analysis of whole cell lysates derived from NT/ShcA and NT/ShcR175Q cells using pSrc(Y416), Src and Tubulin-specific antibodies. (B) Immunoblot analysis of whole cell lysates derived from NT/ShcA and NT/ShcR175Q cells using pFAK(Y576/577), FAK, α5-integrin, β1-integrin and Tubulin-specific antibodies. (C) Relative Fibronectin mRNA levels in NT/ShcA and NT/ShcR175Q cell lines normalized to GAPDH (n=4). (D) Fibronectin protein levels secreted from NT/ShcA and NT/ShcR175Q cell lines in vitro as determined by ELISA (n=4). (E) The rate of adhesion of NT/ShcA and NT/ShcR175Q cells onto Fibronectin coated plates over a two hour period is shown. The data is representative of six wells and triplicate experiments. All statistical analysis was performed using a two-tailed student’s t test.

Figure 5: The ShcA R175Q PTB mutant promotes VEGF production in a Src-dependent manner. (A) VEGF ELISA on conditioned media from NT/ShcA or NT/ShcR175Q cells transfected with scrambled or Src-specific siRNAs (n=8 wells
each). (B) VEGF ELISA on conditioned media from NT/ShcA and NT/ShcR175Q cells were treated with DMSO alone or 20μM PP2 for 12 or 24 hours (n=6 wells each). (C, D) Whole cell extracts were generated from the cells described in panels A and B and were subjected to immunoblot analysis with the indicated antibodies. All statistical analysis was performed using a two-tailed student’s t test.

**Figure 6:** A 100 gene ShcR175Q signature stratifies patients with high CD31 positivity. Primary breast tumors from six publically available datasets, comprising a total of 3102 patients, were first stratified based on 100 genes that are differentially expressed between NT/ShcA and NT/ShcR175Q breast cancer cells. Following this stratification, relative CD31, PDGFRα and PDGFRβ mRNA levels are shown for each patient. The red line indicates the slope for each graph.

**Figure 7:** The ShcA PTB domain is essential for breast tumor initiation but also negatively regulates growth of emerging mammary tumors. The ShcA PTB domain binds to the majority of activated receptor tyrosine kinases in breast cancer cells, including ErbB2, which stimulates the transduction of pro-tumorigenic signals that promote breast cancer initiation, growth and angiogenesis. Given that ShcA can also bind phospho-tyrosine residues via the SH2 domain, we propose that the PTB domain also serves to limit SH2-driven oncogenic signaling from intracellular pools, other than the transforming oncogene. Therefore, expression on a PTB mutant that is no longer able to bind phospho-tyrosine (R175Q) would increase SH2-driven ShcA signal transduction to further amplify tumor growth and angiogenesis. Expression of a
ShcR175Q mutant potentiates integrin signaling leading to increased Src activation. Src, in turn, phosphorylates tyrosine residues on key receptor tyrosine kinases, including PDGFR, Met and FGFR, which have been shown to bind the SH2 domain of ShcA. This permits recruit of the ShcR175Q mutant to cytoplasmic (Src) and receptor tyrosine kinases (PDGFR, Met, FGFR) via the SH2 domain, leading to increased ShcA-mediated signal transduction.
References


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Ahn et al., Figure 1
**A**

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**C**

- NT/ShcA
- NT/ShcR175Q

Relative Fibronectin mRNA Levels

- P = 0.029

**D**

- NT/ShcA
- NT/ShcR175Q

Fibronectin Levels (ng/ml)

- P < 0.01

**E**

- NT/ShcA
- NT/ShcR175Q

Rate of Cell Adhesion onto Fibronectin

- P = 0.038

Ahn et al., Figure 4
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The ShcA PTB Domain Functions as a Biological Sensor of Phospho-tyrosine Signaling During Breast Cancer Progression

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