SHP2 Potentiates the Oncogenic Activity of β-Catenin to Promote Triple-Negative Breast Cancer
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ABSTRACT
Previous studies have reported dysregulated cytoplasmic and nuclear expression of the β-catenin protein in triple-negative breast cancer (TNBC) in the absence of Wnt signaling pathway dysregulation. However, the mechanism that sustains β-catenin protein dysregulation independent of Wnt signaling is not understood. In this study, we show that Src homology phosphotyrosyl phosphatase 2 (SHP2) is essential for β-catenin protein stability and for sustaining the cytoplasmic and nuclear pools in TNBC cells. The first evidence for this possibility came from immunofluorescence (IF) and immunoblotting studies that showed that inhibition of SHP2 induces E-cadherin expression and depletion of cytoplasmic and nuclear β-catenin, which in turn confers adherence junction mediated cell–cell adhesion. We further show that SHP2 promotes β-catenin protein stability by mediating the inactivation of GSK3β through its positive effect on Akt and ERK1/2 activation, which was confirmed by direct pharmacologic inhibition of the PI3K-Akt and the MEK-ERK signaling pathway. Finally, we show that SHP2-stabilized β-catenin contributes to TNBC cell growth, transformation, cancer stem cell (CSC) properties, and tumorigenesis and metastasis. Overall, the findings in this report show that SHP2 mediates β-catenin protein stability to promote TNBC.

Implications: Data presented in this article demonstrates that SHP2 positively regulates β-catenin protein stability, which in turn promotes triple-negative breast cancer (TNBC) cell transformation, tumorigenesis, and metastasis.

Introduction
Of all breast cancer subtypes, triple-negative breast cancer (TNBC) causes disproportionately high mortalities in women particularly in racial minorities and younger women of all races (1, 2). The major factors for the poor clinical outcome are the aggressive nature of the disease and the lack of targeted therapies. Developing targeted therapies against TNBC has been very difficult primarily because of the multiplicity and heterogeneity of dysregulated molecules and their overlapping functions. One of the major molecular aberration in TNBC is the increased expression of multiple receptor tyrosine kinases (RTK), including EGFR, cellular MET receptor (c-MET), fibroblast growth factor receptor 1 and 2 (FGFR1 and FGFR2), insulin like growth factor-1 receptor (IGF-1R), and vascular endothelial growth factor receptor (VEGFR; refs. 3–5). Because many of these RTKs are potent activators of mitogenic and cell-survival signaling in TNBC, their overexpression is suggested to contribute to tumorigenesis (6) on the basis of which small molecule tyrosine kinase inhibitors and antibody-based drugs that inactivate these molecules are being investigated as potential treatments (7, 8).

The other molecular aberration known to occur in TNBC is the increased expression and transcriptional activity of β-catenin, the major downstream mediator of the Wnt signaling pathway (9). Under physiologic conditions, the Wnt signaling pathway is initiated by the binding of Wnt ligands to the LRPS/6 and frizzled coreceptors at the cell surface. Ligand-activated frizzled receptors disable the β-catenin destruction complex, leading to a temporal increase in β-catenin cytoplasmic pool and translocation to the nucleus where it interacts with the T-cell factor/lymphoid enhancer factor 1 (TCF/LEF1) family of DNA-binding proteins and derepress gene expression (10, 11). In the absence of Wnt signaling, the cytoplasmic pool of β-catenin is kept low by the actions of the β-catenin destruction complex that includes Axin, glycogen synthase kinase-3 β (GSK3β), casein kinase-1 (CK-1), and adenomatous polyposis coli (APC). Dysfunction in this regulatory mechanism can lead to accumulation of cytoplasmic β-catenin and dysregulated gene expression. For instance, mutations of APC that lead to loss of its interaction with β-catenin and hence to dysregulated β-catenin signaling are very common in colon cancer. Accumulating evidence suggests that dysregulated β-catenin signaling also occurs in TNBC, but the mechanism is not clearly understood (12, 13) especially because mutation of APC and other β-catenin destruction complex proteins is rare (14, 15).

Multiple lines of evidence suggest that tyrosine kinase signaling can also lead to β-catenin protein stability. For instance, activated receptor tyrosine kinases (RTK) such as EGFR, c-MET, and RON can directly phosphorylate β-catenin on multiple tyrosine residues, which in turn leads to protein stability and increased transcriptional activity (16). Also, reports show that activated downstream-signaling molecules such as Akt and ERK1/2 stabilize β-catenin by inactivating the activity of GSK3β through phosphorylation on the inhibitory Ser9 residue (17). Since dysregulation of multiple RTKs is known to occur in TNBC and since the Src homology phosphotyrosyl phosphatase 2 (SHP2) is a master regulator of RTK expression and signaling in TNBC (18), we hypothesized that it might be a key mediator of dysregulated β-catenin signaling in TNBC. Additionally, our previous reports in TNBC cells that showed mesenchymal to epithelial transition (MET) upon SHP2 inhibition led us to hypothesize that it might control the mesenchymal
property. In the current report, we show that SHP2 promotes β-catenin protein stability and signaling in TNBC cells. We also show that SHP2-stabilized β-catenin contributes to the transformation, tumorigenesis and metastatic potential of TNBC cells.

Materials and Methods

Cells, cell culture, and reagents
The MDA-MB231 and the MDA-MB468 TNBC cell lines, which were purchased from ATCC, were used in this study. In addition, the nontransformed MCF-10A breast epithelial cells also purchased from ATCC were used as controls in some experiments. In all cases, cells with less than 10 passages were used in this study. Mycoplasma testing was not performed, but we have used normoxin (catalog no. #ant-nr-1, InvivoGen) in our cultures, which is known to suppress mycoplasma contamination. The conditions under which the cells were propagated and used in the various experiments were described previously (19, 20).

The anti-SHP2 (610822) and the anti-GSK3β (610201) antibodies were from BD Biosciences, the anti-pan-ERK2 (SC-246), and the anti-pan-ERK1 (SC-40), the anti-cyclin D1 (SC-246), and the anti-phospho-ERK1/2 (9101S), anti-phospho-Akt (9271S), anti-phospho-GSK3β (9323), and anti-β-catenin (9561) from Cell Signaling Technology. The antimouse and antirabbit secondary antibodies conjugated with horseradish peroxidase were purchased from Jackson Immuno-Research Laboratories, whereas the fluorochrome-labeled versions were from Life Technologies. The allosteric SHP2 inhibitor SHP099 and a specific inactive-site inhibitor CNBDA were developed by the senior author (21). The SHP2 inhibitor SHP099 (21) was used in the various experiments as described previously (19, 20).

Two independent short hairpin RNA (shRNA) constructs were used to silence the expression of each protein (SHP2 and β-catenin). Silencing SHP2 expression in the MDA-MB231 and MDA-MB468 was described by us previously (23, 24). For β-catenin silencing, two independent shRNA constructs (SHCCLNG-NM_001904 CTRCN0000314991 and TRCN0000314921, Sigma) were used. The shRNA constructs and supporting plasmids were transfected into 293T packaging cells using FuGene (Roche) transfection reagent. Viral particle collection and supporting plasmids were transfected into 293T packaging cells using IScript reverse transcription Supermix and IQ SYBR Green Supermix (Bio-Rad Laboratories). The state of β-catenin mRNA expression in the control and the SHP2-silenced cells was determined by qRT-PCR using the BioRad iScript reverse transcription Supermix and IQ SYBR Green Supermix following the manufacturers’ protocol. The state of GAPDH mRNA expression was used as a control. The qPCR probes for both β-catenin and GAPDH were purchased from Integrated DNA technologies, Inc.

Silencing SHP2 and β-catenin expression

Cells, cell culture, and reagents

The SHP2 and β-catenin silencing were performed as described above. For SHP2 silencing, a specific shRNA construct targeting SHP2 mRNA expression levels was used. The state of GAPDH mRNA expression in the control and the SHP2-silenced cells was determined by qRT-PCR using the BioRad iScript reverse transcription Supermix and IQ SYBR Green Supermix following the manufacturers’ protocol. The state of GAPDH mRNA expression was used as a control. The qPCR probes for both β-catenin and GAPDH were purchased from Integrated DNA technologies, Inc.

Determining cell proliferation rate

For determining effect of β-catenin silencing on cell proliferation, we used a direct cell counting method from randomly collected bright-light microscopic images. Cells were thinly seeded in 6-cm cultures dishes, and pictures were collected at 4x objective from 10 random fields immediately after attachment, and then every 24 hours thereafter for a total of 3 days. Number of cells in fixed quadrants in each image was used for comparing cell proliferation rate. For each time point, average of cells in 10 images were counted using the Adobe Photoshop software to arrive at an average. Growth rate (duplication rate) was determined by dividing the averages at each time point to the average of the initial time point (number of cells immediately after attachment). The results were plotted as line graph using fold over initial value versus time in hours.

Anchoragel-independent growth assay

The importance of β-catenin in TNBC cell transformation was determined by colony formation assay in soft agar as described previously (20, 25). In this assay, the bottom of 6-cm cell-culture plates was first covered with 0.5% agar in growth medium and allowed to solidify by incubation at room temperature for 15 minutes. Next, approximately 105 cells were suspended in 2 mL growth medium, mixed with melted agar to a final concentration of 0.3%, and immediately poured onto the agar overlay. After 5 minutes of incubation in the luminal flow hood, plates were transferred into a 37°C and 5% CO2 incubator and maintained for 10 days. The plates were fed with growth medium containing 0.3% soft agar twice, and after that, once
with growth medium only. Colony formation was monitored by visualization under a microscope, and bright-light images were taken under an Olympus IX-71 microscope equipped with DP80 digital camera.

Mammosphere formation and ALDEFLUOR assays
We have used modified versions of the previously described protocols (26) for mammosphere (tumorisphere) formation and for assaying aldehyde dehydrogenase (ALDH) A1 levels (for ALDEFLUOR assay) to

Figure 1.
Effect of SHP2 silencing on adherence junction-mediated cell–cell adhesion and expression of E-cadherin and β-catenin. A, IF staining of β-catenin and E-cadherin in control and SHP2-silenced (sh1) cells derived from the MDA-MB231 cells. B, IF staining of β-catenin and E-cadherin in control and SHP2-silenced (sh1) cells derived from the MDA-MB468 cells. C, IB analysis of total cell lysates for state of E-cadherin and β-catenin protein expression in the MDA-MB231 cell derivatives. D, IB analysis of total cell lysates for state of E-cadherin and β-catenin protein expression in the MDA-MB468 cell derivatives. E, IB analysis of cytoplasmic and nuclear protein fractions for β-catenin. PARP was used as a positive control for nuclear fractions, while β-actin was used as a control to cytoplasmic fractions. F, IB analysis of E-cadherin and β-catenin protein levels in the control untreated (Con), in SHP099 treated (Shp), in CNBDA treated (CNB), and in MCF-10A cells. These cells were treated with 250 ng/mL of either compound in the growth medium for at least 5 days.
determine the effect of β-catenin silencing on CSC properties of TNBC cells. Briefly, the control and the β-catenin–silenced cells were seeded in ultra-low adherence culture plates (approximately 5 × 10⁵ cells per 6-cm plate) in a medium, containing serum-free DMEM, 1 µg/ml hydrocortisone, 10 µg/ml insulin, 10 ng/ml EGF, 10 ng/ml FGF, 5 ng/ml heparin, and B27 (Invitrogen). Cells were fed by adding 2 ml of the medium twice in the 10-day experimental period. After 10 days, bright-light images were collected under Olympus IX-71 microscope equipped with DPho digital camera. The ALDEFLUOR assay was used to determine the proportion of ALDH1-high cells in the control and SHP2-silenced TNBC cells. The Stem Cell Technologies kit (catalog no. 01700) and protocol was used for the assay. Cells were sorted using FACSDiva version 6.1.3 (BD Biosciences) to determine the proportion of cells with high ALDH1 activity.

**Tumorigenesis studies by intramammary transplantation**

Approximately 10⁶ MDA-MB31 or 10⁶ MDA-MB468 cells expressing control shRNA or β-catenin shRNA (βsh1) were mixed with matrigel (BD Biosciences) at a 1:1 ratio and injected into the mammary fat pad of 8-weeks-old NOD/SCID mice purchased from The Jackson Laboratory. We used a total of 14 mice for each cell line (7 for control and 7 for βsh1). Tumor growth was monitored by measuring tumor volume with a caliper. 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 \( V = \frac{1}{2}l \times w \times (l+w)/2 \) was used to obtain tumor volume in mm³. These experiments were terminated when the tumor volume reached 1,500 to 2,000 mm³ in the control MDA-MB468 cells and 200 to 300 mm³ in the control MDA-MB231 cells. Tumors, lungs, and liver tissues were harvested for histopathology analysis after sacrificing the mice. The mice studies were approved by the West Virginia University Institutional Animal Care and Use Committee (IACUC), and all experiments were performed following the approved guidelines.

**Results**

**SHP2 is essential for cytoplasmic, nuclear, and total β-catenin levels in TNBC cells**

Inhibition of SHP2 in TNBC cells induces a normal-like epithelial phenotype (24), suggesting occurrence of mesenchymal-to-epithelial transition (MET) in otherwise mesenchymal-like TNBC cells. To obtain insight on the mechanism, we studied state of adherences, junction formation in 2D culture by IF staining for E-cadherin and β-catenin, the commonly used protein markers for epithelial cell–cell adhesions. The expression of SHP2 was silenced with 2 independent shRNA (sh1 and sh2) constructs as described previously (19, 24, 27) in 2 TNBC cells, the MDA-MB231 and the MDA-MB468 cells, which harbor genetic aberrations commonly discovered in TNBC (28–30).

Cells expressing a scrambled form of 1 of the shRNAs was used as a control.

The control and the SHP2-silenced MAD-MB-231 and MDA-MB468 cells were grown on coverslips and processed for IF using a standard protocol. The results showed absence of E-cadherin staining in the control cells and presence in the SHP2-silenced cells (Fig. IA and B). They also showed intense cytoplasmic and nuclear β-catenin staining in the control cells, and only membrane staining in the SHP2-silenced cells. These results were consistent with presence of adherance-free β-catenin in the control cells and only membrane-bound β-catenin in the SHP2-silenced cells.

**The effect of SHP2 on β-catenin expression in TNBC cells is at a protein level**

Data in Fig. 1 show downregulation of β-catenin in SHP2-silenced cells, but could not differentiate whether the effect is at a protein or at a transcription level. To discriminate between these possibilities, we determined β-catenin mRNA level in the control and the SHP2-silenced cells by qRT-PCR. The results showed no significant change in β-catenin mRNA level between the control and the SHP2-silenced cells (Fig. 2A). These findings suggested that the effect of SHP2 on β-catenin in TNBC cells is at a protein level. In other words, they suggested that SHP2 is essential for β-catenin protein stability. To verify this point, we conducted proteasome inhibition studies with MG132 treatment. The control and the SHP2-silenced cells were treated with 5 µg/mL MG132 for 3 hours and total protein extracts were analyzed by IF staining for β-catenin by IB. The single treatment revamped the β-catenin protein level to near control levels (Supplementary Fig. 2), indicating that the effect might indeed be at a protein level. To confirm these findings further, we conducted proteasome inhibition studies in a time-course fashion that included time points ranging from 1 to 6 hours. The control and 1 of the SHP2-silenced cells (sh1) were treated with 5 µg/mL MG132 for the indicated time points and total cell lysates prepared from them were analyzed by IB for β-catenin. The results showed restoration of β-catenin protein levels in less than 3 hours (Fig. 2B). Band density measurements from at least 3 independent experiments confirmed that the β-catenin protein level in the SHP2-silenced cells became comparable with the control cells in less than 3 hours (Fig. 2C). Reblotting with anti-SHP2 antibody showed effective silencing, while reblotting with anti-β-catenin antibody showed...
comparable protein loading in all lanes. These findings confirm that the effect of SHP2 is at a protein level and further suggest that SHP2 is essential for stability of the β-catenin protein in TNBC cells.

In a resting state, cytoplasmic β-catenin or nonadherence junction-associated β-catenin is kept low by proteasomal degradation. This process starts with sequential phosphorylation of the β-catenin degron motif, first by CK1 on Ser-45 and second by GSK3β on Ser-41, Ser-37, and Ser-33 (31). These phosphorylations mediate recognition and ubiquitination by the E3 ubiquitin ligase β-TrCP, and subsequent proteasomal degradation (31). Because proteasome inhibition fully restored β-catenin within 3 hours, we reasoned that SHP2 might prevent phosphorylation of β-catenin to promote its stability. To verify this point, control and SHP2-silenced cells were treated with Mg132 for 3 hours or left untreated, and lysates prepared from them were subjected to anti-β-catenin immunoprecipitation (IP) followed by IB with an antibody that detects phosphorylation on Ser-33 and Ser-37, the GSK3β phosphorylation sites. The results showed hyperphosphorylation of β-catenin in the SHP2-silenced cells that were treated with Mg132 (Fig. 2D). Thus, SHP2 prevents the phosphorylation of β-catenin on its degron motif.

SHP2 mediates GSK3β inactivation to promote β-catenin stability

Because β-catenin is least phosphorylated on GSK3β sites in the control cells, but highly phosphorylated in SHP2-silenced cells, we reasoned that SHP2 might regulate the activity of GSK3β to prevent...

Figure 2.
SHP2 promotes β-catenin expression at a protein level. A, Analysis of β-catenin mRNA expression by qRT-PCR. B, IB analysis for β-catenin in total cell lysates prepared from the control and one of the SHP2-silenced (sh1) cells treated with Mg132 for the indicated time points. This panel also includes IB analysis for SHP2 and for β-actin to show efficiency of silencing and protein loading, respectively. C, Bar graph showing IB band density measurements for β-catenin in the control and the SHP2-silenced (sh1) cells that correspond to the IB data in B. h, hours. Data shown is mean ± SEM adjusted to background from 3 independent analyses in each cell line. D, IP of β-catenin followed by IB analysis for phosphorylated β-catenin on the degron motif (pβ-cat). The bottom β-catenin IB shows efficiency of the IP step.
β-catenin phosphorylation and subsequent degradation. Since the activity of GSK3β itself is regulated by phosphorylation on Ser9 wherein phosphorylation inhibits its kinase activity (31), we determined the effect of SHP2 silencing on this biochemical event by 1B of total cell lysates with an antibody that specifically detects pSer9-GSK3β. The results showed an enhanced GSK3β phosphorylation on Ser9 in the parental and the control cells, but very low in the SHP2-silenced cells (Fig. 3A). The pSer9 antibody also reacts slightly with GSK3α due to epitope similarity surrounding the corresponding Ser residue (Ser21). Re-probing for total GSK3β showed the presence of comparable protein levels in all lanes. To obtain semiquantitative data, we determined band densities of pSer9-GSK3β from 3 independent experiments and found reduction in GSK3β phosphorylation on Ser9 by 60% to 70% in the SHP2-silenced cells when compared with the parental and the control cells (Fig. 3B). These findings suggest that SHP2 mediates inhibitory phosphorylation on GSK3β to prevent phosphorylation of β-catenin and subsequent degradation. Since SHP2 is essential for Akt and ERK1/2 activation, and Akt (31) and ERK1/2 (17, 32) are known to directly or indirectly phosphorylate GSK3β on Ser9, we determined state of Akt and ERK1/2 activation in the same samples. We found that Akt and ERK1/2 activation were augmented in the parental and the control cells and inhibited in the SHP2-silenced cells (Fig. 3A), suggesting an inverse relationship between SHP2-mediated signaling and GSK3β activation.

To directly test whether Akt and ERK1/2 are indeed responsible for GSK3β phosphorylation and β-catenin stability, we conducted pharmacologic inhibition studies by treating cells with LY29499 (L) and PD 0325901 (P), the specific PI3K and MEK inhibitors, respectively. Inhibition of PI3K or MEK alone caused a significant decrease in β-catenin protein levels, which was further enhanced by the combination treatment identified as LP (Fig. 3C). To verify if inhibition of PI3K and MEK renders β-catenin for proteasomal degradation, we added Mg132 in the combination-treatment cells identified as LPM and found a significant restoration. To obtain semiquantitative data, we determined band densities of β-catenin from 3 independent experiments and found reduction by 30% to 40% in the single-treatment cells and approximately 10-fold in 3 days, the β-catenin–silenced cells multiplied by approximately 6-fold over the same period of time, suggesting an approximately 40% reduction in cell proliferation rate (Fig. 4B). Effect of β-catenin silencing on anchorage-independent growth was determined by colony formation assay in soft agar. The results showed formation of relatively larger and many colonies by the Par and Con shRNA cells, but smaller and fewer ones by the β-catenin–silenced (βSh1 and βSh2) cells (Supplementary Fig. 3). Counting colony number under the 4× objective showed formation of 7–9 smaller colonies by the β-catenin–silenced cells and 22–25 relatively larger colonies by the parental and the control cells per field (Fig. 4C). Together, these results suggest that β-catenin is important for the growth and transformation of TNBC cells.

To further characterize the effect of β-catenin–silencing, we conducted mammosphere-formation assay as described in the materials and methods and in our recent reports (23, 33). This is an assay that uses a suspension culture in which cells with CSC properties only can survive, multiply, and form spherical cellular aggregates generally known as tumourosphere, and in case of breast cancer as mammosphere. Equal number of cells were seeded in nonadherent plates and mammosphere formation was monitored by observation under a microscope. After 10 days of incubation, the parental and the control cells formed larger mammospheres, while the β-catenin–silenced cells formed smaller and fewer ones (Supplementary Fig. 4). To confirm the mammosphere findings, we used the ALDEFLUOR assay that measures ALDH1 activity by FACS analysis as described previously (34). This analysis is based on our previous report that showed that CSCs have very high ALDH1 activity (35–37). Based on this method, the control MDA-MB231 and MDA-MB468 cells had 3.48% and 9.38% ALDH1-high cells, respectively, while the β-catenin–silenced counterparts had 0.4% and 0.6% ALDH1-high cells, respectively (Fig. 4D and E). Together, these findings suggest that β-catenin is important for the CSC properties of TNBC cells.

Silencing β-catenin in TNBC cells suppresses xenograft tumorigenesis and metastasis

The cell culture studies described in Fig. 4 were indicative of an essential role for β-catenin in the biology of TNBC cells. To obtain further insight, we sought to investigate the importance of β-catenin under in vivo conditions. Accordingly, we conducted xenograft tumorigenesis studies by intramammary transplantation of approximately 106 control and β-catenin-silenced TNBC cells in NOD/SCID mice. While the control tumors grew to a size of approximately 250 mm3 in 8 days, β-catenin in TNBC cells suppresses xenograft tumorigenesis and metastasis. 

β-catenin contributes to the growth and transformation of TNBC cells

After demonstrating the importance of SHP2 in mediating β-catenin stability, we asked whether β-catenin actually plays a role in the biology of TNBC cells. To address this point, the expression of β-catenin was silenced in the MDA-MB231 and MDA-MB468 cells using 2 independent shRNA constructs referred to as βSh1 and βSh2. Total cell lysates prepared from the parental, the control shRNA, and the two β-catenin shRNA cells were analyzed by IB for β-catenin. The results showed silencing of β-catenin by 90% to 95% in both cell types (Fig. 4A). In the same preparations, we also determined the levels of cyclin D1 and c-Myc, the β-catenin transcriptional target genes, and found a substantial decrease in both proteins. These results show that we were able to efficiently silence β-catenin in 2 TNBC cells, which in turn led to downregulation of the β-catenin target genes, cyclin D1 and c-Myc.

To determine the biological significance of β-catenin, we studied the effect of silencing on TNBC-cell phenotypes, including effect on cell proliferation, anchorage-independent growth, and CSC properties. Effect on cell proliferation was determined by direct counting of cells growing in 2D culture every 24 hours for a total of 3 days. While the parental (Par) and the control (Con) cells multiplied by approximately 40% reduction in cell proliferation rate (Fig. 4B). Effect of β-catenin silencing on anchorage-independent growth was determined by colony formation assay in soft agar. The results showed formation of relatively larger and many colonies by the Par and Con shRNA cells, but smaller and fewer ones by the β-catenin–silenced (βSh1 and βSh2) cells (Supplementary Fig. 3). Counting colony number under the 4× objective showed formation of 7–9 smaller colonies by the β-catenin–silenced cells and 22–25 relatively larger colonies by the parental and the control cells per field (Fig. 4C). Together, these results suggest that β-catenin is important for the growth and transformation of TNBC cells.
Figure 3.
Effect of SHP2 silencing on GSK3β activation and β-catenin silencing on cell growth and transformation. A, Silencing SHP2 activates GSK3β, while inactivating ERK1/2 and Akt. B, Bar graph showing band density measurements for pGSK3β in parental (Par), control shRNA (Con), and SHP2 shRNA (sh1 and sh2) cells expressed as percent of the bands in Par cells. Data shown is mean ± SEM adjusted to background from 3 independent analyses in each cell line. C, Pharmacologic inhibition of PI3K and MEK leads to activation of GSK3β (reduced pGSK3β) and downregulation of β-catenin. D, Bar graph showing band density measurements for β-catenin levels in the control-untreated (c), in the LY294249-treated (L), in the PD-0325901 treated (P), in the combination-treated (LP), and in the combination-treated and Mg132-added (LPM) cells. E, Bar graph showing band density measurements for pGSK3β levels in the control-untreated (c), in the LY294249-treated (L), in the PD-0325901 treated (P), in the combination-treated (LP), and in the combination-treated and Mg132-added (LPM) cells.
shown is mean of colony number from 3 independent experiments, using the parental (P), the control (C), and the β-catenin-silenced (βsh1 and βsh2) cells. D and E, Effect of β-catenin silencing on proportion of ALDH1-high cells in MDA-MB231 and the MDA-MB468 cells, respectively. Cells used were control shRNA (Control) and βsh1 and βsh2.

Figure 4. Effect of β-catenin silencing on the biology of TNBC cells. Cell proliferation, anchorage-independent growth and mammosphere formation, A, IB analysis for β-catenin silencing with 2 independent shRNA constructs (βsh1 and βsh2) in the MDA-MB231 and MDA-MB468 cells. Samples used in the analysis were from Par, Con, and βsh1 and βsh2 cells. The IB analysis also includes cyclin D1 and c-Myc, the β-catenin transcriptional target genes. B, Line graph showing effect of β-catenin silencing on cell proliferation in the MDA-MB231 and the MDA-MB468 cells. C, Bar graph showing effect of β-catenin silencing on colony formation in soft agar. Data shown is mean ± SEM of colony number from 3 independent experiments, using the parental (P), the control (C), and the β-catenin-silenced (βsh1 and βsh2) cells. D and E, Effect of β-catenin silencing on proportion of ALDH1-high cells in MDA-MB231 and the MDA-MB468 cells, respectively. Cells used were control shRNA (Control) and βsh1 and βsh2.

To gain further insight on tumor-growth property, tumor slices were stained by the hematoxylin and eosin (H&E) method and examined under a microscope. The control MDA-MB231 tumors showed a highly invasive carcinoma, while the β-catenin-silenced counterparts were noninvasive (Fig. 5B, top). Simlar analysis of lung and liver sections showed multiple pulmonary and liver metastases in the controls and no such lesions in the β-catenin–silenced mice (Fig. 5B, middle and bottom, respectively). On the other hand, the control MDA-MB468 tumors showed only clustered local push into the mammary gland stroma with no sign of metastasis to the lungs and the liver (Supplementary Fig. 5B). As expected, the corresponding
β-catenin–silenced MDA-MB468 tumors did not show any local push into the stroma. Rather, they were surrounded by a thick connective tissue (Supplementary Fig. 5B). These results demonstrate that silencing β-catenin in TNBC cells blocks local invasiveness and/or distant metastasis.

Discussion

We have previously shown that inhibition of SHP2 either by shRNA-based silencing or dominant-negative expression induces a normal-like epithelial phenotype in TNBC cells (24). Those findings were indicative of SHP2 promoting the mesenchymal phenotype in TNBC cells, and its inhibition inducing MET. However, the mechanism was unclear. To address this important biological question, we first investigated state of adherence junction–mediated cell–cell interaction by IF in the control and in the SHP2-silenced TNBC cells. These studies clearly demonstrated that silencing SHP2 induces E-cadherin expression that localizes to the plasma membrane to mediate adherence junctions between neighboring cells (Fig. 1A and B). In addition, the IF findings showed plasma membrane localization of β-catenin, but downregulation in the cytoplasm and the nucleus. These findings show that inhibition of SHP2 induces E-cadherin and β-catenin–mediated cell–cell adhesion in TNBC cells, which in turn lead to acquisition of a normal-like epithelial phenotype.

One of the interesting observations in these studies was the paucity of β-catenin in the cytoplasm and the nucleus of SHP2-silenced cells, which was confirmed by IB analysis of cytoplasmic and nuclear fractions (Fig. 1C). Data presented in Fig. 1D–F and Supplementary Fig. 1 further showed reduction in total β-catenin upon SHP2 inhibition. These findings are consistent with a positive role for SHP2 in β-catenin expression in TNBC cells. This is significant since the Wnt/β-catenin signaling pathway is dysregulated in TNBC (12, 38) and β-catenin cytoplasmic stability and nuclear localization is at the core of its oncogenic property (16).

The qRT-PCR data in Fig. 2 A together with the proteasome inhibition data in Supplementary Fig. 2 indicated that the effect of SHP2 on β-catenin expression in TNBC cells is at a protein level. A further support for this conclusion came from time-course proteasome inhibition experiments that showed restoration of β-catenin protein levels in less than 3 hours in the SHP2-silenced cells (Fig. 2B and C). These findings are consistent with enhanced β-catenin proteasomal degradation upon SHP2 inhibition. In support of this possibility, we have found that Mg132-stabilized β-catenin in the SHP2-silenced cells was highly phosphorylated on its degron motif (Fig. 2D). Together, these findings suggest that SHP2 promotes the dysregulated expression of β-catenin by preventing proteasomal degradation. Since GSK3β is the major kinase that makes the final phosphorylations on β-catenin before ubiquitination by β-TRCP and subsequent degradation (17, 31), we reasoned that the positive role of SHP2 on β-catenin protein stability might be related to regulation of GSK3β. Indeed, data presented in Fig. 3 show that GSK3β is least phosphorylated on Ser9, the regulatory phosphorylation site (17, 31), in the SHP2-silenced cells, suggesting that SHP2 promotes GSK3β inactivation; note that phosphorylation of GSK3β on Ser9 inactivates its function.

Previous studies have shown that Akt is one of the kinases that phosphorylates GSK3β on Ser9 (31). Also, ERK1/2 kinases are known to prime GSK3β on Thr-43 for subsequent phosphorylation by p90Rsk on Ser9 (17, 32). Since RTK dysregulation is the primary mechanism for activation of the Ras-ERK and PI3K-Akt signaling pathways and since SHP2 is a master regulator of RTK signaling, it is highly likely that SHP2 orchestrates RTK-induced β-catenin stability through this mechanism. Although not in the context of SHP2, previous studies have shown a positive role for RTKs in stabilizing β-catenin in the absence of Wnt signaling (39, 40). Since activation of Akt and ERK1/2 by multiple RTKs is SHP2 dependent and since RTK dysregulation is a common phenomenon in TNBC, the enhanced degradation of β-catenin in the SHP2-inhibited cells suggests that SHP2 acts, at least in part, through Akt and ERK1/2 to promote β-catenin stability. The pharmacologic inhibition studies described in Fig. 3C provide direct evidence that the PI3K-Akt and the MEK-ERK signaling cascades mediated by SHP2 are important for β-catenin protein stability. Additionally, SHP2 may promote β-catenin protein stability by mediating RTK-induced β-catenin Tyr phosphorylation. This is possible since the expression and activation of multiple RTKs in TNBC cells is...
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In summary, we have demonstrated that inhibition of SHP2 in TNBC cells induces E-cadherin expression and depletion of cytoplasmic and nuclear β-catenin, which in turn confers adherence function mediated cell-cell adhesion. We have also shown that SHP2 promotes β-catenin protein stability in TNBC cells by mediating the inactivation of GSK3β through its positive effect on Akt and ERK1/2 activation. Finally, we have shown that SHP2-stabilized β-catenin is essential for cell growth, transformation, CSC properties, and tumorigenesis and metastasis in TNBC cells. Overall, the findings in this report show that SHP2 mediates β-catenin protein stability to promote TNBC.

Authors’ Disclosures
No disclosures were reported.

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