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Conditional knockout of SHP2 in ErbB2 transgenic mice or inhibition in HER2-amplified breast cancer cell lines blocks oncogene expression and tumorigenesis

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Abstract

Overexpression of the human epidermal growth factor receptor 2 (HER2) is the cause of HER2-positive breast cancer (BC). Although HER2-inactivating therapies have benefited BC patients, development of resistance and disease recurrence have been the major clinical problems, pointing to a need for alternative therapeutic strategies. For that to happen, proteins that play critical roles in the biology of HER2-induced tumorigenesis have to be identified and characterized. Here, we show that the Src homology phosphotyrosyl phosphatase 2 (Shp2) encoded by the *Ptpn11* gene is a requisite for ErbB2-induced tumorigenesis. We report that conditional knockout of *Shp2* alleles in the *ErbB2* BC model mice abrogates mammary tumorigenesis by blocking the expression of the *ErbB2* transgene. We also show that inhibition of SHP2 encoded by the *PTPN11* gene in the HER2-amplified BC cells induces a normal-like cellular phenotype and suppresses tumorigenesis and metastasis by blocking HER2 overexpression. These findings demonstrate that ErbB2-induced tumors in mice or xenograft tumors induced by transplantation of HER2-amplified BC cells are vulnerable to SHP2 inhibition since it abrogates the expression of the very oncogene that causes of the disease. This report paves the way for developing SHP2-targeting therapies for BC treatment in the future.

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AUTHOR CONTRIBUTION

HZ carried out the genetic studies and tissue processing, including H&E and IF staining, and microscopic imaging. EM was responsible for cell-based studies, including mammosphere formation and RT-PCR analyses. FM contributed in cell-based studies, including immunoblotting, cell transformation and cancer stem cell studies. AI contributed in the RT-PCR analyses and manuscript editing. J. MR contributed in the genetic studies and manuscript editing. NS and PL contributed in xenograft tumorigenesis studies and manuscript editing. YMA was responsible for designing, overseeing, directing the whole project, and preparation of the manuscript. In addition, YMA participated in data acquisition in the genetic and cell culture studies.

CONFLICT OF INTEREST

The authors do not have any conflict of interest to declare.

Keywords

ErbB2; HER2 overexpression; SHP2 knockout; breast cancer; tumorigenesis

INTRODUCTION

Approximately 15–20% of breast cancer (BC) is caused by overexpression of the human epidermal growth factor receptor 2 (HER2), primarily due to gene amplification (1, 2). HER2 belongs to the EGFR family of receptor tyrosine kinases (RTKs) which consist of four members, EGFR1–4 or HER1–4 (3, 4). HER2 is peculiar in that it does not require ligand binding for activation (5). As such, it can spontaneously undergo homodimerization or heterodimerization with ligand-activated family members (5–7) for transphosphorylation in the C-terminal region (8) and formation of signaling complexes (9).

The oncogenic property of HER2 was first observed when a chicken erythroblastosis virus expressing a truncated ortholog known as v-ErbB2 transformed fibroblasts (10). Later on, formation of spontaneous mammary tumors by transgenic mice overexpressing the rodent ErbB2 in the mammary gland confirmed the oncogenic property of HER2 (11). The discovery of HER2 overexpression in BC then led to the suggestion that it might play a causative role (1, 2). Multiple lines of evidence confirmed that HER2 is indeed the cause of HER2-positive BC. These findings led to the development of several HER2-targeting drugs (12) that benefited BC patients, but development of resistance has been a major clinical problem (12, 13). These challenges signify the need for alternative strategies to treat HER2-positive BC. Accumulating evidence suggests that the Src homology phosphotyrosyl phosphatase 2 (SHP2) might be another key molecule in cancer in general and BC in particular since it functions as a master regulator of RTK signaling, including HER2 (14–17). However, its mechanism in promoting cell transformation and tumorigenesis induced by RTK overexpression is poorly understood.

SHP2 is a cytoplasmic protein composed of two tandemly-arranged SH2 domains in the N-terminal, a phosphotyrosyl phosphatase (PTP) domain in the C-terminal and Tyr phosphorylation sites in the extreme C-terminal regions (18, 19). The activity of SHP2 is regulated by interaction with Tyr phosphorylated proteins via its SH2 domains. These interactions, not only activate SHP2, but also recruit SHP2 to biological substrates (18, 19). Under normal conditions, Tyr kinase signaling is a highly regulated process and so is the activity of SHP2, but dysregulation of Tyr kinase signaling as in HER2 overexpression in BC can lead to dysregulation of the SHP2 PTPase activity.

Previous reports by us (20–23) and others (24) show that SHP2 is overexpressed in BC and plays essential roles in cell transformation and xenograft tumorigenesis. However, the role of SHP2 in a disease-relevant BC model particularly in HER2-positive BC wherein hyperactivated RTK signaling is responsible for causing the disease is not clear. To address this point, we conditionally knocked out the *Ptpn11* gene that codes for the Shp2 tyrosine phosphatase in the mammary glands of the *MMTV-ErbB2* transgenic BC model mice. We show that Shp2 is essential for overexpression of the *ErbB2* transgene and induction of tumorigenesis and metastasis. These findings were corroborated in four HER2-amplified BC

cells wherein interfering with SHP2 function either by shRNA-based silencing or dominant-negative expression blocked HER2 overexpression and suppressed the transformation and tumorigenic property. In conformity to the commonly used gene annotations, we have used *Shp2* (*Ptpn11*) and *ErbB2* to refer to the rodent versions, and *SHP2* (*PTPN11*) and *HER2* to the human versions. The corresponding protein products are not italicized.

RESULTS

Generation of mice to determine importance of Shp2 in ErbB2-induced tumorigenesis

To determine the role of Shp2 in ErbB2-induced tumorigenesis, we crossed the *Ptpn11*-floxed mice (25) with the *MMTV-ErbB2* transgenic mice (11). These mice were further crossed with the *MMTV-Cre* (26) mice to conditionally delete the *Ptpn11* alleles in the mammary gland. Because the *Ptpn11*-floxed mice, hereinafter referred to as *Shp2^{f/f}* mice, were mixed lineage (129SvJ/C57BL/J) (25), it was necessary to backcross them with wild-type FVB (WT-FVB) so that they would have a similar genetic background as that of the *MMTV-ErbB2* and the *MMTV-Cre* mice, which are pure FVB, to properly compare the strains. The backcrossing scheme and the steps followed are presented in Figure 1a. Briefly, the *Shp2^{f/f}* mice were crossed with WT-FVB and the F1 progenies that were heterozygous for Shp2 alleles (*Shp2^{+/f}*) were backcrossed to WT-FVB five time. As outlined, the backcrossing steps were repeated for a total of five generations, and then the *Shp2^{+/f}* mice with an approximately 98.5% FVB background were inbred to obtain the *Shp2^{f/f}* mice (Figure 1a1). Next, the *Shp2^{f/f}* mice were crossed with the *MMTV-ErbB2* mice to obtain the *MMTV-ErbB2;Shp2^{f/f}* strain (Figure 1a2). Finally, the *MMTV-ErbB2;Shp2^{f/f}* strain was crossed with the *MMTV-Cre* mice to conditionally delete Shp2 alleles in the mammary gland. We also crossed the *MMTV-ErbB2* with the *MMTV-Cre* mice to obtain double-transgenic mice with wild type Shp2 alleles (Figure 1a3). Multiple inbreeding steps involving the F1 and the F2 generations (as denoted by successive arrows between Figure 1a3 and Figure 1a4) were conducted to obtain the four strains listed in Figure 1a4. Crossing the *Shp2^{f/f}* mice with the *MMTV-ErbB2* and the *MMTV-Cre* mice, which were pure FVB, was expected to increase the FVB background further.

As shown in Figure 1b, we had successfully generated the required strains for genetically testing the importance of Shp2 in ErbB2-induced tumorigenesis. Mice carrying the *MMTV-ErbB2* transgene and floxed Shp2 alleles, but lacking the Cre recombinase (*MMTV-ErbB2;Shp2^{f/f}*), designated as control mice 1 (CM1), were used as positive controls (lanes 6 and 7) for ErbB2-induced tumorigenesis. The double-transgenic mice with wild type Shp2 alleles (*MMTV-ErbB2;MMTV-Cre;Shp2^{+/+}*), designated as control mice 2 (CM2), were used as a second positive control (lanes 9 and 10) to show that Cre expression did not interfere with ErbB2-induced tumorigenesis. Double-transgenic mice with floxed Shp2 alleles (*MMTV-ErbB2;MMTV-Cre;Shp2^{f/f}*), designated as knockout mice (KM), were used as a test strain (lanes 3, 4 and 11), while double-transgenic mice with one floxed and one wild type Shp2 alleles (*MMTV-ErbB2;MMTV-Cre;Shp2^{+/f}*), designated as haplo-insufficient mice (HM), were used as a heterozygote strain for Shp2 (lanes 1, 2, 5 and 8). Genotyping data in Supplementary Figure 1a–d shows generation of at least 10 female mice in each category to determine the effect of Shp2 on HER2-induced tumorigenesis.

Knockout of Shp2 suppresses ErbB2-induced mammary tumorigenesis

To determine the importance of Shp2 in ErbB2-induced tumorigenesis, 10 female mice from each category were monitored for tumor development. The sample size was chosen based on prior xenograft tumorigenesis studies in which SHP2 silencing had a drastic effect (20). Just before the reported time for tumor formation by the *MMTV-ErbB2* mice (11), the females were mated continuously with the male counterparts to activate the MMTV promoter that drives ErbB2 and Cre expression. No palpable mammary tumors were observed in any of the four groups by the time the first pups were born (~21 weeks of age). However, some CM1 and CM2 mice started to develop palpable mammary tumors during the second lactation cycle (~24 weeks of age). By about 36 weeks of age, all of the control mice (CM1 and CM2) had developed palpable tumors, while only a few HM mice had small tumors (Figure 1c). Clearly, Shp2 haplo-insufficiency led to a delayed tumor onset in the HM mice. Even then, 4 out of the 10 HM mice did not form tumors by 52 weeks. With regard to the KM group, no mouse developed palpable tumors within this timeframe. Representative pictures taken at the age of 36 weeks in case of the CM and the HM and at 52 weeks in case of the KM mice show formation of larger tumors by the CM, smaller tumors by the HM, and absence of palpable tumor formation by the KM mice (Figure 1d). Overall, these results demonstrate for the first time that conditional knockout of the *Ptpn11* gene that codes for the Shp2 protein in the mammary glands of the *MMTV-ErbB2* mice blocks tumorigenesis. They also show that reduction in Shp2 expression suppresses ErbB2-induced tumorigenesis as evidenced by the reduced tumor size and delayed onset in the HM mice.

Shp2 haplo-insufficiency leads to eventual tumor regression

Because tumor development in the CM1 and the CM2 mice was similar, we have used data from the CM1 mice only, hereinafter referred to as CM, to determine tumor growth behavior. Since tumor onset was variable within each group, we used averages of three CM and three HM mice that had similar tumor onset time points to compare tumor growth rate. We also included three KM mice as negative controls. While the CM tumors were growing steadily, reaching an average of 2300 mm³ in size in a relatively shorter time, the HM tumors were growing very slowly, reaching an average of only 750 mm³ in size in a relatively longer time and regressing thereafter (Figure 1e). To show tumor growth behavior further, we compared the CM and the HM tumors by ultrasound imaging and found a linear progression in the CM and a gradual regression in the HM mice. Representative images from one CM and one HM mice are shown in Figure 1f. These results suggest that elevated Shp2 expression is required to sustain ErbB2-induced tumor growth and progression to malignancy.

Shp2 is essential for neoplastic tissue development

Although we have not detected development of palpable tumors during the first pregnancy and lactation cycle, we suspected development of microscopic neoplastic lesions. As such, mammary gland sections harvested during the first lactation cycle from extra female mice were analyzed by hematoxylin and eosin (H & E) staining. A lactating wild type mammary gland (WM) was included as a normal control. The results showed formation of comparable alveolar structures in the HM, the KM and the WM. However, the CM mammary glands were relatively denser, suggesting increased cell proliferation (Supplementary Figure 2a–d).

In fact, it was possible to detect hyperplastic lesions particularly in the CM and to some extent in the HM mammary glands as indicated by blue arrows in Supplementary Figure 2a and b particularly in the expanded outset.

To confirm development of neoplastic tissue following the first lactation cycle, we harvested mammary glands four days after weaning and analyzed sections by H & E staining. The results showed complete involution in the WM and the KM, but formation of ductal hyperplasia in the CM and to some extent in the HM mammary glands (Figure 2a, left panel, indicated by blue arrows). Similar analyses were performed after the second lactation cycle. While invasive neoplastic lesions were present in the CM mammary glands, only ductal carcinoma *in situ* (DCIS) lesions were present in the HM mammary glands (Figure 2a, right panel, indicated by blue arrows). On the other hand, the KM mammary glands did not show any obvious neoplastic lesions except a few slightly thickened ducts (Figure 2a, indicated by blue arrows), which did not progress to neoplastic tissue.

We also performed histopathology analyses on CM and HM tumors and on normal-appearing KM mammary glands harvested at the end of the experiments in each category. The results showed formation of locally invasive carcinomas in the CM, non-invasive tumors in the HM, and absence of neoplastic lesions in the KM mammary glands except a slight thickening of ducts as indicated by blue arrows (Figure 2b, left panel). Analysis of corresponding lung sections showed presence of metastatic lesions in the CM (indicated by blue arrows), but not in the HM and KM lungs (Figure 2b, right panel). Similar analyses of liver sections showed no metastatic lesions even in the CM mice (not shown). Hence, optimal Shp2 levels are needed for ErbB2-induced tumorigenesis and metastasis.

Shp2 is essential for ErbB2 expression *in vivo*

Although the KM mice carried the ErbB2 transgene, they did not form tumors. These findings led us determine the state of ErbB2 expression. Initially, we performed immunofluorescence (IF) microscopy analyses for ErbB2 and Shp2 expression in the CM and the HM tumors and in lactating KM mammary glands. Note that the KM mice did not form palpable tumors, but their lactating mammary glands had abundant mammary epithelial cells. As expected, the Shp2 signal was intense in the CM tumors, reduced in the HM tumors, and very low in the KM mammary glands (Figure 2c and Supplementary Figure 2e). We also found a similar staining pattern for ErbB2, which was unexpected. These findings suggested that Shp2 might be essential for expression of the ErbB2 transgene.

To confirm the IF findings, we conducted immunoblotting (IB) studies on protein extracts of the corresponding tumor and mammary gland tissue samples. To make conclusive comparisons, samples from five mice in each category were analyzed. Protein extracts from lactating WM mammary glands were included as controls. In agreement with the IF findings, the ErbB2 protein was elevated in the CM, reduced in the HM, and very low in the KM and the WM mammary glands (Figure 2d and Supplementary Figure 2f). With regard to Shp2, its expression was very low in the KM mammary glands, which is expected, but elevated in the CM tumors when compared to the WM normal control tissues. Interestingly, the Shp2 protein in the HM tumors was comparable to that of the WM samples despite expression from a single allele, suggesting enhancement by oncogene priming. Furthermore,

consistent with the ErbB2 and the Shp2 levels, Akt and ERK1/2 were hyperactivated in the CM, reduced in the HM, and inhibited in the KM. These findings provide the first evidence for Shp2 regulation of ErbB2 expression and downstream signaling *in vivo*. They also show that Shp2 is correspondingly elevated in ErbB2 overexpressing control tumors, suggesting interdependence between ErbB2 and SHP2 expression.

Because the IB data on protein extracts could not show whether the effect of Shp2 is at a protein or at an mRNA level, we performed quantitative reverse transcriptase PCR (qRT-PCR) analyses for ErbB2. We have used mRNA extracts from three CM and three HM tumors, and three lactating KM mammary glands. The results showed an approximately 4-fold and 10-fold reduction in ErbB2 mRNA in the HM tumors and the KM mammary glands, respectively, when compared to the CM tumors (Figure 2e). These findings confirm the IB findings that Shp2 is essential for expression of the ErbB2 transgene and further show that the effect of Shp2 is at an mRNA level.

SHP2 is essential for HER2 overexpression in human BC cell lines

Here, we sought to corroborate the mouse *in vivo* findings in human BC cell lines, including the BT474, the SUM225, the Skbr-3 and the JIMT-1 cells, that overexpress the human epidermal growth factor receptor 2 (HER2) protein. Of these, the Skbr-3 and the JIMT-1 cells also overexpress the EGFR protein (Supplementary Figure 3). In addition, these BC cells have elevated SHP2 protein and hyperactivated Akt and ERK1/2 when compared to the MCF-10A cells (Supplementary Figure 3a). To test the importance of SHP2, its expression was silenced using two independent shRNA constructs (sh1 and sh2) as described previously (21, 27). Analysis of total cell lysates showed effective silencing of SHP2 (Figure 3a). The results also showed a drastic reduction in HER2 and/or EGFR protein levels in SHP2-silenced cells (Figure 3a). Consequently, activation of Akt and ERK1/2 was reduced. Band density measurements for HER2 confirmed downregulation by 45–50 fold in the SHP2 silenced cells when compared to the background (Figure 3b). To rule out the possibility that the low level of ErbB2 in Shp2-knockout mammary glands or SHP2-silenced BC cells was not related to epitope masking caused by SHP2 loss, we used two additional anti-HER2 antibodies with epitopes in the C-terminal region. As shown in Supplementary Figure 3b, both antibodies showed similar results, suggesting that the observed change in HER2 protein was not caused by epitope masking.

To rule out the possibility of any shRNA-based artifact on the HER2 protein level, we inhibited SHP2 function by stable expression of the PTPase-dead dominant-negative SHP2 (C459S-SHP2) as described previously (28). The results showed a similar decrease in the HER2 protein level in cells that expressed C459S-SHP2, but not in those that expressed WT-SHP2 or vector alone (Figure 3c). Hence, the PTPase activity of SHP2 is required for effecting HER2 overexpression.

To determine whether the effect of SHP2 on HER2 overexpression in BC cells is at a protein or an mRNA level, we conducted qRT-PCR on RNA extracts. The results showed an approximately 10-fold reduction of in HER2 mRNA level in SHP2-silenced cells (Figure 3d). These results confirm the findings in the *MMTV-ErbB2* mice that SHP2 is essential for overexpression of the HER2 protein at an mRNA level.

SHP2 inhibition-induced loss of HER2 expression confers a normal-like phenotype

Having shown that inhibition of SHP2 blocks HER2 and/or EGFR overexpression in multiple HER2-positive BC cells, we conducted a series of assays to determine effect on cellular phenotypes. First, we observed that SHP2-silenced cells growing in 2D culture had acquired a flattened and a cobblestone-like epithelial morphology (Figure 4a and Supplementary Figure 4a). Next, we seeded the cells in soft agar to observe effect of SHP2 silencing induced loss of HER2 and/or EGFR expression on anchorage-independent growth. The results showed suppression of colony formation under this culture condition (Figure 4b and Supplementary Figure 4b). Finally, cells were tested for growth behavior in laminin-rich basement membrane (LRBM) culture, which allows morphogenesis of breast epithelial cells to form structures reminiscent of milk producing alveoli in the breast (23, 29, 30). Consistent with the acquisition of a normal like epithelial cell morphology, the SHP2-silenced cells formed acini-like structures in this culture system (Figure 4c and Supplementary Figure 4c). These findings suggest that SHP2 inhibition-induced loss of HER2 and/or EGFR expression reverses cell transformation and induces a normal-like epithelial phenotype.

SHP2 inhibition-induced loss of HER2 expression depletes cancer stem cells (CSCs)

Differentiation of the SHP2-silenced cells into a normal-like epithelial phenotype in 2D and 3D cultures was an indication for loss of CSC populations. This property was first tested by the mammosphere formation assay. While the control cells formed many and larger mammospheres in the primary culture, the SHP2-silenced cells formed fewer and smaller ones (Figure 5a and 5b, and Supplementary Figure 5a and 5b). Further passaging from a primary to a secondary culture led to an enrichment in the controls and to an exhaustion in the SHP2-silenced cells. Hence, SHP2 inhibition-induced loss of HER2 and/or EGFR expression in HER2-amplified BC cells leads to loss of mammosphere forming ability.

To confirm the mammosphere findings, we used the ALDEFLUOR assay that utilizes aldehyde dehydrogenase 1 (ALDH1) activity to estimate proportion of CSCs (31). For the ALDEFLUOR-based FACS analysis, only the control and one of the SHP2-silenced (sh2) cells from each cell line were used. A geometric median of ALDEFLUOR intensity in the MCF-10A cells (~600) was used as a baseline to compare ALDH1 levels (Supplementary Figure 5c). An ALDEFLUOR intensity 5 times higher than the MCF-10A was considered as medium and 10 times higher as high. Based on this criterion, the control BT474, JIMT-1, SUM225, and Skbr-3 cells had 10%, 14%, 6%, and 8% ALDH1-high cells, respectively (Figure 5c and 5d and Supplementary Figure 5d and 5e). On the other hand, the SHP2-silenced counterparts had very little or no ALDH1-high cells. In the presence of the inhibitor, all cells had very low level of ALDH1 activity, indicating the effectiveness of the assay. Hence, SHP2 inhibition-induced loss of HER2 and/or EGFR expression in HER2-amplified BC cells depletes CSC populations.

SHP2 inhibition-induced loss of HER2 expression blocks xenograft tumorigenesis

To confirm the cell culture findings, we conducted *in vivo* xenograft tumorigenesis studies. Approximately 10^6 control and SHP2-silenced cells derived from the BT474 and the JIMT-1 lines were transplanted into the mammary glands of NOD/SCID mice and tumor growth rate

was monitored by caliper measurement and ultrasound imaging. The control tumors grew steadily, reaching an average of 2000 mm³ in 16 weeks in case of the BT474 cells (Figure 6a) and in 8 weeks in case of the JIMT-1 cells (Figure 6b). On the other hand, tumors induced by the SHP2-silenced counterparts reached to approximately 250 mm³ in 16 weeks in case of the BT474 and to 350 mm³ in 8 weeks in case of JIMT-1 cells. Ultrasound images taken at the end of each experiment also showed formation of larger tumors by the controls and smaller tumors by the SHP2-silenced cells (Supplementary Figure 6a and 6b). Hence, silencing SHP2 suppresses the tumorigenic property of HER2-amplified BC cells.

To examine tumor growth behavior, sections were analyzed by H & E staining. The results showed variations in tumor growth behavior depending on the cell type. While tumors induced by the control BT474 cells exhibited a collective push into the stroma (Figure 6c, blue arrow), the control JIMT-1 tumors mainly showed an invasive phenotype (Figure 6d, blue arrows). On the other hand, the SHP2 shRNA tumors were rounded and non-invasive, and often surrounded by connective tissue (Figure 6c and d, red arrows). To determine the occurrence of metastasis, lung sections were examined by H & E staining. The results showed metastatic lesions only in the control JIMT-1 lungs (Figure 6c, right panel blue arrow). Lower magnification images that show the whole lung tissue sections harvested from mice transplanted with the control as well as the SHP2-silenced cells are presented in Supplementary Figure 6c–6f. These pictures also show extensive metastatic lesions in the lungs of mice transplanted with the control JIMT-1 cells (Supplementary Figure 6e, blue arrows). Hence, inhibition of SHP2 blocks the tumorigenic and/or metastatic property of HER2-overexpressing BC cells.

To determine the state of SHP2 and HER2 expression and associated signaling in xenograft tumors, we analyzed protein extracts by immunoblotting. The results showed SHP2 silencing is maintained at approximately 90% *in vivo*, which in turn led to downregulation of HER2 and/or EGFR expression and suppression of Akt and ERK1/2 activation (Figure 6e). Hence, SHP2 silencing, HER2 and/or EGFR downregulation, and downstream signaling inactivation were maintained *in vivo*.

DISCUSSION

We have shown that conditional knockout of *Shp2* in the mammary glands of the *MMTV-ErbB2* mice abolishes tumorigenesis (Figure 1c and 1d). Equally important were the longer latency, the reduced tumor incidence, and the eventual tumor regression observed in the *Shp2* haplo-insufficient (HM) mice (Figure 1e and 1f). These findings imply that inhibition of SHP2, even partially, can lead to remission of HER2-positive breast tumors in patients. A recent report has also shown that Knockout of *Shp2* in the *PyMT* transgenic mice suppresses tumorigenesis (32), supporting the findings of the current study.

It was previously reported that conditional knockout of *Shp2* in the mammary glands of wild type mice reduces lobule-alveolar development (33). But, our data in the *ErbB2* transgenic model mice show comparable mammary gland development (Supplementary Figure 2a–2d), suggesting that this physiological process was not compromised in our model, at least not in the first pregnancy and lactation cycle. The differences in the two studies might be related to

the genetic backgrounds of the mice used in the respective studies. While a transgenic BC mouse model with FVB background (*MMTV-ErbB2*) was used in the current study, a wild type BALB/c strain was used in the other report. In support of our findings, a previous study has shown that knockout of Gab2, an adaptor protein essential for signaling downstream of EGFR and HER2, delays tumor development induced by the activated form of ErbB2 (34). Since Gab2 is essential for SHP2-mediated activation of the Ras-ERK and the PI3K-Akt signaling pathways, the observed delay in tumor development phenocopies, at least in part, the Shp2 knockout findings of this study. The relatively less effective nature of the Gab2 knockout might be related to lack of effect on ErbB2 expression as opposed to the SHP2 knockout, which affects receptor expression.

Analysis of mammary gland sections harvested during the first lactation cycle showed presence of hyperplastic lesions in the CM and to some extent in the HM, but not in the KM mammary glands (Supplementary Figure 2a–2d), suggesting that oncogene priming has occurred as early as the first lactation cycle. Induction of mammary gland involution confirmed the progression of hyperplastic lesions had to neoplasms primarily in the CM and secondarily in the HM, but not in the KM (Figure 2a). Similar analysis of late-stage tumors revealed presence of local invasiveness and metastasis to the lungs in the CM, but only DCIS in the HM mice (Figure 2b). These findings show that optimal SHP2 expression is required for ErbB2-induced tumorigenesis and progression to malignancy.

Lack of tumor development in the KM mice regardless of the presence of the ErbB2 transgene was puzzling. The immunofluorescence (IF) findings of mammary gland sections (Figure 2c and Supplementary Figure 2e) combined with the immunoblotting (IB) data on corresponding protein extracts (Figure 2d and Supplementary Figure 2f) provided the first clue as to the role of SHP2 on ErbB2 transgene expression. Both methods showed a drastic reduction in ErbB2 protein level in Shp2 knockout mammary glands. Finally, qRT-PCR analyses of mRNA levels showed a more than 10-fold reduction in the KM mammary glands (Figure 2e), confirming that Shp2 is essential for expression of the ErbB2 transgene and this effect is primarily at an mRNA level. However, the IF as well as the IB data showed presence of a low level of the Shp2 protein in the KM mammary glands (Figure 2c and d, and Supplementary Figure 2e and f). This might come from basal cells, fibroblasts, and immune cells in which the MMTV promoter that drives Cre expression is not active. The mosaic nature of conditional gene deletion in general might also contribute to the presence of a low amount of the Shp2 protein. But, the trace amount of SHP2 in the KM mammary glands was insufficient to support ErbB2 expression to the level that can induce tumorigenesis. Also, a low level of the ErbB2 protein was detected in the KM mammary glands, which might be related to the effect of the remnant SHP2. The colocalization of the ErbB2 and the Shp2 IF staining signals supports this possibility (Figure 2c and Supplementary Figure 2e, white arrows). However, our results cannot rule out the possibility of other factors (other than Shp2) playing some role in low level ErbB2 expression. For instance, a previous study has shown a role for the estrogen receptor related α (ERR α) protein in positively effecting ErbB2 expression in BC model mice (35).

Consistent with the findings in the HER2 transgenic mice, silencing SHP2 or inhibition by dominant-negative expression in four HER2-amplified BC cells blocked HER2

overexpression (Figure 3a–c). The qRT-PCR data (Figure 3d) further revealed that the effect of SHP2 on HER2 overexpression was at the mRNA level. Together, these findings confirm that SHP2 is a master regulator of ErbB2 overexpression in mice and HER2 in BC cell lines at an mRNA level. Because both ErbB2 expression from the artificial MMTV promoter and HER2 from the endogenous promoter were affected by SHP2 inhibition, the likely mechanism might be that SHP2 stabilizes the mRNA transcripts or modulates the function of regulatory sequences within the mRNA. These are novel findings, but the molecular mechanism of SHP2 in promoting HER2 overexpression is yet to be discovered. It will be interesting to address these questions in future studies. However, our results cannot rule out the possibility that SHP2 loss-induced protein instability might also contribute to the low level of the HER2 protein especially given our recent report on EGFR in which we showed effect of SHP2 on both protein stability and mRNA level (20). Future studies shall address these points.

Previous studies, including our own, focused on downstream signaling often using specific ligand stimulation for a few minutes to a few hours. For instance, we have previously shown that SHP2 mediates ligand-induced EGFR and HER2 signaling by dephosphorylating RasGAP binding sites on both receptors (14, 17). Another group has also shown that the *Drosophila* homologue of SHP2, *Corkscrew*, promotes *Torso* (the PDGFR homologue) signaling by blocking RasGAP (36). It is now becoming clear that SHP2 also regulates the very expression of the receptors themselves, which is consistent with our recent report in triple-negative breast cancer cells (20).

Using multiple cellular assays in culture, we have demonstrated that SHP2 inhibition-induced loss of HER2 and/or EGFR expression reverses the cancerous phenotype of HER2-positive BC cells (Figure 4a–4c and Supplementary Figure 4a–4c). We have also demonstrated that SHP2 inhibition-induced loss of HER2 and/or EGFR expression leads to loss of mammosphere forming ability (Figure 5a and 5b and Supplementary Figure 5a 5b) and depletion of CSCs (Figure 5c and 5d, and Supplementary Figure 5d and 5e). These findings demonstrate that SHP2 inhibition-induced loss of HER2 and/or EGFR expression induces a normal-like epithelial phenotype.

The intramammary xenograft studies in NOD/SCID mice also showed that SHP2 is important for the tumorigenic and metastatic property of HER2-amplified BC cells (Figure 6a and 6b, and Supplementary Figure 6a and 6b). From the *in vivo* studies, it was possible to discern differences in the metastatic property of HER2-positive BC cells. For instance, the BT474 cells that primarily overexpress HER2 and are more epithelial-like were tumorigenic, but much less metastatic. On the other hand, the mesenchymal-like JIMT-1 cells that co-overexpress HER2 and EGFR were highly tumorigenic and metastatic. These findings suggest that co-overexpression of HER2 and EGFR might serve as an indicator of a metastatic disease. In either case, SHP2 is a critical player in HER2-positive BC. These findings demonstrate that SHP2 silencing-induced loss of HER2 and/or EGFR expression is responsible for the observed suppression of tumorigenic and metastatic property.

MATERIALS AND METHODS

Genetic studies

The SHP2-floxed mice (25) were obtained from Dr. Benjamin Neel, the HER2/*Neu* transgenic mice (11) were purchased from the Jackson Laboratory, and the MMTV-Cre (26) were obtained from Dr. Timothy Lane. A standard crossbreeding protocol as outlined in Figure 1a was used in the study. Genomic DNA isolated from tail snips was used for PCR genotyping of mice. The forward and the reverse primers in a respective order include 5'-TAGCTGCTTTAACCCTCTGTGT-3' and 5'-CATCAGADCAGGCCATATTCC-3' for SHP2, 5'-TTTCCTGCAGCAGCCTACGC-3' and 5'-CGGAACCCACATCAGGCC-3' for HER2, and 5'-ATCCGAAAAGAAAACGTTGA-3' and 5'-ATCCAGCTTACGGATATAGT-3' for Cre. The internal control primers for the HER2 transgene were the forward 5'-CAAATGTTGCTTGTCTGGTG-3' and the reverse 5'-GTCAGTCGAGTGCACAGTTT-3'. All genetic studies were conducted by strictly following the approved protocol by the West Virginia University Institutional Committee for animal Use and Care (WVU ICUC).

Caliper-based tumor volume measurement

Tumor growth rate in each mouse was determined by measuring tumor volume with a caliper every week. The length (l) and the width (w) were measured directly while the height was estimated by calculating the average of the two measurements. Hence, the formula $l \times w \times (l+w)/2$ was used to determine tumor volume in mm³.

Ultrasound imaging

Tumor imaging was conducted using a Visual Sonics Vevo 2100 high-resolution micro-ultrasound imaging system (VisualSonics, Toronto, ON, Canada). Briefly, the mice were anesthetized with an isoflurane-oxygen mixture, applied with hair remover, and imaged by placing a 40 mHz transducer over the mammary tumor. The tumors were scanned to generate a series of images with 0.076 step size between images. The image with the maximum radius was used for comparison among the experimental groups.

Histopathology and immunofluorescence (IF) studies

Formalin-fixed and paraffin-embedded tissues were sectioned at a thickness of 5 µm for hematoxylin and eosin (H & E) and IF studies. The standard protocol for both H & E and IF staining is described in our recent report (20). Pictures were collected under an Olympus IX71 microscope equipped with DP30 and DP80 digital cameras and cellSense software.

Cells, cell culture and reagents

The BT474, the Skbr-3 and the SUM225 cells were purchased from ATCC, and the JIMT-1 cells were purchased from DSMZ, Germany. All cells were grown as recommended by the respective companies. Cells with as few as 10 culture passages were used for most of the experiments. Mycoplasma contamination was monitored by PCR analysis from time to time using commercially available kits. In addition, normoxin (NC9273499, Fisher Scientific) that has an anti-mycoplasma effect is used in the cell growth media. The anti-HER2

antibodies used were a monoclonal antibody (610162) from BD Biosciences, and a rabbit anti-pTyr1221/1222-HER2 (2243) and a rabbit anti-HER2 (2165) from Cell Signaling Technologies. While the BD antibody recognizes the extracellular region (amino acids 182–373), the Cell Signaling antibodies recognize the cytoplasmic autophosphorylation site. The other antibodies used were anti-SHP2 (610822) from BD Biosciences, rabbit anti-pan-ERK2 (SC-81457) from Santa Cruz Biotechnology, anti- β -actin (A5441) from Sigma-Aldrich, anti-phospho-ERK1/2 (9101S), anti-phospho-Akt (9271S), and anti-pan-Akt (11E7) from Cell Signaling. All the antibodies are extensively characterized as evidenced by a large body of literature, including our own (17, 20–23, 37).

Inhibition of SHP2 in HER2-positive breast cancer cells

Two approaches were used to inhibit SHP2 in HER2-positive breast cells. First, we used shRNA-based SHP2 silencing as described by us and others previously (21, 38). And second, we employed retrovirus-based dominant negative SHP2 (C459S-SHP2) expression as described by us previously (28). To discriminate from the endogenous, FLAG-tagging at the C-terminus of SHP2 was used.

Preparation of tissue and cell lysates and immunoblotting

Tissue samples were first minced and then suspended in cell lysis buffer containing 20 mM Tris-HCl, pH7.2, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 10% glycerol, 1% triton-X-100, 1 mM sodium orthovanadate and a protease inhibitor cocktail. The suspensions were sonicated on ice, incubated at 4°C for 4 hours with rocking, centrifuged at 12,000 rpm for 10 minutes, and used for immunoblotting analyses. Cell lysates were prepared in the same buffer without vigorous sonication. Electrophoretic and immunoblotting analyses were conducted as described previously (21, 38). Specific antibodies were used one-after-another for detection of different proteins on the same membrane. For this, some full-length blots contain bands for different proteins (e.g. pAkt and pERK1/2). X-ray films were digitalized by scanning at 600 dpi resolution using Adobe Photoshop software.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The ErbB2 mRNA in transgenic mice and the HER2 mRNA in HER2 amplified BC cell lines were determined by qRT-PCR using the BioRad IScript reverse transcription Supermix and IQ SYBR Green Supermix following the manufacturers' protocol. All the primers were purchased from Integrated DNA technologies Inc. For HER2, the forward primer was 5'-TTTCATCCTCATCATCTTCACATTG-3' while the reverse primer was 5'-GTAGAACCTTTGCTGTCCTGT-3. The probe sequence is included in the primer mix. For human glyceraldehyde phosphate dehydrogenase (GAPDH), the forward primer was 5'-ACAGCCTCAAGATCATCAGCAATG-3' while the reverse primer was 5'-TGTGGTCATGAGTCCTTCCACGATAG-3. The HER2 mRNA expression level was corrected against GAPDH mRNA in all cell lines. The corresponding primers for rat ErbB2 were a forward 5'-AGATCCTGAAGGGAGGAGTT-3' and a reverse 5'-CAGGAGCCAGTTGGTTATTCT-3'. For rat GAPDH, the forward primer was 5'-GTGGAGTCATACTGGAACATGTAG-3', while the reverse primer was 5'-AATGGTGAAGGTCCGGTGTG-3'.

Anchorage-independent growth assay

To determine the effect of SHP2-silenced downregulation of HER2 on cell transformation, we conducted anchorage-independent growth in soft agar as described previously (17, 23). Ability of cells to form colonies was monitored by visualization under a microscope. Pictures were collected under the IX71 Olympus microscope equipped with a digital camera.

Laminin-rich basement membrane (LRBM) culture

Acini-like structure formation in 3D laminin-rich basement membrane (LRBM) culture as was performed as described previously (23). For this assay, four-well chamber slides (Falcon) were first overlaid with 80 μ l of growth factor reduced LRBM medium (BD Biosciences) and then approximately 10^3 cells suspended in regular growth medium were seeded per well and incubated at 37°C for about two weeks. Bright-field pictures were collected under Olympus IX71 microscope.

Mammosphere formation assay

The mammosphere assay was conducted as described previously (39). Briefly, cells were cultured in serum-free DMEM, 1 μ g/ml hydrocortisone, 10 μ g/ml insulin, 10 ng/ml EGF, 10 ng/ml FGF, 5 ng/ml heparin, and B27 (Invitrogen) in ultra-low adherence culture plates. For passaging, the primary spheres were collected by centrifugation, dissociated to single cells by trypsinization and pipetting, and seeded in new ultra-low attachment plates. Both primary and secondary mammospheres were pictured after 10 days of incubation.

ALDUFLUOR assay

The ALDEFLUOR assay was used to determine the effect of SHP2 inhibition-induced loss of HER2 expression on the cancer stem cell phenotypes. The kit was purchased from the Stem Cell Technologies (catalog number 01700) and the manufacturer's protocol was strictly followed to prepare the cells for fluorescent activated cell sorting (FACS). Finally, the cells were sorted using FACS Diva Version 6.1.3 to determine proportion of cells with variable ALDH1 activity.

Intramammary xenograft tumorigenesis studies

The intramammary xenograft studies were conducted as per the approved institutional Animal Care and Use Committee protocol (WVU #1603001644) and using the procedure described by us recently (20). Female NOD/SCID mice were purchased from the Jackson laboratories and approximately 10^6 cells mixed in a 1:1 ratio with matrigel (BD Biosciences) were injected into the mammary fat pad of each mouse using a hypodermic needle. After transplantation, tumor growth monitoring was conducted as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Pegram MD, Konecny G, Slamon DJ. The molecular and cellular biology of HER2/neu gene amplification/overexpression and the clinical development of herceptin (trastuzumab) therapy for breast cancer. *Cancer Treat Res* 2000;103:57–75. [PubMed: 10948442]
2. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987;235(4785):177–82. [PubMed: 3798106]
3. Brandt BH, Roetger A, Dittmar T, Nikolai G, Seeling M, Merschjann A, et al. c-erbB-2/EGFR as dominant heterodimerization partners determine a motogenic phenotype in human breast cancer cells. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 1999;13(14):1939–49. [PubMed: 10544177]
4. Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas CF, 3rd, Hynes NE. The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. *Proceedings of the National Academy of Sciences of the United States of America* 2003;100(15):8933–8. [PubMed: 12853564]
5. Garrett TP, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, et al. The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. *Molecular cell* 2003;11(2):495–505. [PubMed: 12620236]
6. Garrett TP, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, et al. Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha. *Cell* 2002;110(6):763–73. [PubMed: 12297049]
7. Ogiso H, Ishitani R, Nureki O, Fukai S, Yamanaka M, Kim JH, et al. Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains. *Cell* 2002;110(6):775–87. [PubMed: 12297050]
8. Pawson T, Gish GD. SH2 and SH3 domains: from structure to function. *Cell* 1992;71(3):359–62. [PubMed: 1423600]
9. Schlessinger J SH2/SH3 signaling proteins. *Current opinion in genetics & development* 1994;4(1):25–30. [PubMed: 8193536]
10. Difiore PP, Pierce JH, Kraus MH, Segatto O, King CR, Aaronson SA. Erbb-2 Is a Potent Oncogene When Overexpressed in Nih/3t3 Cells. *Science* 1987;237(4811):178–82. [PubMed: 2885917]
11. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proceedings of the National Academy of Sciences of the United States of America* 1992;89(22):10578–82. [PubMed: 1359541]
12. Dent S, Oyan B, Honig A, Mano M, Howell S. HER2-targeted therapy in breast cancer: a systematic review of neoadjuvant trials. *Cancer treatment reviews* 2013;39(6):622–31. [PubMed: 23434074]
13. Verma S, Miles D, Gianni L, Krop IE, Welslau M, Baselga J, et al. Trastuzumab emtansine for HER2-positive advanced breast cancer. *The New England journal of medicine* 2012;367(19):1783–91. [PubMed: 23020162]
14. Agazie YM, Hayman MJ. Molecular mechanism for a role of SHP2 in epidermal growth factor receptor signaling. *Molecular and cellular biology* 2003;23(21):7875–86. [PubMed: 14560030]
15. Ahmed Z, George R, Lin CC, Suen KM, Levitt JA, Suhling K, et al. Direct binding of Grb2 SH3 domain to FGFR2 regulates SHP2 function. *Cellular signalling* 2010;22(1):23–33. [PubMed: 19735729]

16. Frearson JA, Alexander DR. The phosphotyrosine phosphatase SHP-2 participates in a multimeric signaling complex and regulates T cell receptor (TCR) coupling to the Ras/mitogen-activated protein kinase (MAPK) pathway in Jurkat T cells. *The Journal of experimental medicine* 1998;187(9):1417–26. [PubMed: 9565634]
17. Zhou X, Agazie YM. Molecular mechanism for SHP2 in promoting HER2-induced signaling and transformation. *The Journal of biological chemistry* 2009;284(18):12226–34. [PubMed: 19261604]
18. Feng GS, Shen R, Heng HH, Tsui LC, Kazlauskas A, Pawson T. Receptor-binding, tyrosine phosphorylation and chromosome localization of the mouse SH2-containing phosphotyrosine phosphatase Syp. *Oncogene* 1994;9(6):1545–50. [PubMed: 8183548]
19. Hadari YR, Kouhara H, Lax I, Schlessinger J. Binding of Shp2 tyrosine phosphatase to FRS2 is essential for fibroblast growth factor-induced PC12 cell differentiation. *Molecular and cellular biology* 1998;18(7):3966–73. [PubMed: 9632781]
20. Matakah F, Martin E, Zhao H, Agazie YM. SHP2 acts both upstream and downstream of multiple receptor tyrosine kinases to promote basal-like and triple-negative breast cancer. *Breast cancer research : BCR* 2016;18(1):2. [PubMed: 26728598]
21. Zhao H, Agazie YM. Inhibition of SHP2 in basal-like and triple-negative breast cells induces basal-to-luminal transition, hormone dependency, and sensitivity to anti-hormone treatment. *BMC cancer* 2015;15:109. [PubMed: 25885600]
22. Zhou X, Coad J, Ducatman B, Agazie YM. SHP2 is up-regulated in breast cancer cells and in infiltrating ductal carcinoma of the breast, implying its involvement in breast oncogenesis. *Histopathology* 2008;53(4):389–402. [PubMed: 18643929]
23. Zhou XD, Agazie YM. Inhibition of SHP2 leads to mesenchymal to epithelial transition in breast cancer cells. *Cell death and differentiation* 2008;15(6):988–96. [PubMed: 18421299]
24. Muenst S, Obermann EC, Gao F, Oertli D, Viehl CT, Weber WP, et al. Src homology phosphotyrosyl phosphatase-2 expression is an independent negative prognostic factor in human breast cancer. *Histopathology* 2013;63(1):74–82. [PubMed: 23672411]
25. Fornaro M, Burch PM, Yang W, Zhang L, Hamilton CE, Kim JH, et al. SHP-2 activates signaling of the nuclear factor of activated T cells to promote skeletal muscle growth. *The Journal of cell biology* 2006;175(1):87–97. [PubMed: 17015617]
26. Li G, Robinson GW, Lesche R, Martinez-Diaz H, Jiang Z, Rozengurt N, et al. Conditional loss of PTEN leads to precocious development and neoplasia in the mammary gland. *Development* 2002;129(17):4159–70. [PubMed: 12163417]
27. Hartman ZR, Schaller MD, Agazie YM. The tyrosine phosphatase SHP2 regulates focal adhesion kinase to promote EGF-induced lamellipodia persistence and cell migration. *Molecular cancer research : MCR* 2013;11(6):651–64. [PubMed: 23512980]
28. Burks J, Agazie YM. Modulation of alpha-catenin Tyr phosphorylation by SHP2 positively effects cell transformation induced by the constitutively active FGFR3. *Oncogene* 2006;25(54):7166–79. [PubMed: 16767162]
29. Debnath J, Muthuswamy SK, Brugge JS. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods* 2003;30(3):256–68. [PubMed: 12798140]
30. Muthuswamy SK, Li D, Lelievre S, Bissell MJ, Brugge JS. ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. *Nature cell biology* 2001;3(9):785–92. [PubMed: 11533657]
31. Kim YS, Jung MJ, Ryu DW, Lee CH. Clinicopathologic characteristics of breast cancer stem cells identified on the basis of aldehyde dehydrogenase 1 expression. *Journal of breast cancer* 2014;17(2):121–8. [PubMed: 25013432]
32. Lan L, Holland JD, Qi J, Grosskopf S, Vogel R, Gyorffy B, et al. Shp2 signaling suppresses senescence in PyMT-induced mammary gland cancer in mice. *The EMBO journal* 2015.
33. Ke Y, Lesperance J, Zhang EE, Bard-Chapeau EA, Oshima RG, Muller WJ, et al. Conditional deletion of Shp2 in the mammary gland leads to impaired lobulo-alveolar outgrowth and attenuated Stat5 activation. *The Journal of biological chemistry* 2006;281(45):34374–80. [PubMed: 16959766]

34. Bentires-Alj M, Gil SG, Chan R, Wang ZC, Wang Y, Imanaka N, et al. A role for the scaffolding adapter GAB2 in breast cancer. *Nature medicine* 2006;12(1):114–21.
35. Deblois G, Chahrour G, Perry MC, Sylvain-Drolet G, Muller WJ, Giguere V. Transcriptional control of the ERBB2 amplicon by ERRalpha and PGC-1beta promotes mammary gland tumorigenesis. *Cancer research* 2010;70(24):10277–87. [PubMed: 20961995]
36. Cleghon V, Feldmann P, Ghiglione C, Copeland TD, Perrimon N, Hughes DA, et al. Opposing actions of CSW and RasGAP modulate the strength of Torso RTK signaling in the Drosophila terminal pathway. *Molecular cell* 1998;2(6):719–27. [PubMed: 9885560]
37. Hartman Z, Zhao H, Agazie YM. HER2 stabilizes EGFR and itself by altering autophosphorylation patterns in a manner that overcomes regulatory mechanisms and promotes proliferative and transformation signaling. *Oncogene* 2013;32(35):4169–80. [PubMed: 23027125]
38. Hartman ZR, Schaller MD, Agazie YM. The tyrosine phosphatase SHP2 regulates focal adhesion kinase to promote EGF-induced lamellipodia persistence and cell migration. *Molecular cancer research : MCR* 2013.
39. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, et al. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes & development* 2003;17(10):1253–70. [PubMed: 12756227]

Figure 1: Generating mice strains and testing the importance of Shp2 in ErbB2-induced mammary tumorigenesis.

(a1) Crossbreeding the *Shp2*^{fl/f} mice with pure wild type (WT) FVB 5 times (5×) to eventually obtain *Shp2*^{fl/f} mice with ~98.5% FVB background. These steps involved initial crossing and inbreeding the F1 generation. (a2) Crossing the *Shp2*^{fl/f} mice with the *MMTV-ErbB2* transgenic mice with FVB background to obtain the *MMTV-ErbB2;Shp2*^{fl/f} strain which served as control mice 1 (CM1). These steps also involved initial crossing and inbreeding the F1 generation. (a3) Crossing the *MMTV-ErbB2;Shp2*^{fl/f} or the *MMTV-ErbB2* mice with the *MMTV-Cre* mice to finally obtain the second control (*MMTV-ErbB2;MMTV-Cre;Shp2*^{+/+}) designated as CM2, the *Shp2* haplo-insufficient strain (*MMTV-ErbB2;MMTV-Cre;Shp2*^{+/f}) designated as HM, and the *Shp2* knockout or test strain (*MMTV-ErbB2;MMTV-Cre;Shp2*^{fl/f}) designated as KM. (a4) List of the four experimental mice strains used in the study. The successive arrows between Figure 1a3 and 1a4 denote inbreeding the F1 and the F2 generations to obtain the required strains. (b) Genotyping PCR

data showing the generation of founder mice strains in each category. (c) Line graph showing tumor development in the control (CM1 and CM2), in the haplo-insufficient (HM), and in the *Shp2* knockout (KM) mice. (d) Representative pictures showing presence of tumors in the CM and the HM and absence in the KM mice. The dotted line represents the approximate boundary of tumors. (e) Tumor growth rate in the CM (CM1), the HM and the KM mice as determined by tumor volume measurement in live animals every week. Data shown is mean $\pm$ SEM from three mice in each group. (f) Representative ultrasound images of one CM and one HM mouse taken at 3 weeks interval.

Figure 2: Analysis of tissue samples collected from the genetic studies.

(a) Representative pictures of H & E stained CM, HM, KM, and WM mammary gland sections harvested after inducing involution following the first (left panel) and the second (right panel) lactation cycles. The scale bar at the bottom right of each panel is 100 μ m. (b) H & E stained CM and HM tumors sections, KM mammary gland sections (left panel), and corresponding lung sections (right panel). (c) Immunofluorescence (IF) staining for HER2 and SHP2 in tissue sections that correspond to panel **b**. (d) Immunoblotting (IB) analysis of protein extracts from the CM and the HM tumors and from lactating KM mammary glands for Shp2, ErbB2, β -actin, activated Akt (pAkt), total Akt (panAkt), activated ERK1/2 (pERK1/2), and total ERK2 (panERK2). IB images were grouped together by cropping from full-length exposures. (e) Bar graph showing qRT-PCR analysis of the ErbB2 mRNA level in the CM and KM tumors and lactating KM mammary glands.

Figure 3: SHP2 is required for HER2 expression in HER2-amplified BC cells.

(a) Immunoblotting (IB) analysis of protein extracts from the indicated BC cell lines. Total cell lysates from parental (P), control (c) and the two SHP2 silenced derivatives (sh1 and sh2) were analyzed for SHP2, HER2, EGFR, β -actin, activated Akt (pAkt), total Akt (panAkt), activated ERK1/2 (pERK), and total ERK2 (panERK2). IB images were grouped together by cropping from full-length exposures. (b) Bar graphs showing IB band density measurements for HER2 in the four HER2-positive BC cell lines adjusted to background. Data shown is mean $\pm$ SEM of three independent analyses in each cell line. (c) Inhibition of SHP2 by dominant-negative (C459S-SHP2) expression in four HER2-amplified breast cancer cells blocks HER2 overexpression. (d) Bar graphs showing relative HER2 mRNA levels in the control and the corresponding SHP2-silenced (sh2) cells in the four HER2-amplified BC cell lines as determined by qRT-PCR.

Figure 4: SHP2 is required for the growth and transformation phenotypes of the HER2 gene-amplified BC cell lines.
 (a) Silencing SHP2 in the BT474 and the JIMT-1 cells induces a cobblestone-like epithelial phenotype. (b) Silencing SHP2 in BT474 and JIMT-1 cells abrogates anchorage-independent growth (colony formation) in soft agar. (c) Silencing SHP2 in the BT474 and the JIMT-1 cells induces acini-like structure formation in laminin-rich basement membrane (LRBM) culture. While the 2D and the LRBM cultures were collected under the 10× objective, that of the soft agar was under the 5× objective.

Figure 5: Silencing SHP2 expression leads to loss of CSC populations.

(a and b) Effect of SHP2 silencing on the mammosphere (tumorisphere) forming capacity of the BT474 and the JIMT-1 cells. Note that silencing SHP2 leads to exhaustion of mammosphere forming capacity on passaging from primary to secondary cultures. Con: control; sh1: SHP2 shRNA-1; sh2: SHP2 shRNA-2. These pictures were collected under the 5× objective. (c and d) Effect of SHP2 silencing on ALDH1 activity as determined by the ALDEFLUOR assay in the control and the SHP2 silenced BT474 and JIMT-1 cells. The three gated channels from left to right represent low, medium, and high intensity and the numbers above each channel represent percent of cells. Note that the ALDEFLUOR intensity in both the control and the SHP2-silenced cells in the presence of ALDH1 inhibitor was very low.

Figure 6: Silencing SHP2 suppresses tumorigenesis and blocks metastasis.

(a and b) Line graphs showing effect of SHP2 silencing on xenograft tumor growth rate induced by intramammary transplantation of the BT474 and the JIMT-1 cells. Data shown is mean $\pm$ SEM of tumor volume from 5 mice in each category (5 controls and 5 SHP2 shRNA) as determined by caliper measurement. (c and d) Representative H and E stained tumor (left panels) and lung (right panels) sections from control and SHP2 shRNA mice transplanted with derivatives of the BT474 and the JIMT-1 cells. The pictures were collected at 5 $\times$ magnification. The bar graph at the bottom right hand side represents 100 μ m. (e) Immunoblotting analysis of protein extracts from control (Con Tumors) and SHP2 silenced (sh Tumors) tumors for SHP2, HER2, EGFR, pAkt, panAkt, pERK1/2, panERK2, and β -actin.