ShcA signalling is essential for tumour progression in mouse models of human breast cancer

Josie Ursini-Siegel¹, W Rod Hardy²,
Dongmei Zuo¹, Sonya HL Lam¹, Virginie
Sanguin-Gendreau¹, Robert D Cardiff³,
Tony Pawson² and William J Muller^{1,*}

¹Departments of Medicine and Biochemistry, McGill University, McGill University Health Center, Montreal, Quebec, Canada, ²Samuel Lunenfeld Research Institute, Mount Sinai Hospital, University of Toronto, Toronto, Ontario, Canada and ³Center for Comparative Medicine, University of California Davis, Davis, CA, USA

To explore the *in vivo* significance of ShcA during mammary tumorigenesis, we used mice expressing several phosphotyrosine-deficient ShcA alleles under the control of their endogenous promoter. We show that all three ShcA tyrosine phosphorylation sites are involved in the early stages of mammary tumour progression, including loss of the myoepithelial cell layer surrounding hyperplasias and during progression to carcinoma. We have determined that signals emanating from Y13 are important for tumour cell survival, whereas Y239/240 transduce signals promoting tumour vascularization. We further demonstrate that loss of ShcA expression in mammary epithelial cells abrogates tumour development. This study is the first to directly demonstrate that signalling downstream from the ShcA adaptor protein is critical for breast cancer development.

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Introduction

The mammalian *ShcA* gene (also termed *Shc1*) encodes three proteins (p46, p52 and p66) (Pelicci *et al*, 1992; Migliaccio *et al*, 1997) that contain tyrosine phosphorylation sites at residues 239/240 and 317 (analogous to residue 313 in mice) (Ravichandran, 2001). While deletion of the *ShcA* gene is embryonic lethal (Lai and Pawson, 2000), germline deletion of *p66-ShcA* results in increased longevity and resistance to apoptosis following tissue oxidative stress (Migliaccio *et al*, 1999). It has recently been demonstrated that mid-gestational heart development requires the ShcA phosphotyrosine-binding (PTB) domain but not the CH1 phosphotyrosine residues, while ShcA must retain its PTB and SH2 domains, along with

its three tyrosine phosphorylation sites, to regulate muscle spindle formation (Hardy *et al*, 2007). This demonstrates that ShcA differentially utilizes its interaction domains and motifs to contribute to the development of distinct tissues.

Clinical studies have suggested an important role for engagement of ShcA signalling in breast cancer development. Increased ShcA tyrosine phosphorylation, combined with reduced p66ShcA levels, is a strong predictor for nodal status, disease stage and relapse in breast cancer patients (Daval *et al*, 2003; Frackelton *et al*, 2006). Furthermore, several transgenic mouse models have suggested an important role for ShcA during mammary tumour progression (Webster *et al*, 1998; Dankort *et al*, 2001b).

An excellent model system to study receptor tyrosine kinase-dependent mammary tumorigenesis is the Polyomavirus middle T antigen (MT). Mammary-specific expression of MT results in the rapid induction of multi-focal, metastatic mammary tumours (Guy *et al*, 1992), which recapitulates numerous stages of human breast cancer progression (Lin *et al*, 2003). Mammary-specific expression of a mutant MT (MT-Y250F), which no longer binds ShcA, significantly delays mammary tumour onset and reduces tumour burden relative to parental MT mice (Webster *et al*, 1998). Strikingly, subsets of primary mammary tumours and lung metastases in MT-Y250F mice have, through somatic mutation, restored a functional ShcA-binding site (Webster *et al*, 1998).

A clinically relevant oncogene in breast cancer is the Neu/ErbB2 receptor tyrosine kinase. Transgenic mouse models have clearly established that ErbB2 overexpression is sufficient to transform the mammary epithelium (Ursini-Siegel *et al*, 2007b). ShcA has been suggested to contribute to ErbB2-mediated mammary tumorigenesis, as reconstitution of a ShcA-binding site to a mutant receptor, lacking its major tyrosine phosphorylation residues, is sufficient to restore the kinetics of mammary tumour development to levels observed with wild-type ErbB2 (Siegel *et al*, 1999; Dankort *et al*, 2001a,b).

While these observations suggest that recruitment of ShcA to MT and ErbB2 is critical for their ability to transform the mammary gland, the precise molecular mechanism by which ShcA exerts these effects remains poorly understood. Here we demonstrate that retention of ShcA expression and phosphotyrosine-dependent signalling is critical for mammary tumour progression. Our data also suggest that distinct ShcA tyrosine phosphorylation sites provide critical angiogenic and survival signals during mammary tumour outgrowth.

Results

MT-induced mammary tumorigenesis requires tyrosine phosphorylation-dependent ShcA-signalling pathways

To directly establish a role for ShcA signalling in mammary tumour progression, we interbred MMTV/MT transgenic

*Corresponding author. Departments of Medicine and Biochemistry, McGill University, McGill University Health Center, 687 Pine Avenue West, Room H5-21, Montreal, CDN-Montreal, Quebec, Canada H3A 1A1. Tel.: +1 514 934 1934; ext. 36383; Fax: +1 514 843 1478; E-mail: william.muller@mcgill.ca

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mice (Guy *et al*, 1992) with animals expressing targeted *ShcA* 'knock-in' alleles, harbouring phenylalanine substitutions of the Y313 or Y239/240 phosphotyrosine residues (*ShcA*^{313F}, *ShcA*^{2F} or *ShcA*^{3F}), under the control of the endogenous *ShcA* promoter (Hardy *et al*, 2007). While we were able to derive cohorts of MMTV/MT mice that were homozygous for the *ShcA*^{313F} and *ShcA*^{2F} alleles, we only generated *ShcA*^{3F} heterozygotes, as the majority of *ShcA*^{3F/3F} mice die perinatally (Hardy *et al*, 2007). The strength of these mouse models relies on the fact that the *ShcA* mutants are expressed at physiologically relevant levels, and that all cell types in *ShcA*^{313F/313F} and *ShcA*^{2F/2F} animals lack wild-type *ShcA*. Moreover, *ShcA*^{+/3F}, *ShcA*^{313F/313F} and *ShcA*^{2F/2F} female mice undergo normal mammary gland development, precluding the possibility that any observed effects on transformation result from reduced epithelial content (data not shown).

We first examined mammary tumour onset between the different cohorts of animals. While the kinetics of tumour onset was modestly delayed in *MT/ShcA*^{+/313F} and *MT/ShcA*^{+/2F} mice, tumour latency was more strongly increased in *MT/ShcA*^{+/3F} animals (Figure 1A; Table I). Furthermore, MMTV/MT mice that were homozygous for the *ShcA*^{313F} or *ShcA*^{2F} alleles only developed mammary tumours after a long latency period (132 days; *MT/ShcA*^{313F/313F} and 155 days; *MT/ShcA*^{2F/2F} versus 66 days; *MT/ShcA*^{+/+}) (Figure 1A; Table I).

We next compared the incidence of lung metastasis between wild-type MT mice and those expressing the *ShcA* tyrosine-deficient mutants. While the majority of MT mice developed lung metastases, expression of a single *ShcA*^{2F}, *ShcA*^{313F} or *ShcA*^{3F} allele resulted in a 1.5- to 2-fold decrease in the percentage of animals with lung lesions (Table I). The metastatic potential of mammary tumours in *MT/ShcA*^{313F/313F} (threefold reduction) and *MT/ShcA*^{2F/2F} (fourfold reduction) bigenic animals was even further attenuated (Table I). These results demonstrate that the three *ShcA* tyrosine phosphorylation sites play important and non-redundant roles during MT-induced mammary tumour development and metastatic spread.

We also examined the pathological profiles of mammary tumours derived from each of the crosses. MT mammary tumours are divided equally into papillary and glandular subtypes, with a very small percentage demonstrating histological features of adenosquamous carcinoma (Figure 1B). Although these subtypes were present in *MT/ShcA*^{+/3F}, *MT/ShcA*^{313F/313F} and *MT/ShcA*^{2F/2F} tumours, a significant proportion of the mammary tumours displayed a solid/nodular phenotype, which is atypical for MMTV/MT tumours but reminiscent of the pathological profile observed with MMTV/Neu mouse models (Cardiff *et al*, 2000). In particular, over 60% of *MT/ShcA*^{2F/2F}-induced mammary

Figure 1 MT-induced mammary tumorigenesis is attenuated by loss of phosphotyrosine-dependent *ShcA* signalling. (A) Percentage of tumour free mice over time for the indicated genotypes. *N* represents the number of animals analysed. (B) (Upper panel) Percentage of tumours categorized into the following histological tumour types: papillary, solid/nodular, adenosquamous or glandular. For the *MT/ShcA*^{+/+} genotype, 85 tumours were analysed, while nine individual tumours were examined for the *MT/ShcA*^{+/3F}, *MT/ShcA*^{313F/313F} and *MT/ShcA*^{2F/2F} genotypes. (Lower panel) Images depicting the tumour phenotypes: the arrow indicates laminar keratin. (C) Immunoblot analysis on tumour cell lysates from the indicated genotypes using MT-, Shc-, ErbB2-, ErbB3- and E-cadherin-specific antibodies. The numbers indicate individual mice.

tumours exhibited this nodular phenotype (Figure 1B). These differences in tumour latency and histopathology are attributed neither to consistently altered MT or ShcA levels, nor to the extent of ErbB2 or ErbB3 overexpression within the mammary tumours. However, minor variations in the expression levels of MT, ShcA, ErbB2 and ErbB3 can be observed within individual tumours (Figure 1C). These observations suggest that the three phosphotyrosine residues within the CH1 domain of ShcA contribute to the development of metastatic mammary tumours with distinct histopathological features.

Loss of ShcA phosphorylation sites results in the induction of mammary epithelial hyperplasias that retain an intact myoepithelial lining

The delay in mammary tumour induction within the various ShcA-mutant backgrounds indicates that the ShcA tyrosine phosphorylation sites contribute to mammary tumour progression. Previous studies have demonstrated that the

MMTV/MT model proceeds through well-defined stages of tumour progression proceeding from early mammary epithelial hyperplasia to non-invasive adenoma and finally invasive carcinoma (Lin *et al*, 2003).

To directly assess whether disruption of ShcA signalling impacts the early stages of MT-induced mammary tumour development, we performed whole-mount analyses on 8-week-old mammary glands from female mice. Although the *MT/ShcA*^{+/+}, *MT/ShcA*^{+2F} and *MT/ShcA*^{+313F} strains contained numerous tumour nodules throughout the entire mammary gland, *MT/ShcA*^{+3F} mammary glands possessed a combination of both tumour and hyperplastic structures. This effect was even more pronounced in mammary glands from *MT/ShcA*^{313F/313F} and *MT/ShcA*^{2F/2F} mice, which were entirely hyperplastic in nature (Supplementary Figure 1). Histological examination revealed the presence of extensive adenomas in the parental MT strain, but a combination of adenomas and cystic hyperplasias in mice that are heterozygous for the *ShcA*^{313F}, *ShcA*^{2F} or *ShcA*^{3F} alleles. In contrast, mammary glands from *MT/ShcA*^{313F/313F} and *MT/ShcA*^{2F/2F} mice were comprised primarily of a single layer of epithelial cells surrounding a dilated lumen (Figure 2). This correlates with a fivefold decrease in the percentage of cytokeratin-8 (CK-8)-positive luminal epithelial cells in mammary glands of *MT/ShcA*^{313F/313F} and *MT/ShcA*^{2F/2F} mice relative to *MT/ShcA*^{+/+} animals (Supplementary Figure 2). These observations suggest that engagement of the ShcA phosphorylation sites play an important role in the conversion of these early hyperplastic lesions to a neoplastic phenotype.

One important event during transition to an invasive phenotype in the MT mouse model is loss of the myoepithelial cell layer surrounding the hyperplastic luminal epithelial structures (Maglione *et al*, 2001; Lin *et al*, 2003). Mammary myoepithelial cells have been called 'natural tumour suppressors' due to their ability to inhibit luminal epithelial cell growth, tumour cell invasion and angiogenesis in a paracrine manner (Polyak and Hu, 2005). Therefore, we assessed

Table 1 Onset of mammary tumour formation and percentage of animals developing lung metastases in MT/ShcA transgenic mice^a

Transgenic line	Sex	Average onset of mammary tumour formation (days) ^b	% Of mice with lung metastases ^c
MT/ShcA ^{+/+}	F	66 ± 10 (n = 28)	91 (19/21)
MT/ShcA ^{+313F}	F	86 ± 12 (n = 20)	58 (11/19)
MT/ShcA ^{+2F}	F	81 ± 11 (n = 23)	53 (8/15)
MT/ShcA ^{+3F}	F	95 ± 14 (n = 24)	60 (12/20)
MT/ShcA ^{313F/313F}	F	132 ± 24 (n = 28)	30 (6/20)
MT/ShcA ^{2F/2F}	F	155 ± 20 (n = 21)	24 (4/17)

^aTransgenic mice were monitored for the appearance of tumours by weekly physical palpation of the mammary glands.

^bAverage time (days) that mammary tumours were first palpated ± s.d. (n = number of animals).

^cPercentage of mice with lung metastases that were tumour bearing for 28–33 days.

Figure 2 Delayed mammary tumour progression in *MT/ShcA*^{313F/313F} and *MT/ShcA*^{2F/2F} mice. Haematoxylin and eosin (H&E) staining of paraffin-embedded mammary glands from 8-week-old *MT/ShcA*^{+/+}, *MT/ShcA*^{+313F}, *MT/ShcA*^{+2F}, *MT/ShcA*^{+3F}, *MT/ShcA*^{313F/313F} and *MT/ShcA*^{2F/2F} mice. The images were taken at × 25 magnification.

Figure 3 Increased myoepithelial content in lesions from *MT/ShcA^{+/3F}*, *MT/ShcA^{313F/313F}* and *MT/ShcA^{2F/2F}* mice. (A) Immunofluorescent staining of 8-week-old *MT/ShcA^{+/+}*, *MT/ShcA^{+/3F}*, *MT/ShcA^{313F/313F}* and *MT/ShcA^{2F/2F}* mammary glands with CK-8 (green) and CK-14 (red)-specific antibodies. The images were taken at $\times 200$ magnification. (B) CK-14 immunohistochemical staining of paraffin-embedded 8-week-old mammary glands. The extent of CK-14 staining was determined for early lesions retaining a hollow lumen. The data are representative of 3–4 independent animals for each genotype, scoring the following number of lesions: FVB, 50; *MT/ShcA^{+/+}*, 50; *MT/ShcA^{+/3F}*, 85; *MT/ShcA^{313F/313F}*, 146; *MT/ShcA^{2F/2F}*, 100, and is depicted as CK-14-positive pixels/ μm^2 total epithelial area $\pm$ s.e.m. *FVB versus *MT/ShcA^{+/+}*, $P = 3.2 \times 10^{-13}$; ***MT/ShcA^{+/+}* versus *MT/ShcA^{+/3F}*, $P = 2.4 \times 10^{-14}$; ****MT/ShcA^{+/+}* versus *MT/ShcA^{313F/313F}*, $P = 1.0 \times 10^{-15}$; *****MT/ShcA^{+/+}* versus *MT/ShcA^{2F/2F}*, $P = 7.1 \times 10^{-12}$. (C) Representative images for the graph shown in panel B.

whether the delayed transition from a hyperplastic to invasive state in the various *MT/ShcA* phosphotyrosine-deficient mutant mice resulted from prolonged retention of the myoepithelial layer surrounding the hyperplastic lesions. We subjected mammary glands from 8-week-old virgin females to immunofluorescent staining using CK-8 (green) and CK-14 (red)-specific antibodies, which stain luminal epithelial and myoepithelial cells, respectively. While tumour nodules in the parental MMTV/MT strain contained very few CK-14-positive cells that were scattered throughout the lesion, hyperplastic structures within the mammary glands of *MT/ShcA^{+/3F}*, *MT/ShcA^{313F/313F}* and *MT/ShcA^{2F/2F}* mice retained a uniform myoepithelial layer surrounding the luminal epithelial cells (Figure 3A). We also performed immunohistochemical staining of the mammary glands with CK-14-specific antibodies to quantitatively assess the myoepithelial content within the hyperplastic lesions. These analyses revealed a 2- to 3-fold increase in the number of CK-14-positive cells surrounding mammary epithelial structures with hollow lumens in *MT/ShcA^{+/3F}*, *MT/ShcA^{313F/313F}* and *MT/ShcA^{2F/2F}* mice (Figure 3B and C). This correlated with a significant reduction in the percentage of proliferating cells, but not a dramatic increase in the number of apoptotic cells, in hyperplastic lesions from *MT/ShcA^{313F/313F}* and *MT/ShcA^{2F/2F}* mice (Supplementary Figure 3). These observations suggest that delayed transition to an invasive tumour phenotype in the various *ShcA* phosphotyrosine-deficient strains correlates with prolonged retention of the myoepithelial layer surrounding the hyperplastic structures.

The *ShcA* phosphorylation sites have distinct and non-overlapping roles on tumour cell survival and angiogenesis

Despite the importance of the *ShcA* phosphotyrosine residues during hyperplastic transition, lesions derived from *MT/ShcA^{+/3F}*, *MT/ShcA^{313F/313F}* and *MT/ShcA^{2F/2F}* mice eventually progress to carcinoma. We therefore determined whether mutation of the *ShcA* tyrosine phosphorylation sites affected tumour cell growth or viability. Ki67 immunohistochemical staining revealed similar proliferative rates for breast tumours derived from each genotype (Supplementary Figure 4). By contrast, *MT/ShcA^{313F/313F}*-induced tumours were highly apoptotic compared with those derived from control MT mice (Figure 4A). These observations indicate that phosphorylation of Y13 provides critical survival signals in established mammary tumour cells.

It has been previously demonstrated that recruitment of *ShcA* to the Neu/ErbB2 and Met receptor tyrosine kinases is sufficient to induce an angiogenic response (Saucier *et al*, 2004). Therefore, we performed CD31 immunohistochemical staining on tumours from the various genotypes. Microvessel density reflects the percentage of CD31+ endothelium per area of tumour epithelium. Tumours derived from all *ShcA* phosphotyrosine-deficient mutant genotypes exhibited a decrease in tumour angiogenesis relative to parental MT tumours (Figure 4B). However, only *MT/ShcA^{+/3F}* and *MT/ShcA^{2F/2F}* tumours demonstrated increased levels of ischaemic necrosis (data not shown) and a significant decrease in their microvessel density (Figure 4B). This suggests that signals emanating from tyrosines 239/240

Figure 4 Y313 of ShcA is required for tumour cell survival, whereas tyrosines 239/240 are required for tumour angiogenesis in MMTV/MT mice. (A) TUNEL staining of paraffin-embedded tumours for the indicated genotypes. The data are representative of four independent tumours (10 fields/tumour at $\times 20$ magnification) and is depicted as % TUNEL-positive cells $\pm$ s.e.m. (B) Immunohistochemical staining of OCT-embedded tumours with a CD31-specific antibody for the indicated genotypes. The data are representative of four independent tumours (10–20 fields/tumour at $\times 20$ magnification) and is depicted as % CD31-positive area/total epithelial area $\pm$ s.e.m. (* $P = 0.02$ and ** $P = 0.037$). (C) Immunoprecipitation of tumour lysates using a ShcA-specific antibody, followed by immunoblot analysis with MT, Grb2 and ShcA antibodies. (D) Immunoblot analysis of tumour lysates from the indicated genotypes using pMAPK-, MAPK-, pAKT- and AKT-specific antibodies. The numbers indicate individual mice.

are primarily involved in the induction of an angiogenic response.

Tyrosines 313 and 239/240 of ShcA both bind Grb2 and activate the Ras/MAPK and PI3K/Akt-signalling pathways in MT-transformed mammary tumours

Although the PTB domain of ShcA has been shown to bind pY250 of MT (Campbell *et al*, 1994), we confirmed that MT association with the various ShcA tyrosine-deficient mutants was not impaired. Similar levels of MT were co-immunoprecipitated with ShcA in *MT/ShcA^{+/+}*, *MT/ShcA^{+3F}*, *MT/ShcA^{313F/313F}* and *MT/ShcA^{2F/2F}* breast tumours (Figure 4C). This suggests that the phenotypic defects observed with the ShcA tyrosine mutants resulted from a failure to recruit downstream ShcA targets (Nicholson *et al*, 2001; Ong *et al*, 2001). Both Y239 and Y313 of ShcA bind Grb2 in a

phosphorylation-dependent manner (Rozakis-Adcock *et al*, 1992; van der Geer *et al*, 1996). To ascertain whether the observed biological effects on tumour outgrowth, survival or angiogenesis resulted from differential recruitment of Grb2 to the various ShcA-mutant proteins, tumour lysates were immunoprecipitated with a ShcA antibody and immunoblotted with Grb2 antisera. We did not observe any differences in the ability of ShcA to bind Grb2 from *MT/ShcA^{+/+}*, *MT/ShcA^{313F/313F}* and *MT/ShcA^{2F/2F}* breast tumours, but did see a modest reduction from *MT/ShcA^{+3F}* tumours (Figure 4C). This likely reflects the fact the ShcA3F allele has lost all Grb2-binding sites. Following ShcA binding, Grb2 can activate both the Ras/MAPK and phosphatidylinositol-3'-kinase/Akt-signalling pathways through its ability to recruit Sos and the Gab proteins, respectively (Rozakis-Adcock *et al*, 1992; Holgado-Madruga *et al*, 1996). We observed reduced

levels of phosphorylated mitogen-activated protein kinase (MAPK) in all *MT/ShcA*^{+/3F} breast tumours, and in 50% of *MT/ShcA*^{2F/2F} tumours, but not in any *MT/ShcA*^{313F/313F} tumours (Figure 4D). In contrast, MEK1/2 expression and/or phosphorylation is not strictly linked to phospho-MAPK levels in all samples (data not shown). This raises the possibility that diminished MAPK phosphorylation may result, in part, from increased expression/activation of one or more MAPK phosphatases (Owens and Keyse, 2007). Activation of the Akt pathway was also similar in the majority of breast tumours, with variability seen within each genotype examined (Figure 4D). These results suggest that Y313 and Y239/240 are both capable of binding Grb2 and loss of the Y239/240 sites partially attenuates MAPK activation.

One complication in the interpretation of these distinct tumour phenotypes results from the germline nature of the ShcA knock-in mutants. Thus, ShcA signalling is compromised in both the tumour epithelium and adjacent stroma. To address this issue, we transplanted MT breast tumour cells into cleared mammary fat pads of *Shc*^{+/+}, *ShcA*^{2F/2F} and *ShcA*^{313F/313F} animals. Mammary tumour outgrowth of wild-type MT tumour cells was comparable in *ShcA*^{+/+} and *ShcA*^{2F/2F} cleared fat pads, but significantly reduced in *ShcA*^{313F/313F} stroma (Supplementary Figure 5). Collectively, these data indicate that reduced tumour outgrowth in *MT/ShcA*^{313F/313F} mice is due, in part, to defective ShcA signalling in the stroma, whereas the requirement for ShcA Y239/240 signalling is tumour cell autonomous.

ShcA tyrosine phosphorylation sites are important for Neu/ErbB2-induced mammary tumour outgrowth and angiogenesis

To explore the role of the ShcA tyrosine phosphorylation sites in the tumour epithelium, we ectopically expressed the ShcA mutants in an immortalized mammary epithelial cell line that was transformed by activated Neu/ErbB2 (NMuMG-NT2197) (Ursini-Siegel *et al*, 2007a). We employed Neu/ErbB2-transformed mammary epithelial cells, as overexpression of this receptor tyrosine kinase is observed in 20–30% of human breast cancer and is inversely correlated with patient survival (Slamon *et al*, 1987, 1989).

Pooled ShcA-expressing populations were generated by combining 3–4 clonal cell lines, each expressing similar levels of the various FLAG-tagged ShcA mutants. We consistently observed lower FLAG-ShcA levels in the ShcA313F- and ShcA3F-expressing cells relative to the NT2197/ShcA2F population. Neu/ErbB2 expression levels were comparable in all cell lines examined (Supplementary Figure 6A). We further demonstrated that the ShcA313F, ShcA2F and ShcA3F mutants retained their ability to bind activated Neu (Supplementary Figure 6B). We also confirmed that the ShcA313F protein was only phosphorylated on Y239/Y240, ShcA2F retained only the Y313 site and ShcA3F was not phosphorylated on any of these tyrosine residues in NT2197 cells (Supplementary Figure 6B).

We determined the impact of the ShcA mutants on Neu/ErbB2-mediated tumorigenesis by injecting these cell populations into the mammary fat pads of immunocompromised mice. In contrast to parental cells, which rapidly formed tumours to endpoint, mammary tumour outgrowth was dramatically impaired in NT2197 cells expressing the

Figure 5 Overexpression of ShcA tyrosine phosphorylation mutants suppresses tumourigenesis and spontaneous metastasis. (A) NeuNT-transformed NMuMG cells (NT2197) along with populations stably expressing ShcA313F, ShcA2F or ShcA3F alleles were injected into the mammary fat pads of nude mice and tumours were monitored by bi-weekly caliper measurements. The data are represented as tumour volumes (mm³) $\pm$ s.d. ($n = 6$ mice). (B) Left panel: Percentage of mice ($n = 6$) with spontaneous lung metastases following 36–48 days of tumour growth in the mammary fat pad (MFP). Right panel: Cells (1×10^6) were injected into the tail veins of nude mice and mice were killed 28 days later. The data are presented as the number of lung lesions $\pm$ s.d., $n = 4$ mice.

ShcA mutants (Figure 5A). This is consistent with the argument that all three tyrosine phosphorylation sites in the CH1 region of ShcA are required for Neu/ErbB2-mediated mammary tumourigenesis, and suggests that the ShcA mutants function in a dominant-negative manner by interfering with the ability of endogenous ShcA to bind Neu.

We next assessed whether expression of these ShcA tyrosine phosphorylation mutants interfered with the ability of NT2197 cells to metastasize to the lung either from the mammary fat pad or following injection into the bloodstream. Expression of the ShcA phosphorylation mutants severely impaired the metastatic spread of the mammary tumour cells from the orthotopic site, but had no effect on their ability to colonize the lung once they were given access to the bloodstream (Figure 5B). This suggests that the main defect in the metastatic cascade is in tumour cell intravasation from the primary site.

One potential mechanism that may explain the decreased tumour burden and intravasation with the mutant ShcA-expressing NT2197 cells is a reduced ability to recruit tumour vasculature. Matrigel plug assays using both parental NT2197 cells and those stably expressing the different ShcA phosphorylation mutants were performed to determine their angiogenic potential. Mutation of either the Y313 or Y239/240 phosphorylation sites in ectopically expressed ShcA

Figure 6 Overexpression of ShcA tyrosine phosphorylation mutants suppresses tumour angiogenesis. (A) H&E-stained section of Matrigel plugs using NMuMG, NT2197, NT2197/ShcA313F, NT2197/ShcA2F and NT2197/ShcA3F cells injected into nude mice. The inset represents a photograph of the Matrigel plug taken immediately following removal from the animal. (B) Matrigel plugs were subjected to CD31 immunohistochemical staining and the number of CD31-positive pixels was quantified. The data are representative of four independent Matrigel plugs for each genotype/five independent fields. (C) Quantification of secreted VEGF levels from the indicated cell lines. Cells were serum starved overnight, media was harvested and assayed for secreted VEGF levels (pg/ml) by enzyme-linked immunosorbent assay, normalized to total protein levels (mg/ml). The data are representative of triplicate wells; *NMuMG versus NT2197, $P=0.003$; **NT2197 versus NT2197/ShcA313F, $P=0.03$; ***NT2197 versus NT2197/ShcA2F, $P=0.09$; NT2197 versus NT2197/ShcA3F, $P=0.0009$. (D) Engagement of the Neu ShcA-binding site elicits the strongest angiogenic response. Matrigel plug assay using primary tumour cells derived from NDL2-5, NYPD, YB, YC, YD and YE transgenic animals. CD31 immunohistochemical staining was performed on Matrigel plugs. The data are representative of 3–4 individual mammary tumours for each genotype, injected into two independent mice; *NDL2-5 versus NYPD, $P=5.8 \times 10^{-5}$; **NYPD versus YB, $P=0.25$; ***NYPD versus YC, $P=0.11$; ****NYPD versus YD, $P=0.0002$; *****NYPD versus YE, $P=0.03$.

severely impaired the ability of NT2197 tumour cells to induce an angiogenic response (Figure 6A and B). This correlated with a complete loss of vascular endothelial growth factor (VEGF) secretion by ShcA3F-expressing cells and a partial attenuation of VEGF production in cells expressing either the ShcA313F or ShcA2F mutants (Figure 6C). The observation that expression of the ShcA313F and ShcA2F mutants impaired tumour angiogenesis despite only moderate effects on VEGF production, suggests that these tyrosine phosphorylation sites likely regulate the expression of additional angiogenic or angiostatic factors.

These xenograft studies suggested that ShcA is important for tumour angiogenesis. We therefore examined the contribution of ShcA in the angiogenic response induced by Neu/ErbB2-transformed primary breast cancer cells. To this end, we performed Matrigel plug assays on explanted primary mammary tumour cells derived from transgenic mice expressing oncogenic Neu/ErbB2 receptors that either retain (NDL2-5) or lack (NYPD) all five autophosphorylation sites (Siegel *et al*, 1999; Dankort *et al*, 2001b). Primary mammary tumour cells derived from the NDL2-5 line demonstrated a fourfold enhancement in the recruitment of CD31+ vasculature relative to NYPD tumour cells (Figure 6D). Transgenic mice have been generated in which individual tyrosine residues in the Neu/ErbB2 cytoplasmic tail, termed YB through YE, were reconstituted to restore binding of distinct adaptor proteins (Dankort *et al*, 2001b; Schade *et al*, 2007). Significantly, restoration of the major ShcA-binding site (YD) to the NYPD mutant (Dankort *et al*, 2001a,b) rescued the angiogenic response induced by the mammary tumour cells

to levels observed with the wild-type receptor (Figure 6D). It is notable that a partial rescue was observed in tumours expressing the YE phosphorylation site, as this residue has also been reported to recruit ShcA *in vitro* (Figure 6D; Marone *et al*, 2004; Jones *et al*, 2006; Smith *et al*, 2006). Therefore, the ShcA-binding sites on Neu/ErbB2 appear to transduce angiogenic signals that are required for tumour growth. Taken together, these observations suggest that engagement of the ShcA phosphorylation sites within mammary epithelial cells is involved in mammary tumour outgrowth, intravasation and induction of an angiogenic response.

ShcA expression is essential for ErbB2/Neu-induced mammary tumour development

To investigate whether ShcA has a direct role within mammary epithelial cells in the development of Neu/ErbB2-induced mammary tumours, we assessed whether mammary-epithelial disruption of ShcA could interfere with ErbB2-induced tumour progression. One complication with Cre-mediated deletion of conditional alleles results from the stochastic nature of transgene expression in MMTV/Cre animals. Thus, Cre-negative mammary epithelial cells retain expression of the conditional allele and may be selectively targeted for oncogenic transformation (White *et al*, 2004). To circumvent this issue, we generated transgenic mice co-expressing both activated ErbB2 (NDL2-5) (Siegel *et al*, 1999) and the Cre recombinase from the same bicistronic transcript due to the presence of an internal ribosome entry site (IRES) between the two cDNA sequences (MMTV/NIC). Expression of Neu/ErbB2 and Cre is coupled within the same

Figure 7 ShcA expression in the mammary gland is required for Neu-mediated transformation. (A) Percentage of tumour-free mice over time for the following genotypes: *NIC/ShcA^{+/+}* (n = 26) and *NIC/ShcA^{fl/fl}* (n = 10). (B) Immunohistochemical staining of paraffin-embedded 1-year-old mammary glands with a Cre-specific antibody for the indicated genotypes. (C) PCR analysis for the excised *ShcA* allele (Δ FlxShc), the unexcised floxed-*ShcA* allele (FlxShc), the endogenous *ShcA* allele (ShcWT) and Cre on genomic DNA isolated from mammary glands obtained from 1-year-old FVB and *NIC/ShcA^{fl/fl}* mice. The numbers indicate the individual mice. (D) (Left panel) Immunohistochemical staining of paraffin-embedded 1-year-old mammary glands with a Ki67-specific antibody for the indicated genotypes. The data are representative of four independent animals for each genotype scoring the following number of ducts: FVB, 36; *NIC/ShcA^{fl/fl}*, 26, and is depicted as % Ki67-positive cells/duct $\pm$ s.e.m. (Right panel) TUNEL staining of paraffin-embedded 1-year-old mammary glands for the indicated genotypes. The data are representative of four independent animals for each genotype scoring the following number of ducts: FVB, 27; *NIC/ShcA^{fl/fl}*, 26, and is depicted as % TUNEL-positive cells/duct $\pm$ s.e.m.

mammary epithelial cell, precluding the possibility of obtaining Neu/ErbB2-expressing, Cre-negative 'escapee' cells. MMTV/NIC mice develop mammary tumours with an average latency of 146 days (Figure 7A), which is comparable to the tumour kinetics observed with MMTV/NDL2-5 transgenic animals (Siegel *et al*, 1999). Moreover, both NIC and NDL2-5 tumours express elevated levels of Neu/ErbB2 and histologically resemble solid/nodular adenocarcinomas (data not shown) (Siegel *et al*, 1999). We observed high levels of NIC transgene expression in the mammary gland, with lower levels in the adrenal gland, lung, ovary, pancreas and salivary gland (Supplementary Figure 7). Moreover, MMTV/NIC mice only develop mammary tumours.

To determine the role of ShcA signalling in Neu/ErbB2-mediated mammary tumorigenesis, we interbred MMTV/NIC mice with animals expressing a floxed *ShcA*-3xFLAG cDNA (under the control of the endogenous *ShcA* promoter) (Hardy *et al*, 2007). Strikingly, mammary tumour development was completely ablated in all *NIC/Shc^{fl/fl}* mice examined (Figure 7A). This was associated with retained Cre expression

and the presence of the excised *ShcA* allele in mammary glands from 1-year-old *NIC/Shc^{fl/fl}* mice (Figure 7B and C). Histological analysis revealed the absence of any detectable hyperplastic or neoplastic lesions within the mammary epithelial cells of *NIC/Shc^{fl/fl}* mice. Instead, the ducts were either morphologically normal or underwent ductal ectasia, which is characterized as a single layer of luminal epithelial cells surrounding a dilated lumen (data not shown). This was further associated with a significantly reduced proliferative capacity and increased apoptotic index (Figure 7D) in *NIC/Shc^{fl/fl}* mammary epithelial structures relative to FVB controls. This suggests that sustained ShcA expression in mammary epithelial cells is critical for their proliferation, survival and transformation by the Neu oncogene.

Taken together, we demonstrate that ShcA signalling is important during numerous stages of mammary tumour progression, including transition from hyperplasia to neoplasia and finally invasive carcinoma. We further demonstrate non-overlapping roles for the ShcA phosphotyrosine residues in the development of metastatic mammary tumours. Finally,

retention of ShcA within mammary epithelial cells is absolutely critical for mammary tumourigenesis induced by the Neu/ErbB2 oncogene.

Discussion

Phosphotyrosine-dependent ShcA signalling is critical for communication between the mammary tumour cell and its microenvironment

We show that phosphorylation of the ShcA adaptor protein plays a critical role during MT- and Neu/ErbB2-induced mammary tumour progression. These results clearly establish an important role for the ShcA tyrosine phosphorylation sites in transition from hyperplasia to overt carcinoma. One of the critical events that must occur during this transition, that is, disruption of the surrounding myoepithelial cell layer (Polyak and Hu, 2005). Retention of the myoepithelial barrier in MT/ShcA^{+/3F}, MT/ShcA^{313F/313F} and MT/ShcA^{2F/2F} hyperplastic structures may reflect the fact that they are not as advanced in the transformation process relative to age-matched, wild-type MT-expressing mammary glands. While normal mammary ducts retain a myoepithelial cell layer composed of flat cells with equal spacing, the myoepithelial cells surrounding hyperplastic lesions from 4-week-old MT mice are more rounded in structure and unevenly spaced around the acinar structures (Lin *et al*, 2003). Morphologically, the myoepithelial cell layer surrounding the hyperplasias in 8-week-old MT/ShcA^{+/3F}, MT/ShcA^{313F/313F} and MT/ShcA^{2F/2F} mice most resembles that observed in the normal mammary gland. However, it remains possible that prolonged retention of the myoepithelial cell layer surrounding the hyperplastic structures is the result of delayed tumour progression. It is also conceivable that phosphotyrosine-dependent ShcA signalling may permit disruption of the myoepithelial cell layer, which contributes to the transition from hyperplasia to carcinoma. Given that the ShcA mutations are germline in nature, delayed disruption of the surrounding myoepithelium may result from defects in either cellular compartment. Indeed, it has been demonstrated that myoepithelial cells present in human breast ductal carcinoma *in situ* (DCIS) upregulate the expression of chemokines, which function in a paracrine manner to stimulate tumour cell growth and angiogenesis (Allinen *et al*, 2004).

Another important function of the ShcA tyrosine phosphorylation sites during mammary tumour progression is in promoting tumour angiogenesis. In established mammary tumour cells expressing activated Neu/ErbB2, ectopic expression of ShcA313F, ShcA2F and ShcA3F mutants profoundly impaired the ability of these Neu/ErbB2-transformed tumour cells to induce an angiogenic response. Moreover, germline introduction of these ShcA phosphorylation mutants also impaired the ability of MT tumours to recruit tumour vasculature. However, in the case of the MT model, engagement of the 239/240 tyrosine phosphorylation sites was most important for induction of an angiogenic response. The differences observed between these two models may reflect the higher levels of ShcA expression achieved in ErbB2-transformed cell lines compared with the MT model, in which ShcA is expressed at physiologically relevant levels. It is also possible that ShcA (pY239/240)-dependent signalling is also required within the endothelial cells themselves for establishment of a tumour vasculature. Indeed, ShcA expression within the

vascular compartment is required for blood vessel maturation during embryogenesis (Lai and Pawson, 2000), and ShcA binding to the Tie2 receptor has been shown to promote endothelial cell migration and sprouting (Audero *et al*, 2004). Collectively, these observations suggest that engagement of ShcA signalling plays a critical role in allowing the mammary tumour cell to communicate with both the myoepithelial and endothelial cell compartments during tumour progression.

The observation that ShcA plays a critical role in paracrine signalling between the tumour cells and components of the microenvironment is reminiscent of the spindle defects observed in ShcA^{3F/3F} mice (Hardy *et al*, 2007). In these animals, loss of ShcA tyrosine phosphorylation-dependent signalling resulted in a profound defect in communication between the muscle spindle cell and sensory neuron that modulates limb movements. Indeed, conditional deletion of ShcA in the muscle cell compartment produced a similar spindle defect (Hardy *et al*, 2007). In an analogous manner, disruption of ShcA signalling impairs the ability of MT tumour cells to disrupt the myoepithelial cell layer and recruit vasculature. The identification of ShcA-dependent factors involved in communication between the tumour cell with the endothelial and myoepithelial cell compartments will help define the mechanisms by which ShcA controls the tumour microenvironment.

Mammary epithelial expression of ShcA is essential for ErbB2-induced mammary tumour progression

Whereas this study argues that tyrosine phosphorylation of ShcA is important for mammary tumour induction, it does not preclude the involvement of phosphotyrosine-independent ShcA signals in this process. Given that ShcA tyrosine phosphorylation sites are not essential for embryonic cardiovascular development in the mouse (Hardy *et al*, 2007), and that comparable phosphorylation sites are absent from some invertebrate ShcA proteins (Luzi *et al*, 2000), it is likely that other conserved ShcA domains play an equally important role in ShcA signalling. To address this issue, we utilized a conditional ShcA allele and a unique transgenic mouse model where expression of activated ErbB2 and Cre in the mammary gland is coupled. Remarkably, ablation of ShcA expression in the mammary gland resulted in a complete block in ErbB2-induced tumour induction. These observations argue that ShcA function is critical for ErbB2-mediated transformation of the mammary epithelium.

The importance of ShcA in ErbB2-mediated mammary tumour progression is also reflected in the phenotypic similarities between ErbB2- and ShcA-knockout mice. Germline deletion of both ShcA and ErbB2 is embryonic lethal as a result of cardiovascular defects (Lee *et al*, 1995; Lai and Pawson, 2000). In addition, conditional ablation of ErbB2 or ShcA in the muscle leads to identical spindle cell defects (Andrechek *et al*, 2002; Hardy *et al*, 2007). These similarities argue that ShcA is a principal physiological signalling output for ErbB2.

The observation that mammary epithelial expression of ShcA is critical for ErbB2-mediated mammary tumour induction may also reflect its importance in coordinating signals between ErbB2 and other receptor pathways. In this regard, the β 4-integrin receptor, which couples to ShcA, is also involved in ErbB2-induced mammary tumour progression (Mainiero *et al*, 1995; Guo *et al*, 2006). In addition, reduced

dosage of the ShcA-binding protein, Grb2, in MT-transformed tumour cells delays mammary tumour progression (Cheng *et al*, 1998). Future identification of these ShcA-coupled signalling networks will provide potential targets for therapeutic intervention in the treatment of breast cancer.

Materials and methods

Cells

NMuMG-NT2197 cells were generated as described previously (Ursini-Siegel *et al*, 2007a) and transfected with pMSCV/ShcA, pMSCV/ShcA313F, pMSCV/ShcA2F or pMSCV/ShcA3F plasmids followed by selection with 500 µg/ml hygromycin. The stable cell lines represent pools of 3–4 clonal populations expressing similar levels of the FLAG-tagged ShcA expression constructs.

Mice

MMTV/MT has been described by Guy *et al* (1992). Mice expressing a targeted *ShcA^{flx}* allele or mutant ShcA proteins harbouring tyrosine-to-phenylalanine point mutations at residues 239/240 (ShcA2F), 313 (ShcA313F) or 239/240/313 (ShcA3F) under the control of the endogenous *shcA* promoter have been described (Hardy *et al*, 2007) and were interbred for six generations onto an FVB background before their utilization in bigenic crosses. The excised *ShcA^{fl}* allele was detected by polymerase chain reaction (PCR) analysis on genomic DNA as described (Hardy *et al*, 2007).

A construct encoding a bicistronic transcript expressing an oncogenic *Neu* allele (NDL2-5) (Siegel *et al*, 1999) followed by the Cre recombinase, under the translation control of an IRES element, was generated (NIC). MMTV/NIC transgenic mice were generated by pronuclear injection as described (Siegel *et al*, 1999).

At necropsy, mammary glands, tumours or lungs were fixed in 10% buffered formalin, embedded in optimal cutting temperature (OCT) compound or frozen in liquid nitrogen. The number four mammary glands were subjected to whole-mount staining as described (Siegel *et al*, 1999).

In vivo tumorigenesis assays were performed by injecting 5×10^4 cells as described (Ursini-Siegel *et al*, 2007a). For the experimental metastasis assays, 1×10^6 cells were injected into the lateral tail vein of Ncr nude mice (NIH) and the lungs were collected 4 weeks later. All animal studies were approved by the Animal Resources Centre (ARC) at McGill University and complied with the guidelines set by the Canadian Council of Animal Care.

Matrigel plug assay

The Matrigel plug assay was performed as described by Saucier *et al* (2004). A total of 1×10^5 primary mammary tumour cells were used and the animals were killed 10 days later. For the NMuMG-derived cell line studies, 1×10^5 cells were injected subcutaneously into the Matrigel plug and the mice were killed 7 days later. Paraffin-embedded sections were stained with a CD31-specific antibody (1:300; cat. no. sc-1506; Santa Cruz). The number of CD31-positive pixels for each Matrigel plug was calculated from five independent fields ($\times 400$ magnification) using Image J software.

Immunohistochemistry

Staining was performed on paraffin-embedded sections as described by Ursini-Siegel *et al* (2007a). Sections were incubated first with a Ki67-specific antibody (1:100; cat. no. ab15580; Abcam), CK-14 antibody (1:800; cat. no. PRB-155P; Covance) or Cre antibody (1:600; MMS-106P; Covance), and then processed with the Elite IgG Vectastain ABC kit (Vector). For Cre staining, endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol and antigen

retrieval was performed in a pressure cooker. Sections were blocked with Power Block Universal Blocking Agent (Biogenex). Paraffin-embedded sections were also subjected to TdT-mediated dNTP nick end labelling (TUNEL) staining (Apoptag Detection kit; Chemicon). CD31 staining of mammary tumors was performed on OCT-embedded sections (1:200; cat. no. 550274; BD Biosciences) as previously described (Maglione *et al*, 2001). Slides were scanned using a ScanScope XT Digital Slide Scanner (Aperio) and data were analysed with positive pixel count or nuclear algorithms.

Whole-mount immunofluorescence

Mammary glands were fixed for 30 min in 2% paraformaldehyde, permeabilized for 20 min in phosphate-buffered saline (PBS)/0.05% Triton X-100 and incubated in 100 mM glycine/PBS. The mammary glands were blocked in immunofluorescence buffer (PBS/0.2% Triton X-100/0.05% Tween-20) plus 10% goat serum for 2 h followed by overnight incubation with the CK-8 (1:200; cat. no. RDI-PROGP11; Fitzgerald Industries) and CK-14 (1:200; cat. no. PRB-155P; Covance) antibodies. The mammary glands were incubated with anti-guinea pig AlexaFluor-488 and anti-rabbit AlexaFluor-555 secondary antibodies (1:500; Molecular Probes), mounted and visualized using an Axiovision 200 microscope (Zeiss).

Immunoblotting/immunoprecipitation

Cells in flash-frozen tumour tissue were lysed in phospholipase-C-γ lysis buffer (Dankort *et al*, 2001b). Lysates were analysed by immunoblot analysis using the following antibodies: ShcA (cat. no. S14630 or cat. no. 610878; BD Biosciences), FLAG (cat. no. F3165; Sigma), pShc(Y239/240) (cat. no. sc18074R; Santa Cruz), pShc(Y313) (cat. no. sc23765R; Santa Cruz), Neu (sc-284; Santa Cruz), ErbB3 (cat. no. sc285; Santa Cruz), MT (Dilworth and Griffin, 1982), E-cadherin (cat. no. C20820; BD Biosciences), pAKT (cat. no. 9271; Cell Signaling), AKT (cat. no. 9272; Cell Signaling), pMAPK (cat. no. 9106; Cell Signaling), MAPK (cat. no. 9102; Cell Signaling), Grb2 (cat. no. 610112; BD Biosciences), and α-tubulin (T9026; Sigma). The blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson Laboratories) and visualized by enhanced chemiluminescence (Amersham). Immunoprecipitations were performed using 1 µg of antibody per 500 µg total cell lysate with the following antibodies: Neu (cat. no. OP15; Calbiochem), FLAG (cat. no. F3165; Sigma) and Shc (cat. no. S14630; BD Biosciences).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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