The p52 isoform of SHC1 is a key driver of breast cancer initiation

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Abstract

Background: SHC1 proteins (also called SHCA) exist in three functionally distinct isoforms (p46\textit{SHC}, p52\textit{SHC}, and p66\textit{SHC}) that serve as intracellular adaptors for several key signaling pathways in breast cancer. Despite the broad evidence implicating \textit{SHC1} gene products as a central mediator of breast cancer, testing the isoform-specific roles of \textit{SHC1} proteins have been inaccessible due to the lack of isoform-specific inhibitors or gene knockout models.

Methods: Here, we addressed this issue by generating the first isoform-specific gene knockout models for p52\textit{SHC} and p66\textit{SHC}, using germline gene editing in the salt-sensitive rat strain. Compared with the wild-type (WT) rats, we found that genetic ablation of the p52\textit{SHC} isoform significantly attenuated mammary tumor formation, whereas the p66\textit{SHC} knockout had no effect. Rats were dosed with 7,12-dimethylbenz(a)anthracene (DMBA) by oral gavage to induce mammary tumors, and progression of tumor development was followed for 15 weeks. At 15 weeks, tumors were excised and analyzed by RNA-seq to determine differences between tumors lacking p66\textit{SHC} or p52\textit{SHC}.

Results: Compared with the wild-type (WT) rats, we found that genetic ablation of the p52\textit{SHC} isoform significantly attenuated mammary tumor formation, whereas the p66\textit{SHC} knockout had no effect. These data, combined with p52\textit{SHC} being the predominant isoform that is upregulated in human and rat tumors, provide the first evidence that p52\textit{SHC} is the oncogenic isoform of \textit{Shc1} gene products in breast cancer. Compared with WT tumors, 893 differentially expressed (DE; FDR < 0.05) genes were detected in p52\textit{SHC} KO tumors compared with only 18 DE genes in the p66\textit{SHC} KO tumors, further highlighting that p52\textit{SHC} is the relevant \textit{SHC1} isoform in breast cancer. Finally, gene network analysis revealed that p52\textit{SHC} KO disrupted multiple key pathways that have been previously implicated in breast cancer initiation and progression, including ESR1 and mTORC2/RICTOR.

Conclusion: Collectively, these data demonstrate the p52\textit{SHC} isoform is the key driver of DMBA-induced breast cancer while the expression of p66\textit{SHC} and p46\textit{SHC} are not enough to compensate.

Keywords: DMBA, Shc proteins, Breast cancer, Signaling, Rat model

Background

The progression of breast carcinomas is controlled by the aberrant expression of a variety of signaling molecules expressed in the mammary tumor itself, or in cells of the tumor microenvironment \cite{1, 2}. The principal factors that control mammary tumor formation remain unclear and insufficiently defined. Mediating some of these signals are the SHC adaptor proteins. Many studies have implicated \textit{SHC1} involvement in signaling by epidermal growth factor receptor-2 (HER-2), estrogen receptor (ER), and prolactin (PR) signaling, three well-established biological markers for prognosis and response to breast cancer therapy \cite{3–5}. More recently, increased expression of the p66\textit{SHC} isoform was shown to occur in cell lines with increased metastatic potential and in patient samples with metastatic breast tumors \cite{5–7}. However, to date, there has been no direct in vivo

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examination of the role of specific SHC1 protein isoforms in the progression of mammary tumorigenesis.

The ubiquitously expressed adaptor protein SHC1 [8] exists in three isoforms with relative molecular masses of 46 kDa, 52 kDa, and 66 kDa (p46SHC, p52SHC, and p66SHC) encoded by single gene SHC1 (also known as SHCA). p46SHC and p52SHC arise from the use of alternative translation initiation sites within the same transcript, whereas p66SHC isoform contains a unique N-terminal region and is believed to be generated as a result of activation of an alternative promoter [9]. Although all three Shc proteins contain a recognition site for the SH2 domain of the adaptor protein GRB2, a number of studies have suggested distinct physiological roles for the three isoforms [10–12]. Additionally, different intracellular localization patterns have been reported for each (reviewed in [12]). Moreover, it has been shown that p66Shc and p46Shc/p52Shc exert counter-regulatory effects on the c-fos promoter [11]. This has given rise to the general consensus that isoform p52SHC functions as an adaptor protein linking receptor tyrosine kinases and G-protein-coupled receptors with the RAS signaling cascade, while p66SHC isoform contributes to reactive oxygen species (ROS) production as well as regulation of vascular contraction [12]. While early studies suggested p46SHC functions to be like p52SHC, recent data indicate a distinct role for p46SHC in the mitochondria [12, 13].

In mice, the polyomavirus middle T antigen (MT) model is frequently used to study mammary tumor progression. Several lines of evidence suggest the direct involvement of products of the Shc1 gene in the regulation of breast cancer initiation and progression in this model [14, 15]. Shc1 products have been shown to directly bind to the MT antigen protein, serving as an adaptor protein leading to activation of downstream signaling cascades such as RAS/MAPK and PI3K, all of which have a positive effect on tumorigenesis [14]. Furthermore, increased expression of Shc1 gene products have been shown to accelerate tumorigenesis in the MT antigen model, indicating its importance in this model of breast cancer [16]. More recently, studies have shown that Shc1 gene products in this model further function in a paracrine manner to promote a pro-tumorigenic state by inhibiting the adaptive immune response [17, 18]. These data clearly indicate both cell autonomous function, as well as microenvironment effects of Shc1 products in this model of mammary tumorigenesis. However, in these studies, the role of individual SHC1 isoforms have not been tested, as many of the mutations in the previous studies will alter all three Shc1 protein isoforms, or overexpression systems will produce multiple SHC1 protein isoforms. Furthermore, this is an activated model in which increased expression of several well-known signaling pathways promotes tumorigenesis. This model does not address the role of SHC1 proteins in spontaneous lesions and tumorigenesis that is more typical of human populations that develop mammary cancer.

For these reasons, we generated the first animal models for Shc1 isoform-specific knockout of the p52SHC (p52SHCKO) isoform and first rat knockout of p66SHC (p66SHCKO) isoform [19] in the SS/JrHsdMcwi (SS) genetic background, which is a well-studied rat parental background for breast cancer risk [20, 21]. Mammary tumors were induced by the oral administration of 7,12-dimethylbenz(a)anthracene (DMBA), a known inducer of breast cancers that introduce random mutations into the genome and develop spontaneous mammary tumors [22–24]. DMBA-induced tumors are typically ER+ and PR+ lesions and serve as excellent models to study these types of tumors, as these represent a clinically needed model [25]. In humans, ER+ tumors represent tumor types successfully targeted by hormone therapy; however, few clinical options remain in case of cancer relapse. Accordingly, analysis of tumor incidence, latency, and molecular characteristics of mammary tumors in the rat DMBA model could provide data relevant for breast cancer treatment in humans. Here, we show for the first time that p52SHC is the predominant Shc1 isoform that mediates mammary tumorigenesis via multiple key signaling pathways, whereas the p66SHC isoform is expendable in DMBA-induced tumorigenesis.

Methods

Animals

All studies were performed on rats generated and maintained on the SS/JrHsdMcwi (SS) genetic background. Generation of the p66SHC-KO rats has been described previously [19]. Briefly, p52SHC knockout rats (SS-Shc1p52KO) were generated by pronuclear injection of a CRISPR plasmid expressing Cas9 and single-guide RNA targeting the sequence CACTCCCTCCAGGACATTGTCTTCG (protospacer adjacent motif underlined) into one-cell SS/JrHsdMcwi rat embryos. Founder animals were genotyped by Cel-1 assay and confirmed by Sanger sequencing to identify a founder animal harboring a 6-bp deletion including the p52SHC initiation codon. All subsequent litters are genotyped using the following primers: p52ShcKO forward 5′-CCCTCCCTCCAGGACAAAGC-3′; wild-type forward 5′-CTCCCTCCAGGACATGAAACAAGC-3′; common reverse 5′-TATGCACCCCAACGAACAA-3′. p52SHC-KO rats are born in the expected genotypic ratio with normal litter size and able to breed as homozygous knockouts. All rats were fed ad libitum on a low salt (0.4% NaCl, Teklad7034) diet. Animal use and welfare procedures adhered to the NIH Guide for the Care and Use of Laboratory Animals following protocols reviewed and approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.
Mammary tumor model

To induce mammary tumors, 45- to 55-day-old female rats (WT n = 14, p52SHC-KO n = 12, p66SHC-KO n = 13) were administrated 65 mg/kg of body weight of 7,12-dimethylbenz(a)anthracene (DMBA) by oral gavage, as described [20]. Animals were then palpated weekly for tumor formation in the mammary fat pads. The latency of tumor development is defined as the time between DMBA administration and detection of the first tumor, whereas tumor multiplicity is the number of tumors per rat at week 15. At week 15 post-DMBA, rats were euthanized by CO₂ asphyxiation and number of tumors and total tumor burden per rat were recorded. Tumor burden is calculated as the sum total weight of all tumors from one animal.

Whole-mount stain of mammary glands

Carmine alum staining of whole inguinal mammary glands was performed using standard protocols. Briefly, excised fourth and fifth mammary glands from the left and right side of adult approximately three-month-old rats were stretched and pressed onto a 2 × 3 in. glass slide and fixed 24 h in Carnoy’s fixative. Following rehydration to H₂O, slides were stained in Carmin Alum stain 24 h and then serially dehydrated in ethanol. Finally, slides were cleared in Histo-Clear™ and protein was measured using the BCA assay (Thermo Fisher) and frozen at −80 °C before SDS-PAGE western blot analysis. SHC protein expression was analyzed using anti-SHC1 antibody (EMD Millipore 06-203) (dilution 1:1000) followed by IRDye800 secondary antibodies (Li-COR). Total protein was assessed by Bovine serum albumin (BSA) (dilution 1:2000) followed by goat anti-rabbit-HRP and goat anti-mouse-HRP (Biorad) (dilution 1:2000) and protein was measured using the BCA assay (Thermo Fisher). For rats, tumor and distant normal) was obtained from the Medical College of Wisconsin Tissue Bank. For these specimens, normal tissue is defined as being distant from the tumor of the same patient that has no visual tumor present as determined by the pathologist at the time of procurement. Specimens were washed with PBS to remove OCT compound before protein extraction, performed using a Potter-Elvehjem homogenizer followed by sonication in RIPA buffer. Lysates were centrifuged to remove debris, and protein was measured using the BCA assay (Thermo Fisher) and frozen at −80 °C before SDS-PAGE western blot analysis. SHC protein expression was analyzed using anti-SHC1 antibody (EMD Millipore 06-203) (dilution 1:2000) followed by IRDye800 secondary antibodies (Li-COR). Total protein was assessed by REVERT (Li-COR). Human INSTA-Blot™ Breast Tissue OncoPair (Novus Biologicals #NBP2-30128) ready-to-use PVDF membranes were used to assess total SHC proteins expressions in human breast cancer and normal adjacent tissue, 14 µg total protein per sample. Total protein was assessed by amido black stain provided by the manufacturer. Membrane was blotted with anti-SHC1 supplemented with anti-p66SHC antibody ( Biosource 44829M) (dilution 1:1000) followed by goat anti-rabbit-HRP and goat anti-mouse-HRP (Biorad) (dilution 1:2000), according to manufacturer’s protocol. For rats, tumor and distant normal mammary samples from the same rat were also compared. Thirty micrograms of total protein was electrophoresed and transferred to Immobilon-FL membrane (Millipore). Membranes were blocked in Odyssey Blocking Buffer/TBST 1:1 and then blotted with anti-
SHC1 antibody (EMD Millipore) (dilution 1:1000) and anti-β-actin (Sigma) (dilution 1:2000) overnight in 1:1 Odyssey Blocking Buffer/TBST. Membranes were rinsed in TBST and incubated with appropriate IRDye secondary antibodies (Li-COR) at dilution 1:15,000. Membranes were scanned on a LI-COR Odyssey scanner.

**Immunohistochemistry**

Staining for ER, PR, HER2, and SHC1 was performed using Dako EnVision FLEX mini Kit on a Dako Autostainer Omnis (Agilent, Santa Clara, CA). High-resolution digital images were captured at ×20 using a Pannoramic 250 Flash III slide scanner (3DHISTECH Ltd., Budapest, Hungary). ER, PR, HER2, and SHC1 were detected using EnVision FLEX Target Retrieval Solution Low pH (Citrate buffer, pH 6.1, Agilent) and incubation with anti-ER (1:200, ab3575, Abcam, Cambridge, MA), anti-PR (1:2000, ab16661, Abcam), anti-HER2 (1:400, A0485, Agilent), and anti-SHC (1:50, 06-203, Sigma) for 30 min, followed by secondary antibody (Polymer) for 30 min. Counterstaining with hematoxylin, dehydration, clearing, and coverslipping was performed. The Dako HER2 antibody recognizes rat HER2 [28].

**Statistical analysis**

Tumor latency and multiplicity between genotypes were analyzed by a multiple comparison test using Tukey’s method and adjusted using single-step Bonferroni. To compare tumor latency at 5, 10, and 15 weeks, a non-parametric survival regression model was used (with tumors never developed being treated as right-censored observations), while pair-wise comparisons were performed using a two-sample melded BPCP test. Total tumor burden was calculated as the sum of all tumors extracted from each rat. Total tumor burden between genotypes was compared using the non-parametric Kruskal-Wallis test (since the data appeared to be non-normal), followed by testing of pairwise contrasts using Dunn’s test. Multiplicity adjustments for the P values were done using Holm’s method. For western blot semi-quantitative analysis, group means were compared using the Mann-Whitney rank sum test.

**Results**

**Expression of SHC1 gene products are upregulated in mammary tumors**

According to the Human Protein Atlas, in breast carcinomas, there is a difference in expected outcome between patients expressing high levels of SHC1 RNA and patients expressing low levels of SHC1 RNA based on best separation ($P = 0.0412$), but not median separation ($P = 0.0928$) [29]. Even though these data suggest the significance of high levels of SHC1 RNA for the worst expected outcome of breast cancer, they do not address the impact of individual protein isoforms in the progression of breast carcinomas. In mammals, SHC1 is expressed as two distinct transcripts based on the utilization of alternate promoters [9]. The shorter (3062 bp) transcript, termed here the p52SHC/p46SHC transcript, contains in-frame initiation codons for both p52SHC and p46SHC. The longer (3481 bp) transcript, termed the p66SHC transcript, contains initiation codons for all three protein isoforms, but is considered to serve predominately as a source of p66SHC. A broad human TissueScan cancer survey panel was screened for expression of both SHC1 transcripts. Each panel contained cDNA, normalized for β-actin expression, from 9 cancer samples from individual patients of increasing stage from 8 different cancer types and three normal tissues [30]. Expression of p52SHC/p46SHC transcript was upregulated more than 2-fold (*) compared to non-cancer tissue in 5 of the 9 breast cancer samples, with one additional sample being greater than 1.5 times (#) higher (Fig. 1a) when compared to the median non-tumor samples. According to supplied information from OriGene, most of the tumors assayed are estrogen receptor (ER) and prolactin receptor (PR) positive, with mixed Her2 positive staining (Additional file 1: Figure S1). In contrast, no significant changes in p52SHC/p46SHC transcript were observed in colon, ovary, and thyroid cancers. Prostate, lung, kidney, and liver carcinomas exhibited variable expression in p52SHC/p46SHC expression, ranging between 1.5- and 2-fold increase over normal tissue (Fig. 1a). Breast carcinomas were unique in that most samples expressed the p52SHC/p46SHC transcript 2-fold higher than the normal breast tissues. Expression of the p66SHC transcript, the lesser-expressed Shc1 transcript, was below detectable levels in most normal and some breast tumor samples (data not shown). Additionally, p66SHC transcript levels were below detectable levels in many of the tumor samples across all cancer types (data not shown).

To assess differences in expression of all three SHC1 protein isoforms in human breast tumors, we have analyzed 9 independent breast cancer tumor samples (T) paired with matched normal protein samples (N) from tissues obtained from MCW Tissue Bank. It is of note, that in all (five) ER+/PR+/HER2- breast cancer samples, we detected the increased expression of p52SHC and p46SHC whereas the difference in p66SHC expression was not observed (Fig. 1b, c). The tumor/stroma composition (contribution of normal mammary epithelial) was quantified by a pathologist (Additional file 5: Table S1). Although triple negative tumors appeared to have increased SHC1 proteins levels, the differences did not reach significance compared to normal tissues when normalized to the total amount of protein (Fig. 1d, e). Additionally, commercial INSTA-Blot™ Oncopair membranes were blotted with total SHC1 antibody that
recognizes all three isoforms, concurrently with an N-terminal p66Shc antibody. Comparison of seven independent tumor samples of increasing grade and stage with matched normal protein samples from adjacent tissues indicated that p52SHC and p46SHC isoforms were upregulated in 6 out of 7 tumor samples compared to normal-paired samples (Fig. 1f). Likewise, 3 out of 7 tumor samples expressed increased levels of p66SHC protein compared with the normal-paired breast tissues. Similar to observations made with human breast tissue, the analysis of DMBA-induced rat tumors compared with normal rat mammary tissue revealed increased expression of all SHC1 isoforms (Fig. 1g). Immunohistochemical staining of SHC1 proteins showed markedly elevated expression of SHC1 proteins in breast cancer cells compared to adjacent normal breast epithelia or stromal cells, both in human (Fig. 2) and rat tumors (Additional file 2: Figure S2).

The p52SHC isoform is required for DMBA-induced mammary tumor formation

Individual contributions of p66SHC and p52SHC in the development and progression of breast cancer are unclear. Since both p66SHC and p52SHC protein levels were elevated in some human and in rat DMBA-induced tumors (Fig. 1d, e), we utilized rats in which p66SHC or p52SHC proteins were genetically removed to study the consequence of their loss on DMBA-induced mammary tumor formation. The p66SHC knockout (p66SHC-KO) was generated by targeting the unique sequence of p66Shc using Zinc Finger technologies and has been previously described [19]. For the first time in any species, a p52SHC knockout was generated using CRISPR/Cas-9 genomic editing [32]. A single founder was
generated in which 6-base pairs, including the p52SHC ATG initiation codon, were deleted (termed p52SHC-KO, Fig. 3a). This deletion resulted in a 2-amino acid loss from p66SHC protein that was not predicted to alter p66SHC function. The deleted amino acids are not located in any known functional protein domains [12]. Furthermore, p52SHC-KO rats do not exhibit common phenotypes associated with p66SHC loss of function when placed on a high salt diet (data not shown), suggesting a normal p66SHC function in the p52SHC-KO rats. Additionally, the p46SHC sequence was preserved. Importantly, the mutation did not alter expression of either p66SHC or p46SHC protein levels, while p52SHC isoform lacks completely as detected by PCR genotyping (Fig. 3b) and western blot using a total SHC1 antibody (Fig. 3c, Additional file 3: Figure S3). Absence of p66SHC band by western blot analysis in p66SHC-KO, but not in WT or p52SHC-KO rats is also demonstrated (Additional file 3: Figure S3). Additionally, both p52SHC-KO and p66SHC-KO rats can be maintained at homozygous knockouts and give birth to average litter size and pup size (data not shown). There was no difference in the mammary gland structure between WT, p52SHC-KO, and p66SHC-KO rats (Fig. 3d). No masses, cysts or other lesions were identified across all three groups. Thus, changes in the development of mammary glands due to p52SHC or p66SHC ablation are likely not responsible for differences in DMBA-induced mammary tumor formation. Following administration of DMBA, loss of p52SHC resulted in an increase in tumor latency and a decrease in tumor incidence to 58.3% (7 of 12) by 15 weeks, compared to WT (92.9%, 13 of 14) and p66SHC-KO (100.0%, 13 of 13), \( P = 0.038 \) and \( P = 0.003 \), respectively, Fig. 4a). Differences in tumor development were apparent as early as 10 weeks post-DMBA in a pair-wise comparison, 25.0% (3 of 12) p52SHC-KO rats had tumors compared to 78.6% (11 of 14) for WT and 76.9% (10 of 14) p66SHC-KO (\( P = 0.02 \) and \( P = 0.03 \) respectively). There was no difference in latency between WT and p66SHC-KO at any point during the 15-week time course (\( P = 0.4 \)). During the 15 weeks that tumor progression was analyzed, rats were individually palpated for the presence of tumors. It may be possible that very small lesions were missed in the analysis. In addition to increased latency, p52SHC-KO rats also had lower tumor multiplicity, averaging 1.0 ± 0.3 tumors per rat compared to 2.75 ± 0.4 and 4.31 ± 0.3 tumors in WT and p66SHC-KO, respectively (\( P = 0.002 \) and \( P < 10^{-4} \), Fig. 4b). Furthermore, p52SHC-KO rats had a lower overall tumor burden per rat, 0.08 ± 0.04 g, compared to WT, 1.34 ± 0.5 g, and p66SHC-KO rats, 3.10 ± 0.8 g, respectively (\( P < 0.02 \) and \( P < 10^{-5} \), Fig. 4c). In contrast, p66SHC-KO rats trended towards increased multiplicity and tumor burden compared to WT DMBA-induced rats but was not significant (\( P = 0.09 \) and \( P = 0.08 \)). We have carried out immunostaining for ER, PR, and HER2...
of DMBA-induced mammary tumors formed in our genetically modified rats (Additional file 6: Table S2). All analyzed DMBA-induced tumors were ER and PR positive and HER2 negative.

**RNA-seq analysis reveals significant changes in the p52SHC tumor transcriptome**

To begin identifying gene networks that are mediated by p52SHC and p66SHC in breast cancer, RNA-seq was performed on mammary tumors derived from WT (n = 4), p52SHC-KO (n = 5), and p66SHC-KO (n = 5) rats. Compared with the WT tumors, 893 genes were differentially expressed (DE; FDR < 0.05) in the p52SHC-KO tumors, whereas only 18 DE genes were detected in the p66SHC-KO tumors (Fig. 5a, Additional file 7: Table S3). Although the WT and p66SHC-KO tumors were largely indistinguishable, gene ontology (GO) enrichment analysis using IPA revealed multiple enriched GOs in p52SHC-KO tumors compared with WT (Fig. 5b), including the ESR1 pathway (68 DE genes, Z-score = −2.2, P = 1.46 × 10^{-3}) and the RICTOR/mTORC2 pathway (46 DE genes, Z-score = −6.8, P = 6.17 × 10^{-19}). Collectively, these data provide the first in vivo link between p52SHC isoform and well-known drivers of breast cancer.

**Discussion**

Our data that p52SHC-KO rats have increased latency and decreased tumor multiplicity suggest that delayed...
onset of tumorigenesis observed in the mouse model of polyomavirus middle T antigen (MT)-induced mammary tumors in which critical tyrosine phosphorylation residues in SHCA are mutated (termed ShcA3F and ShcA2F) resulting in fewer tumors are likely due to phosphorylation defects of the p52SHC isoform [17]. Indeed, the same phosphorylation of p66SHC can antagonize downstream RTK signaling, whereas phosphorylated p52SHC activate signaling downstream of RTK [11]. In the p52SHC-KO animals, we report that despite being derived from the same mRNA transcript, those p46SHC protein levels are not increased as may be expected. This is likely due to the role p46SHC is performing in normal cells. Recent data suggests that p46SHC expression is restricted to mitochondria where it acts to tightly regulate thiolase and lipid oxidation [13]. As such protein levels are likely to be highly regulated. However, in cancer cells, where p52SHC and p46SHC are overexpressed, studies have indicated p46SHC is able to co-precipitate (along with p52SHC, and sometimes p66SHC) with key factors regulating tumorigenesis and proliferation and are unable to distinguish distinct functions of p46SHC or p52SHC [15, 16, 18, 33–36]. Data presented here (Fig. 4) is the first to show that p52SHC is the

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**Fig. 4** Increased tumor latency and decreased multiplicity in DMBA-induced p52SHC knockout rats. **a** Tumor latency was compared between WT (black, *n* = 14), p66SHC-KO (red, *n* = 13), and p52SHC-KO (blue, *n* = 12) DMBA-induced rats over 15 weeks. *P* < 0.015. **b** The average multiplicity of tumors at 15 weeks for each genotype. Error bars represent standard error of means. *P* < 0.004. **c** Total tumor burden, the sum total of mammary tumors per rat, isolated for each animal. The line indicates average tumor mass for each genotype. *P* < 0.02
predominant \textit{Shc} isoform that mediates mammary tumorigenesis and that p66\textit{Shc} and p46\textit{Shc} are less likely to influence tumorigenesis to a major extent. In p52\textit{Shc}-KO animals, even partial redundancy of either p66\textit{Shc} or p46\textit{Shc} may explain why some tumors do form. Furthermore, it must be mentioned that MT-induced mammary tumor model is a non-random model of breast cancer, in which MT interacts and activates a panel of host signaling molecules components of ERK, AKT and other signaling pathways, including SHC/MAPK cascade [37]. Since DMBA-induced mammary tumors in rats represent a model of random mutagenesis it resembles naturally occurring human ER+/PR+ luminal breast tumors [25, 38]. Evidence suggests that p66\textit{Shc}, while expendable in DMBA-induced tumors (Fig. 4), may function in

\begin{itemize}
  \item \textbf{Fig. 5} RNAseq analysis reveals enriched genes dis-regulated by loss of p52\textit{Shc}.
  \item \textbf{a} Histogram comparing log2 fold change in transcript expression in p52\textit{Shc}-KO tumors compared to WT (X-axis) with fold change in transcript expression in p66\textit{Shc}-KO tumors (Y-axis). Green dots symbolize genes that are differentially expressed in only one knockout group. Blue dots symbolize genes that are differentially expressed in p52\textit{Shc}-KO and p66\textit{Shc}-KO tumors in the opposite directions. Red dots symbolize differentially expressed genes p52\textit{Shc}-KO and p66\textit{Shc}-KO tumors in the same directions.
  \item \textbf{b} Bar graph indicating the top 20 enriched pathways with Z-score greater than ± 2 in p52\textit{Shc}-KO tumors, ranked by Z-score.
  \item \textbf{c}–\textbf{e} Heat map of ESR1 (\textbf{c}) and RICTOR (\textbf{d}) pathway targets identified by RNAseq analysis of wild-type, p52\textit{Shc}-KO, and p66\textit{Shc}-KO DMBA-induced tumors.
\end{itemize}
tumors with higher metastatic potential [5–7], and as such p66SHC-KO rats should be used to study more metastatic models of breast cancer.

Estrogen signaling through ESR1, also known as ERα, is well known in human breast cancers. Several lines of evidence suggest co-regulation of 17β-estradiol (E2, the ligand for ERα and ERβ) and growth factor signaling, such as IGF and EGF, in the traditional genomic regulation of gene transcription [4]. In addition to serving as a co-regulator mediating growth factor signaling with the canonical nuclear action of ERα, recent works have highlighted non-genomic functions of ERα in reciprocal activation of growth factor signaling pathways [4, 39]. Non-genomic activation of membrane ERα has been identified in tumor cells that become hypersensitive to E2 during acquired resistance to hormone deprivation therapies, which appears to be dependent on SHC1 proteins. For example, SHC1 proteins directly interact with ERα and mediate its translocation to the cell membrane for docking with IGF-1R, which subsequently leads to activation of the RAS/MAPK signaling cascade at the plasma membrane [40–42]. Ultimately, the SHC1-mediated crosstalk between ERα and IGF-1R leads to robust activation of proliferation and suppression of apoptosis [40–42]. However, despite the evidence that SHC1 is a key mediator of non-genomic ERα mechanisms of resistant to anti-estrogen therapy, the functionally significant role(s) of SHC1 isoforms were largely unclear. We addressed this question using RNAseq analysis of the p52SHC-KO and p66SHC-KO tumors, which demonstrated that loss of p52SHC disrupts ERα signaling, whereas deletion of p66SHC had no effects (Fig. 5c). Together, these data suggest that genes identified in our RNA-seq analysis of p52SHC-KO tumors represent a partial list of genes that are regulated by non-genomic ERα activation of MAP Kinase/ERK signaling pathways in a p52SHC-dependent manner. Analysis of ERK activation in primary mammary epithelial cells isolated from WT and p52ShcKO rats revealed a slight decrease in ERK activation in p52ShcKO estradiol-treated cells when compared to cells isolated from WT (Additional file 4: Figure S4).

Identification of the RICTOR signaling pathway as being significantly downregulated in p52SHC-KO tumors signifies a potential novel interaction between p52SHC and RICTOR/mTORC2 signaling pathway in mammary carcinogenesis. RICTOR, a defining component of mTORC2 (mammalian target of rapamycin complex 2), is involved in regulating kinases of the AGC group (named after the protein kinases A, G, and C families [43]). Majority of the RICTOR targets identified as being differentially regulated (Fig. 5d) represent genes identified in a microarray of liver-specific conditional RICTOR knockout mice as components of a ribosomal protein complex (EIF signaling) or oxidative phosphorylation pathways negatively regulated by RICTOR/mTORC2 signaling [44]. Coincidently, EIF2 signaling and oxidative phosphorylation represent two canonical pathways significantly dysregulated in p52SHC-KO mammary tumors (P value 2.18 × 10⁻¹² and 2.18 × 10⁻¹⁰, respectively; data not shown). While mTORC2-dependent and mTORC2-independent functions of RICTOR are known [45–48], genes identified in the RNA-seq analysis appear to represent mTORC2-dependent functions of RICTOR signaling pathways, as loss of mTOR itself also causes significant changes in oxidative phosphorylation and mitochondrial dysfunction [49, 50]. Additionally, mTOR is reported to functionally co-regulate RNA polymerase II-dependent genes, such as ribosomal proteins, many of which are also identified in our RNA-seq analysis [51]. These data suggest a previously unrecognized role for p52SHC in the regulation of RICTOR/mTORC2 signaling pathways.

Conclusion

In summary, our data strongly suggest that p52SHC-dependent signaling is required for mammary tumor progression and suggest p52SHC may serve as node interlinking several critical signaling pathways implicated breast cancer progression. Lack of overlap of genes identified as being dysregulated in ESR1 and RICTOR, signaling suggest p52SHC serves distinct functions regulating each of these pathways. Each of these pathways has been targeted therapeutically in order to combat the progression of mammary cancers in women. Our finding that p52SHC appears to be a central hub regulating these pathways provides a rationale for an exciting target for new drug therapies.

Additional files

Additional file 1: Figure S1. Hormone receptor expression status of human breast cancer samples used for qRT-PCR. Quantitative real-time RT-PCR of p52Shc/p46Shc transcript levels from TissueScan cancer survey panel of normal (red bars) and breast cancer (black bars), as seen in Fig. 1. Corresponding expression of Estrogen Receptor (ER), Prolactin Receptor (PR), or Her2 Receptor (Her2) status of breast cancers used in the qRT-PCR assay. NA, not applicable; +, positive staining; +w, weak positive staining; BL, borderline expression; NR, status not reported. (TIF 621 kb)

Additional file 2: Figure S2. Immunohistochemistry of SHC1 proteins shows the increased staining of SHC1 proteins in mammary carcinoma cells compared to adjacent normal breast epithelia or stromal cells in DMBA-induced rat breast cancer. Shown is expression of SHC1 proteins in two wild-type rat DMBA tumors. (TIF 794 kb)

Additional file 3: Figure S3. Western blot analysis of SHC1 isoforms expression in WT and genetically modified rat spleen tissue (A, top panel), freshly isolated mammary tissue fragments (A, bottom panel) and DMBA-induced mammary tumor (B). Longer exposure was used to detect p66Shc expression (A, top panel). Brackets in B indicate that tissues are isolated from the same animal. Upper panel (B) is blotted with anti-SHC1 antibodies. Relative total protein levels (B, lower panel) were determined
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Ethics approval and consent to participate

The animal studies were completed in line with our animal protocol with approval by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

KDW and AS conceptualized the study and provided the overall design of the experiments and writing of the manuscript, with KDW contributing most of the experiments. KDW and SEM analyzed tumor latency and burden. BSM carried out the analysis of expression of SHC1 isoforms in human breast cancer tissues from MCW Tissue Bank and contributed to other research aspects of this project. SWT and MIF performed the RNASeq analysis. AB provided assistance with statistical analysis. YS and KDWo performed morphology analysis of rat mammary glands. BK contributed to establishing the DMBA model. HR and YS carried out the analysis of hormone receptor expression status of DMBA-induced tumors. AMG generated the p52ShcKO rat strain. KDW, HR, MIF, and AS analyzed the data. AS supervised the study. All authors read and approved the final manuscript.

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