The ShcA PTB Domain Functions as a Biological Sensor of Phosphotyrosine Signaling during Breast Cancer Progression

Ryuhjin Ahn1, Valerie Sabourin1, Jacqueline R. Ha1, Sean Cory2,3, Gordana Maric3, Young Kyuen Im1, W. Rod Hardy4, Hong Zhao3, Morag Park3, Michael Hallett2,3, Peter M. Siegel3, Tony Pawson4, and Josie Ursini-Siegel1

Abstract

ShcA (SHC1) is an adapter protein that possesses an SH2 and a PTB phosphotyrosine-binding motif. ShcA generally uses its PTB domain to engage activated receptor tyrosine kinases (RTK), 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 phosphotyrosine residues via its PTB domain. Here, we report that transgenic expression of this mutant delays onset of mammary tumors 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 principal fibronectin receptor. Sustained integrin engagement activated Src, which in turn phosphorylated proangiogenic RTKs, including platelet-derived growth factor receptor, fibroblast growth factor receptor, 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. Cancer Res; 73(14); 4521–32. ©2013 AACR.

Introduction

The ShcA adapter employs distinct domains and motifs to transduce phosphotyrosine-dependent signals downstream of receptor tyrosine kinases (RTK) and non-RTKs (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 neovascularization through a process called angiogenesis. Numerous studies suggest an important role for ShcA during normal and pathologic angiogenesis. In the cardiovascular system, ShcA is required for proper heart development through the angiogenic remodeling of preexisting vessels (5). Moreover, ShcA signaling downstream of RTKs, such as ErbB2, increases VEGF production and promotes an in vivo angiogenic response associated with tumor outgrowth (4).

Following RTK activation, ShcA uses 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 RTKs, including ErbB2, specifically through its PTB domain (6). Moreover, the polyoma virus middle T (MT) antigen binds the ShcA PTB domain via Y250, which resides within a consensus NPXpY motif (7, 8). Activated RTKs, or associated cytoplasmic kinases, subsequently phosphorylate 3 tyrosine residues (Y239/Y240 and Y250, which resides within a consensus NPXpY motif (7, 8). Activated RTKs, or associated cytoplasmic kinases, subsequently phosphorylate 3 tyrosine residues (Y239/Y240 and Y317) within the central collagen homology 1(CH1) domain of ShcA (9, 10). When phosphorylated, these tyrosines serve as docking sites for other PTB- and SH2-containing proteins. Transgenic and orthotopic mouse models of ErbB2- and Polyoma Virus MT antigen-driven breast cancer show that these tyrosine residues transduce critical and nonoverlapping signals to promote breast cancer progression (4). Transgenic studies in MMTV/MT mice further show an important role for the ShcA SH2 domain in promoting breast cancer cell survival by engaging a 14-3-3/phospoinositide-3 kinase (PI3K) 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 show that
the ShcA PTB domain functions as a biological sensor to regulate not only breast cancer initiation but also 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 to 4 clonal populations expressing similar levels of FLAG-tagged ShcA.

Mice

MMTV/MT transgenic mice have been described previously (12). Mice expressing a FLAG-tagged mutant ShcR175Q allele under the control of the endogenous shcA promoter have also been described previously (13) and were interbred for 10 generations onto an FVB background before their use in biogenic crosses. NT2197/ShcA and NT2197/ShcR175Q breast cancer cells (5 × 10⁵) were injected into the fourth mammary fat pad of nude mice (Taconic) and tumor growth was determined biweekly by caliper measurements (4). Tumors were fixed in 10% buffered formalin, embedded in Optimal Cutting Temperature medium (OCT), or frozen in liquid nitrogen. For the Matrigel plug assays, 1 × 10⁵ 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 3 dicer-substrate–duplex siRNAs targeting mouse Src (Ori-gene-SR169299) or with a universal scrambled control (Ori-gene-SR30004) using INTERFERin transfection reagents (Cat# 888888, Polyplus). siRNAs were used at a final concentration of 60 nmol/L per transfection (20 nmol/L for each individual ShcA siRNA). Cells were serially transfected a total of 3 times at 24, 48, and 72 hours post plating. Four hours after the third siRNA). Cells were transferred to low serum (0.5%) at 70% confluency and conditioned media and lysates were harvested 20 hours posttransfection, prior to which the cells were imaged on an OlympusInfinity 1 at ×4 magnification using Infinity Capture software (Lemenera). Cell viability was determined by Trypan blue exclusion.

ELISA

ELISAs were conducted on the conditioned media using commercially available kits directed against mouse PDGF-A, PDGF-AB, PDGF-BB, and 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. The 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

Ki-67 staining was carried out on paraffin-embedded sections as described previously (1:1,000; AB15580, Abcam; ref. 4). Paraffin-embedded sections were also subjected to TUNEL staining (Apoptag Detection Kit, Chemicon) according to the manufacturer’s instructions. CD31 (1:250; 550274, BD Biosciences) and F4/80 (1:250; MF48004-3, Invitrogen) staining was carried out 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 (×20 magnification) were quantified for each section.

Immunoprecipitation/immunoblotting

Whole-cell lysates were generated from cell lines or flash frozen tissue samples as previously described (4). Lysates were separated by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes and probed with the antibodies listed in Supplementary Table S1. Densitometric analysis was conducted using Image J software. Lysates were immunoprecipitated with FLAG-specific antibodies (1:100; Cat# F1804, Sigma Aldrich). For the MT coimmunoprecipitation 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 (Imperial College, London, United Kingdom).

Quantitative real-time reverse transcription PCR

Total RNA was isolated from cell lines using RNasey Midi Kits (Qiagen) according to manufacturer’s instruction, and cDNA was generated using Superscript Reverse Transcriptase II (Invitrogen). Mouse fibronectin mRNA levels were determined quantitatively using the Quantitect SYBR Green RT-PCR kit (Qiagen) and the following primers: Forward: AGCAAATGACCATTGAAGG and Reverse: TGTCGGGAGAAGGTGATT. Mouse GPMBM (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# 432042, 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). Before 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. Thereafter, 80,000 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 instructions using the xCELLigence software. The graph represents data from 3 independent experiments conducted 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 (4 × 44 K) were carried out as described previously (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 < 2.6 × 10−6). Expression of this signature was examined in 6 breast cancer datasets encompassing 3,100 breast cancer patients (15–20). In each dataset, the patients were ordered on the basis of 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 conducted using a 2-tailed, 2-sample equal variance Student t test.

Results

Attenuated PTB-driven ShcA signaling leads to delayed breast tumor onset but enhanced outgrowth

We previously showed 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 (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 phosphotyrosine residues (13). We were unable to generate ShcA−/−/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 show 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 4 weeks after 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), reinforcing the idea that tumor initiation is delayed in MT/ShcR175Q animals (Fig. 1C, top). Strikingly, the average tumor volume is significantly enhanced in MT/ShcR175Q mice at the experimental endpoint, suggesting that attenuated PTB-driven ShcA signaling paradoxically increases mammary tumor growth (Fig. 1C, bottom). We next characterized MT and MT/ShcR175Q mammary tumors with respect to the engagement of signaling pathways known to be downstream of ShcA. We show that the extracellular signal–regulated kinase (ERK), p38 mitogen-activated protein kinase (p38MAPK), e-jun-NH2-kinase (JNK), and AKT signaling pathways were similarly active in both MT and MT/ShcR175Q mammary tumors (Supplementary Fig. S1).

To better understand the mechanisms that enhance tumor growth in MT/ShcR175Q animals, we conducted immunohistochemical (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). However, increased tumor volume correlates with a statistically significant reduction in apoptosis and enhanced neovascularization in MT/ShcR175Q tumors compared with MT control tumors (Fig. 1D, middle panels). Moreover, end-stage MT/ShcR175Q mammary tumors express moderately elevated VEGF levels relative to MT control tumors (Supplementary 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- to -8-week timepoint is not reflective of enhanced macrophage recruitment. Moreover, early-stage MT/ShcR175Q mammary tumors display a statistically significant decrease in macrophage recruitment compared with age-matched MT controls (Fig. 1D, bottom). 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.

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 signaling 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 selected Neu/ErbB2-as the
transforming oncogene, given that it recruits ShcA via its PTB domain (23) and is overexpressed in 20% to 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 phosphotyrosine residues within the CH1 domain of ShcA (9, 10), these data show the specificity of the R175Q mutation within the phosphotyrosine-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 (Supplementary Fig. S3A). We further analyzed NT/ShcA and NT/ShcR175Q mammary tumors at an early stage (<500 mm³) and at the experimental endpoint (1,500 mm³) for their degree of proliferation, survival, and endothelial cell recruitment as assessed by Ki-67, TUNEL, and CD31 IHC staining, respectively (Fig. 2D, top). The degree of tumor cell proliferation and survival is unaltered at either timepoint between
NT/ShcA and NT/ShcR175Q tumors (Supplementary Fig. S3B and 3C). However, we observe a statistically significant increase in microvessel density in NT/ShcR175Q tumors during the early stages of their outgrowth, which normalize at endstage (Fig. 2D, middle; 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, bottom; 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 neovascularization.

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 shown that ShcA signaling promotes tumor angiogenesis by regulating VEGF production (4). We show 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 coincides with the timepoint 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 (Supplementary 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 RTK 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 proangiogenic. In this regard, platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), and Met are 3 RTKs that engage signaling pathways that promote tumor angiogenesis (25, 26). Interestingly, we show that PDGFR, FGFR, and Met signaling is elevated in NT/ShcR175Q breast cancer cells compared with NT/ShcA controls (Fig. 3A). We further show 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 PDGFR, MET, and FGFR phosphorylation in late-stage MT/ShcA and MT/ShcR175Q tumors (Supplementary Fig. S5) and in early- and late-stage NT/ShcA and NT/ShcR175Q tumors (Supplementary 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 RTKs, 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 robust increase in the phosphorylation of Src-dependent phosphorylation sites within focal adhesion kinase (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 show 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 and D). Published studies have shown that 2 fibronectin alternatively spliced variants (EDA and EDB) contribute to tumor neovascularization 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 stromal cell types, including the tumor endothelium (29).

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 cell adhesion onto fibronectin in NT/ShcA and NT/ShcR175Q cells. Relative to scrambled control (Fig. 4E). 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 showed a dramatic reduction in VEGF levels from PP2-treated NT/ShcR175Q cells, both at 12 and 24 hours following inhibitor treatment compared with 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 (Supplementary Fig. S8B and S8C). The amount of VEGF secreted by NT/ShcA cells at the 12-hour timepoint was below the detection threshold of the assay.

We next assessed the consequence of impaired Src activity on phosphorylation of the PDGFR, FGFR, and Met RTKs. We showed 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 showed 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 after 24 hours of PP2 treatment (Supplementary Fig. S8D). Taken together, these data suggest that elevated integrin engagement leads to lateral Src-dependent activation of proangiogenic RTKs, which stimulates VEGF production in breast cancer cells expressing the ShcR175Q mutant.
Acquisition of a ShcR175Q signature is associated with increased microvessel density in primary human breast cancers

We next conducted gene expression profiling to generate a ShcR175Q signature, representing the top 100 most differentially expressed genes between NT/ShcA and NT/ShcR175Q cells (Supplementary Fig. S9A and Supplementary Table S2). We confirmed that 4 of the top most 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 that exhibits reduced tumor growth and angiogenesis (Supplementary Fig. S9B; ref. 4). We used this signature to stratify 3,100 primary breast cancers from 6 publicly available datasets. We show 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 on the basis of FGFR1, FGFR2, or Met mRNA expression levels (Supplementary Fig. S10). These results show 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 show 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 phosphotyrosine-binding properties of the ShcA PTB domain as the ShcR175Q mutant used here lies within the phosphotyrosine-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 show that ShcA/Grb2 binding is unaffected by the PTB mutant (Fig. 2). Previous studies have shown 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 1 ShcA allele with the SheR175Q 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 ShcA R175Q 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 protumorigenic 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 intratumoral 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 show 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+/+ and NT/ShcA), we show enhanced tumor growth and angiogenesis in breast cancer cells expressing both wild-type ShcA and the ShcA PTB mutant (MT/ShcA+/R175Q and NT/ShcR175Q). Moreover, the ShcR175Q-dependent increase in tumor growth and angiogenesis is observed in breast cancer cells that were transformed by oncogenes (MT and 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, phosphotyrosine-dependent ShcA signaling from intracellular pools, other than the transforming oncogene, through its ability to recruit 1 or more growth-inhibitory proteins. 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 (Fig. 7). We propose a unique mechanism whereby ShcA uses 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 phosphotyrosine-independent interactions with proteins such as the cytoskeletal regulator IQ-GAP1 (34). However, the ShcA PTB domain also binds phosphotyrosine-containing proteins that may attenuate RTK signaling. For example, ErbB2 and the protein tyrosine phosphatase PTPe compete for binding to the PTB domain of ShcA. In this manner, PTPe-mediated ShcA recruitment to ErbB2 protects ShcA from PTPe-mediated dephosphorylation and attenuation of signaling. However, PTPe does not contain an NPXY motif and mutations in the ShcA PTB domain that ablate phosphotyrosine binding do not impair PTPe recruitment (35). Given that the R175Q mutant employed herein specifically ablates phosphotyrosine binding to the PTB domain (32), it is unlikely that PTPe 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 shown by increased phosphorylation of the Src autophosphorylation site (Y416) and Src-dependent phosphorylation sites on FAK (Y576/577) in ShcR175Q-expressing cells. In addition, we 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 ShcR175Q 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-t integrin subunit through a trimolecular 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) whereas 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 phosphotyrosine 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 show 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 B1 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 ShcA signaling from intracellular ShcA pools that are not engaged to RTKs.

There is no evidence for point mutations in ShcA. However, we show 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 neovascularization. We suggest that primary breast cancers can acquire such an ShcR175Q signature, either by overexpressing RTKs 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.

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

References

1. The ShcA PTB Domain Regulates Mammary Tumorigenesis

Authors’ Contributions
Conception and design: J. Ursini-Siegel
Development of methodology: V. Sabourin, M. Hallett, J. Ursini-Siegel
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Ahn, V. Sabourin, J.H. Ha, G. Maric, Y. Im, H. Zhao, M. Park, P.M. Siegel
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): R. Ahn, V. Sabourin, J.H. Ha, S. Cory, Y. Im, M. Park, P.M. Siegel, J. Ursini-Siegel
Writing, review, and/or revision of the manuscript: R. Ahn, M. Park, P.M. Siegel, T. Pawson, J. Ursini-Siegel
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Ahn, V. Sabourin, W.R. Hardy, H. Zhao, M. Park
Study supervision: V. Sabourin, J. Ursini-Siegel

Acknowledgments
The authors thank Dr. Antonis Koromilas for critical review of the article.

The authors also thank Dr. Stephen Dilworth for providing the MT-specific antibody.

Grant Support
This work was financially supported by a CIHR grant (MOP-111413) and a Genome Quebec grant to J.U.-S and operating grants from the CSSRI (2011-700790) and AICR (11-0204) to P.M. Siegel and CICR MOP-1346 and MOP-6849, and Terry Fox Foundation/CIHR TFRI 105268 to T. Pawson. We further acknowledge infrastructure support and technical assistance from the Breast Cancer Functional Genomics Group, which is partially supported by funds from the Terry Fox New Frontiers Program (M. Park). J. Ursini-Siegel is the recipient of a CIHR New Investigator Salary Support award. Y.K. Im is the recipient of a TD/LDI studentship and J.R. Ha acknowledges a studentship from the McGill Integrated Program Training Program (MICRTP). G. Maric holds a CIHR doctoral studentship. P.M. Siegel acknowledges salary support from the FRSQ (Junior 2).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
The ShcA PTB Domain Functions as a Biological Sensor of Phosphotyrosine Signaling during Breast Cancer Progression

Ryuhjin Ahn, Valerie Sabourin, Jacqueline R. Ha, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-12-4178

Cited articles
This article cites 49 articles, 26 of which you can access for free at:
http://cancerres.aacrjournals.org/content/73/14/4521.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/73/14/4521.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.