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Inhibition of SHP2 leads to mesenchymal to epithelial transition in breast cancer cells

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The Src homology phosphotyrosyl phosphatase 2 (SHP2) is an essential transducer of mitogenic and cell survival signaling in the epidermal growth factor receptor (EGFR) signaling pathway. However, the role of SHP2 in aberrant EGFR and human EGFR2 (HER2) signaling and cancer, particularly in breast cancer, has not been investigated. Here, we report that SHP2 is required for mitogenic and cell survival signaling and for sustaining the transformation phenotypes of breast cancer cell lines that overexpress EGFR and HER2. Inhibition of SHP2 suppressed EGF-induced activation of the Ras–ERK and the phosphatidylinositol 3 kinase–Akt signaling pathways, abolished anchorage-independent growth, induced epithelial cell morphology and led to reversion to a normal breast epithelial phenotype. Furthermore, inhibition of SHP2 led to upregulation of E-cadherin (epithelial marker) and downregulation of fibronectin and vimentin (mesenchymal markers). These results indicate that SHP2 promotes breast cancer cell phenotypes by positively modulating mitogenic and cell survival signaling, by suppressing E-cadherin expression which is known to play a tumor suppressor role and by sustaining the mesenchymal state as evidenced by the positive impact on fibronectin and vimentin expression. Therefore, SHP2 promotes epithelial to mesenchymal transition, whereas its inhibition leads to mesenchymal to epithelial transition. On the basis of these premises, we propose that interference with SHP2 function might help treat breast cancer.
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Accumulating evidence suggests that the Src homology phosphotyrosyl phosphatase 2 (SHP2) could be important in cancer. First, SHP2 is essential for cell transformation induced by v-Src[1] and the constitutively active form of fibroblast growth factor receptor 3 (K650E-FGFR3).[2,3] And second, SHP2 has been implicated in the development of Noonan syndrome and associated leukemia.[4] This was based on the discovery of activating SHP2 mutations, sometimes referred to as leukemogenic SHP2 mutants, in patients with these diseases.[4] In support of these discoveries, transgenic mice expressing leukemogenic SHP2 mutants develop lymphoid hyperplasia.[5,6] Thus, SHP2 plays a significant role in cancer.

Earlier studies have shown that SHP2 plays a positive role in receptor tyrosine kinase (RTK) signaling.[7] Particularly, SHP2 mediates RTK-induced activation of the Ras-extracellular signal-regulated kinase 1 and 2 (ERK1/2) and the phosphatidylinositol 3 kinase–Akt (also known as protein kinase B) signaling pathways.[7,8] In the epidermal growth factor receptor (EGFR) signaling pathway, SHP2 promotes Ras activation by blocking the Ras GTPase-activating protein (RasGAP)-induced downregulation of Ras, via dephosphorylation of a docking site on the EGFR, Tyr992.[9] Similarly, the *Drosophila* ortholog of SHP2, Corkscrew, transduces the Torso RTK signaling by blocking Ras inactivation.[10] Other reports also show that SHP2 dephosphorylates a RasGAP-binding site on the adapter protein Gab1,[11] leading to the same conclusion that SHP2 promotes Ras activation by blocking RasGAP. Furthermore, SHP2 promotes Ras and ERK1/2 activation by facilitating RTK-induced activation of the Src family kinases (SFKs).[12]

As its acronym indicates, SHP2 possesses two tandemly arranged Src homology 2 (SH2) domains in the N-terminal region, a phosphotyrosyl phosphatase (PTP) domain in the C-terminal region[7,8] and two tyrosine phosphorylation sites and a proline-rich motif in the extreme C-terminal region. The SH2 domains and the PTP domain are essential for the biological activity of SHP2,[13] but its Tyr phosphorylation seems to be dispensable.[14] In a resting state, SHP2 assumes a 'closed conformation' due to interaction between the N-terminal SH2 and the PTP domains. The binding of the SH2 domains to phosphotyrosine-bearing proteins induces an open conformation and enzyme activation.[15] Asp61 and Glu76 of the N-SH2 domain have been shown to be the mediators of the SH2–PTP domain interaction. Mutation of these residues to Ala activates SHP2, and mutations of these residues are suggested to be the causes of Noonan Syndrome and associated leukemia.[4]

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Abbreviations: EGFR, epidermal growth factor receptor; EMT, epithelial to mesenchymal transition; HER2, human EGFR2; MET, mesenchymal to epithelial transition; PTP, phosphotyrosyl phosphatase; RTK, receptor tyrosine kinase; SH2, Src homology 2; SHP2, Src homology phosphotyrosyl phosphatase 2; shRNA, small hairpin RNA
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We have recently reported that the SHP2 protein is elevated in breast cancer cell lines and overexpressed in primary breast tumors.[16] However, the significance of SHP2 overexpression in breast cancer has not been determined. In this report, we show that SHP2 is required for EGF-induced signaling and for maintenance of transformation phenotypes in breast cancer cell lines that overexpress EGFR and human EGFR2 (HER2). In addition, we show that SHP2 is essential for sustaining the mesenchymal state of breast cancer cells, whereas its inhibition leads to mesenchymal to epithelial transition (MET) and reversion to a normal breast epithelial phenotype.

Results

Our recent report has shown that the SHP2 protein is elevated in a heterogeneous group of breast cancer cell lines.[16] Hence, inhibition of SHP2 by dominant-negative expression in these cells might be difficult although not impossible. In addition, the dominant-negative strategy might provide inconclusive results due to insufficient inhibition. Therefore, we employed small hairpin RNA (shRNA)-mediated ablation of the SHP2 protein to fully appreciate the significance of SHP2 inhibition on RTK-induced signaling and transformation. In the current report, we have investigated the consequence of SHP2 inhibition in breast cancer cell transformation. The BT20 and the MDA-MB468 that overexpress EGFR and the BT474 and the SUM225 that overexpress HER2 were used for these experiments.[17,18]

shRNA inhibition of SHP2 and its effect on ERK1/2 and Akt activation. Cells expressing the SHP2 shRNA are referred to as shRNA, parental lines as PAR and cells expressing a scrambled control shRNA as CON. As shown in Figure 1a, expression of the SHP2 protein was inhibited by approximately 75% in the shRNA cells. Reblotting the membrane with anti-β-actin antibody indicated that comparable amount of total protein was loaded to each lane. These results suggest that SHP2 could be constitutively inhibited by shRNA, an approach that provides opportunities for studying the role of SHP2 in cell transformation and xenograft tumor growth.

The proficiency of the shRNA inhibition of SHP2 was confirmed by determining state of EGF-induced ERK1/2 and Akt activations. The EGF-induced ERK1/2 and Akt activation was suboptimal and short lived in shRNA, whereas it was sustained for about 4 h in the PAR and the CON cells of the BT20 and the BT474 line (Figure 1b). Reblotting the membranes with anti-β-actin or anti-ERK2 antibodies showed that there was a comparable amount of total protein in each lane. Similar results were obtained in the SUM225 and the MDA-MB468 cells (data not shown). Therefore, shRNA inhibition of SHP2 expression by approximately 75% was quite sufficient to abolish EGF-induced ERK1/2 and Akt activation.

Breast cancer cell transformation phenotypes are SHP2 dependent. The BT474 and the SUM225 cells typically grow as patchy, multilayered and aggregated colonies with less-defined intercellular boundaries, and they hardly go to confluency. The MDA-MB468 cells are spindle shaped in morphology and thus grow as interspersed and networked jumble at high density. The BT20 cells also exhibit a spindle-shaped morphology at low density with little intercellular adhesion, but form a sheet of elongated cells with islands of multilayered clumps at high density. Inhibition of SHP2 induced a dramatic change in the morphology and transformation phenotypes of all of the breast cancer cells tested in this study. As expected, the CON cells exhibited morphologies reminiscent of the parental PAR lines. In contrast, the shRNA cells grew as highly ordered monolayer

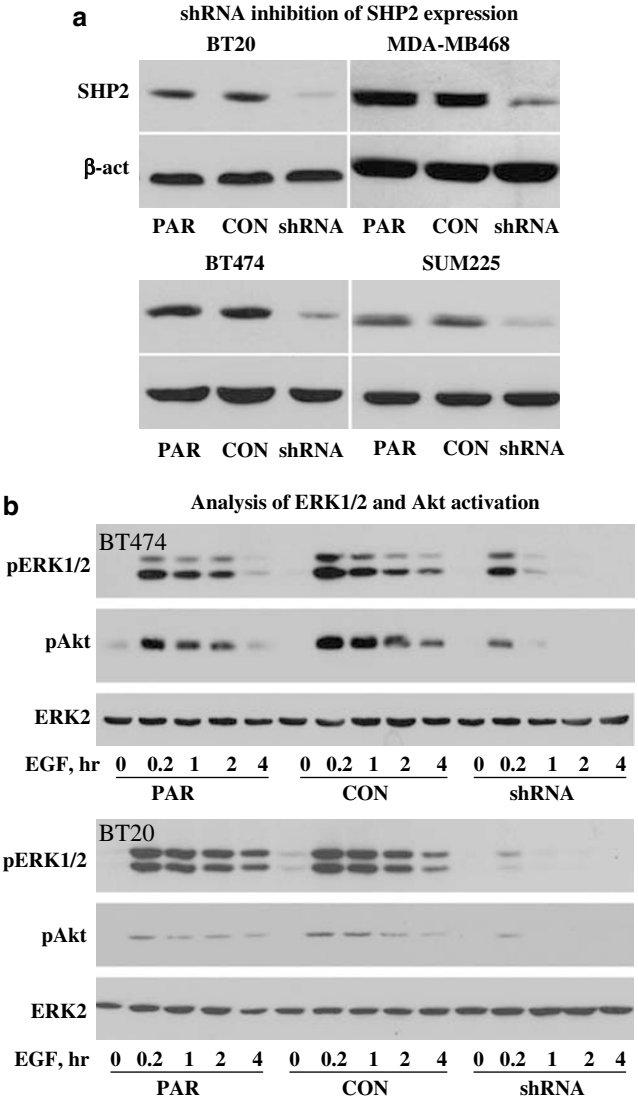

Figure 1 (**a**) Retrovirus-mediated expression of specific anti-Src homology phosphotyrosyl phosphatase 2 (SHP2) small hairpin RNA (shRNA) suppresses SHP2 protein expression by approximately 75% as determined by anti-SHP2 immunoblotting. (**b**) The PAR, the CON and shRNA cell derived from the BT474 and the BT20 lines were serum-starved for $\sim$12 h and then stimulated with 100 ng/ml EGF for the indicated time points. Lysates prepared from these cells were analyzed by immunoblotting with anti-phspho-ERK1/2 and anti-phospho-Akt polyclonal antibodies. β-actin was used as a loading control in (**a**), whereas ERK2 was used for the same purpose in (**b**). PAR, parental cells; CON, cells expressing control shRNA; shRNA, cells expressing the SHP2-specific shRNA

sheet with clearly defined intercellular boundaries, flattened morphology and a characteristic polygonal shape typical of the cobblestone-like appearance of the MCF-10A, the immortalized breast epithelial cell line (Figure 2a). These results suggest that shRNA-mediated ablation of the SHP2 protein induces reversion of breast cancer cells to a phenotype that resembles the normal breast epithelial cell morphology.

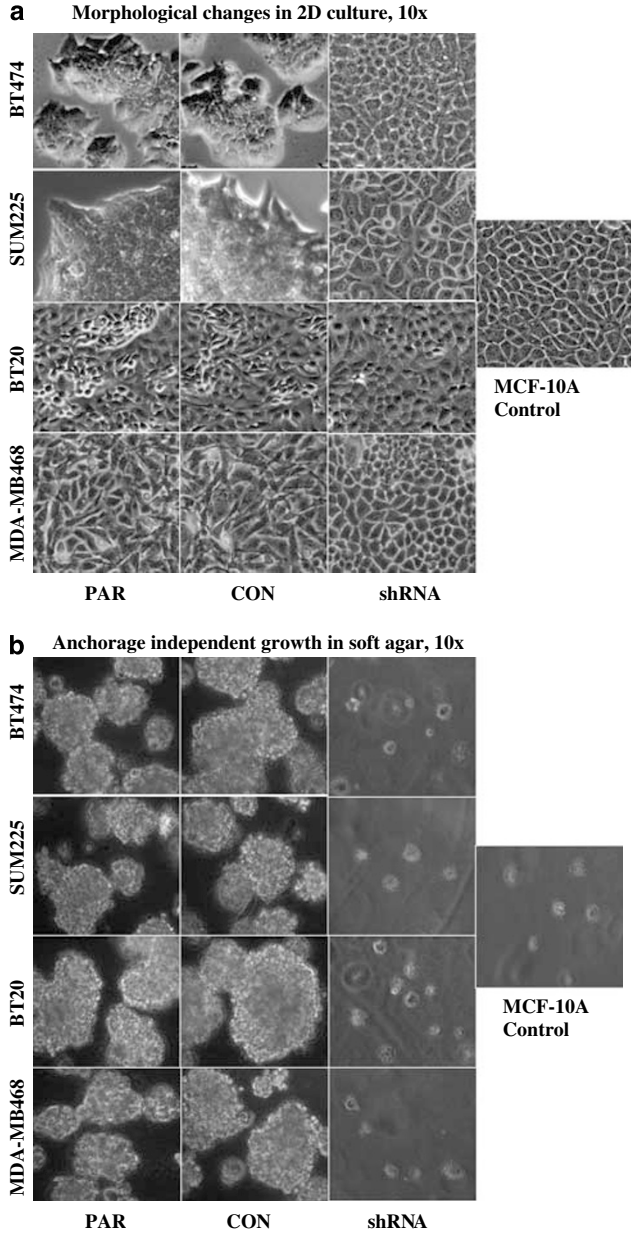

Figure 2 Small hairpin RNA (shRNA) inhibition of Src Homology Phosphotyrosyl phosphatase 2 (SHP2) reverses cell transformation. (**a**) The PAR, the CON and shRNA cells derived from the indicated four breast cancer cells were grown to confluency in culture dishes and phase contrast pictures were collected under Olympus IX70 microscope equipped with a digital camera. (**b**) Anchorage-independent growth assay in agar. Cells were seeded in agar as described in the materials and methods and their ability to form colonies was evaluated by observation under a microscope. Phase contrast pictures were taken after 7 days of incubation. The PAR and the CON cells formed large colonies, whereas the shRNA cells did not, suggesting that inhibition of SHP2 leads to loss of anchorage-independent growth. The MCF-10A cells were used as negative controls in both (**a**) and (**b**)

The four breast cancer cells tested here are proficient in anchorage-independent growth.[19,20] To see if inhibition of SHP2 affects this phenotype, we seeded approximately 10^4 cells from each line in soft agar and monitored colony formation by observation under a microscope. As shown in Figure 2b, the PAR and the CON cells formed large colonies in 7 days, whereas the shRNA cells could not. As expected, the MCF-10A did not form colonies in soft agar. Given that anchorage-independent growth is one of the traits of a cancer cell, the inability of the shRNA cells to form colonies demonstrates that SHP2 is required for the anchorage-independent growth property of breast cancer cells.

Inhibition of SHP2 induces a normal breast epithelial phenotype. The findings in Figure 2 were further corroborated by 3D matrigel assays, sometimes referred to as laminin-rich basement membrane (LRBM) culture. In this system, breast cancer cells continue to grow and form a disorganized mass of cells, whereas breast epithelial cell lines like the MCF-10A multiply a few times, stop growth, acquire a polarized morphology and form acini-like structures.[21] Surprisingly, the shRNA cells formed acini-like structures reminiscent of the MCF-10A cells. In contrast, the PAR and the CON cells formed disorganized mass of cells (Figure 3a). Data shown was from the BT474 and the BT20 lines; similar results were obtained with the SUM225 and MDA-MB468 lines (not shown).

To further characterize the organizational architecture of cells in the matrigel, slides were co-stained with anti-α6 integrin antibody for cell polarity and anti-E-cadherin antibody for lateral cell–cell adhesion and then analyzed by immunofluorescence (IF) confocal microscopy as previously described.[21,22] Similar to the MCF-10A cells, the shRNA cells formed polarized acini with vertical ECM–integrin and lateral cell–cell adhesion with hollow lumen (Figure 3b). On the other hand, the PAR and the CON cells formed nonpolarized, large and disorganized cellular mass with no hollow lumen. Microdissection imaging further confirmed that the shRNA cells formed spheroid structures with hollow lumen, whereas the PAR and CON cells were filled with cellular mass (Figure 3c). Data shown were from cells of BT20 and BT474 origin; similar results were obtained with the MDA-MB468 and SUM225 cells (not shown). By and large, these results demonstrate that SHP2 promotes the transformation phenotypes of breast cancer cells and that its inhibition leads to reversion to a normal breast epithelial phenotype.

SHP2 is required for cell migration and scattering. Previous work has shown that SHP2 suppresses cell adhesion and enhances motility.[23–26] We sough to investigate the significance of SHP2 inhibition on breast cancer cells motility and scattering. Initially, this possibility was tested by the monolayer wound-healing assay. The CON cells of the BT20 (Figure 4a, top panel) and the MDA-MB468 (not shown) lines migrated and filled the gap in less than 36 h, whereas the corresponding shRNA cells were unable to do so even after 48 h. Consistent with

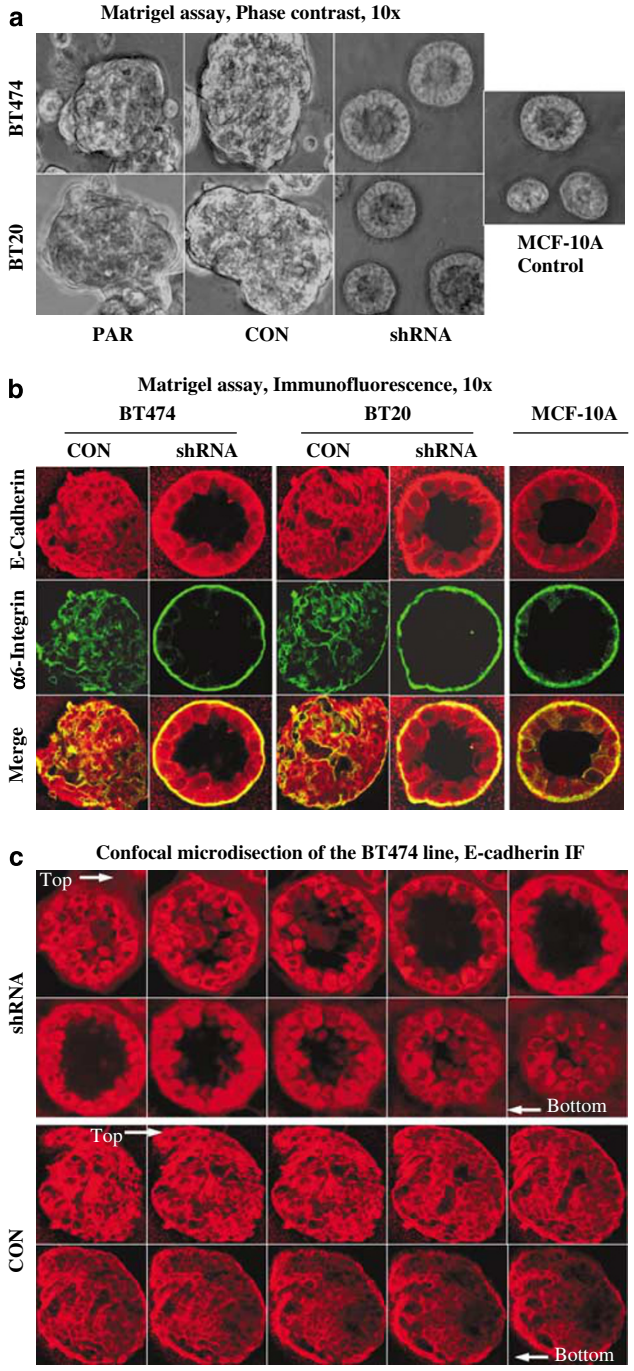

Figure 3 Small hairpin RNA (shRNA)-mediated ablation of the Src Homology Phosphotyrosyl phosphatase 2 (SHP2) protein induces differentiation of breast cancer cells to a normal phenotype. (**a**) Cells were seeded onto laminin-rich basement membrane (LRBM) also called matrigel and incubated for approximately 15 days before phase contrast pictures were taken. (**b**) Immunofluorescence staining of cells grown in LRBM 3D cultures. The shRNA cells formed polarized acini-like structures reminiscent of the MCF-10A cells, the positive controls for acini formation. The shRNA and the MCF-10A cell exhibited integrin-mediated vertical interaction with the basement membrane, adherens junction-mediated lateral cell–cell interaction and a central hollow lumen. In contrast, the PAR and the CON cells formed disorganized cellular mass. (**c**) Comparative confocal slice images of the CON and the shRNA cells depicting the spheroidal structures with a hollow lumen in the shRNA cells and the absence of such a lumen, but amorphous cellular mass in the CON cells

their compact growth property, the CON cells of the BT474 (Figure 4a, bottom panel) and the SUM225 (not shown) lines were slow in filling the gap, but performed significantly better than their shRNA counter parts. Nonetheless, the above results are consistent with a positive role for SHP2 in breast cancer cell migration.

The above results were further corroborated by cell scattering assay to see if the observed cell migration-promoting effect of SHP2 was linked to its ability to modulate adherens junction-mediated cell–cell interaction. As expected, the CON cells of the BT20 line formed dispersed colonies with relatively low intercellular adhesion, whereas those from BT474 line formed highly aggregated colonies. On the other hand, the shRNA cells of both lines formed highly ordered sheet of colonies with defined intercellular boundaries (Figure 4b). Upon EGF stimulation, the CON cells of the BT20 line scattered further, whereas those of the BT474 line rather retracted, became elongated and tended to move as a group. In contrast, EGF stimulation did not cause any significant change in the shRNA cells. Cells derived from the MDA-MB468 line exhibited similar phenotypes to that of the BT20, whereas those from the SUM225 to the BT474 (data not shown). These cells were further analyzed by IF microscopy by co-staining with antibodies against the two major proteins involved in adherens junction, E-cadherin and β-catenin.

In conformity to phase contrast pictures, the CON cells of the BT20 line showed low E-cadherin and β-catenin staining at cell–cell contact sites, whereas those of the BT474 origin showed an intense staining. EGF stimulation induced dissolution of adherens junction, cell scattering and nuclear localization of β-catenin in the BT20 CON cells, but did not cause a similar change in the CON cells of BT474 line (Figure 4c). Rather, the BT474 CON cells exhibited change in colony shape reminiscent of group movement shown in the phase contrast pictures. On the other hand, the shRNA cells derived from both lines exhibited intense E-cadherin and β-catenin staining at cell–cell contact sites both in the absence and presence of EGF with no β-catenin translocation to the nucleus. As a result, colonies of shRNA cells remained virtually intact at least for 2 h following EGF stimulation. The results from the MDA-MB468 were similar to that of the BT20 line, whereas those from the AU565 were to the BT474 line (data not shown). Thus, the EGFR-overexpressing BT20 and MDA-MB468 breast cancer cells migrate individually, whereas the HER2-overexpressing BT474 and SUM225 cells move as a group. In either case, SHP2 is required for mediating cell scattering in EGFR and group movement in HER2-overexpressing breast cancer cells.

SHP2 is essential for EMT and its inhibition leads to MET. The dramatic difference in migratory behavior between the EGFR- and the HER2-overexpressing breast cancer cells led us to first investigate the expression state of E-cadherin, epithelial marker, and fibronectin and vimentin, mesenchymal markers.[27–29] Considering the expression of these proteins in MCF-10A cells as normal, E-cadherin was downregulated, whereas fibronectin and vimentin were upregulated in the BT20 and in the MDA-MB468 cells. In contrast, E-cadherin was upregulated, whereas fibronectin and vimentin were virtually undetectable in the BT474 and

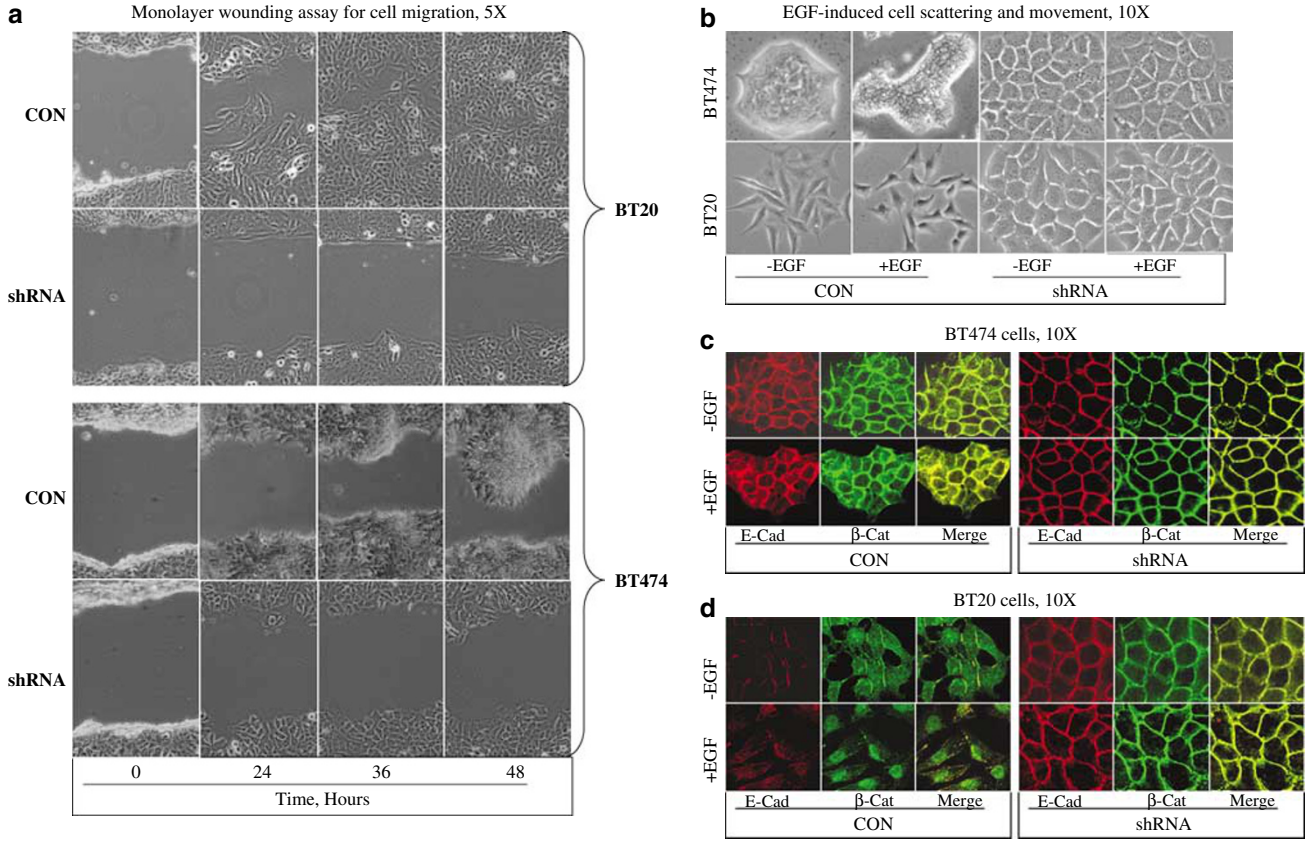

Figure 4 Effect of Src Homology Phosphotyrosyl phosphatase 2 (SHP2) inhibition on cell motility. (**a**) Monolayer wound-healing assay for testing the effect of SHP2 inhibition on cell migration. (**b**) Effect of SHP2 inhibition on EGF-induced cell scattering. Cells were stimulated with 100 ng/ml EGF for 2 h or left unstimulated, and phase contrast pictures were collected under Olympus IX71 microscope. (**c**) Confocal immunofluorescence images of the CON and the small hairpin RNA (shRNA) cells of the BT474 line. (**d**) Confocal immunofluorescence images of the CON and the shRNA cells of the BT20 line. Slides were double-stained with anti-E-cadherin anti-β-catenin antibodies and pictures were collected under Ziess scanning confocal microscope

SUM225 lines (Figure 5a). Band-density measurements showed that the BT20 and the MDA-MB-468 cells had $\sim$3-fold lower, whereas the BT474 and SUM225 cells had approximately 5-fold or more protein than the MCF-10A (Figure 5b). Similar band-density measurement of fibronectin and vimentin showed that the BT20 and the MDA-MB468 cells express at least five times more protein than the MCF-10A cells, whereas the BT474 and the SUM225 cells have a very low amount barely detectable above background (Figure 5b). The low E-cadherin and elevated fibronectin and vimentin in the BT20 and the MDA-MB468 cells explain why these cells are fibroblastic and migratory, and why the *vice versa* is true in the BT474 and SUM225 cells. In addition, the elevated E-cadherin level in the BT474 and SUM225 cells explains why these cells grow as highly aggregated colonies and are unable to scatter upon growth factor stimulation.

Remarkably, shRNA inhibition of SHP2 restored 'normal' E-cadherin and fibronectin levels. As shown in Figure 5c, shRNA cells of the BT20 and the MDA-MB468 lines exhibited an increase, whereas shRNA cells of the BT474 and the SUM225 lines showed a decrease in E-cadherin expression, ultimately resulting in E-cadherin levels that are comparable to the level found in MCF-10A cells. With regard to fibronectin, inhibition of SHP2 led to reduction in the BT20 and the MDA-MB468 lines and an increase in the BT474 and the SUM225 lines, again leading to fibronectin levels comparable to MCF-10A. Even more remarkable was that inhibition of SHP2 led to loss of vimentin expression by the BT20 and the MDA-MB468 cells, which had elevated levels (Figure 5d). Similar to the PAR and the CON counterparts, the shRNA cell of the BT474 and the SUM225 lines did not express vimentin. Reblotting with anti-β-actin antibody showed that total protein loaded to each lane was comparable. These results demonstrate that inhibition of SHP2 restores E-cadherin and fibronectin expression to levels found in the MCF-10A cells. In addition, they show that inhibition of SHP2 abolishes the expression of the mesenchymal marker vimentin by those breast cancer cells that had elevated levels. Therefore, SHP2 promotes epithelial to mesenchymal transition (EMT) and its inhibition leads to MET.

Discussion

Accumulating evidence suggests that SHP2 might play a pivotal role in cancer. First, SHP2 is a positive effector of mitogenic and cell survival signaling.[9,12,30] Second, SHP2 promotes cell transformation induced by v-Src and the constitutively active form of FGFR3.[1–3] Third, activating SHP2 mutations play causative role in Noonan Syndrome

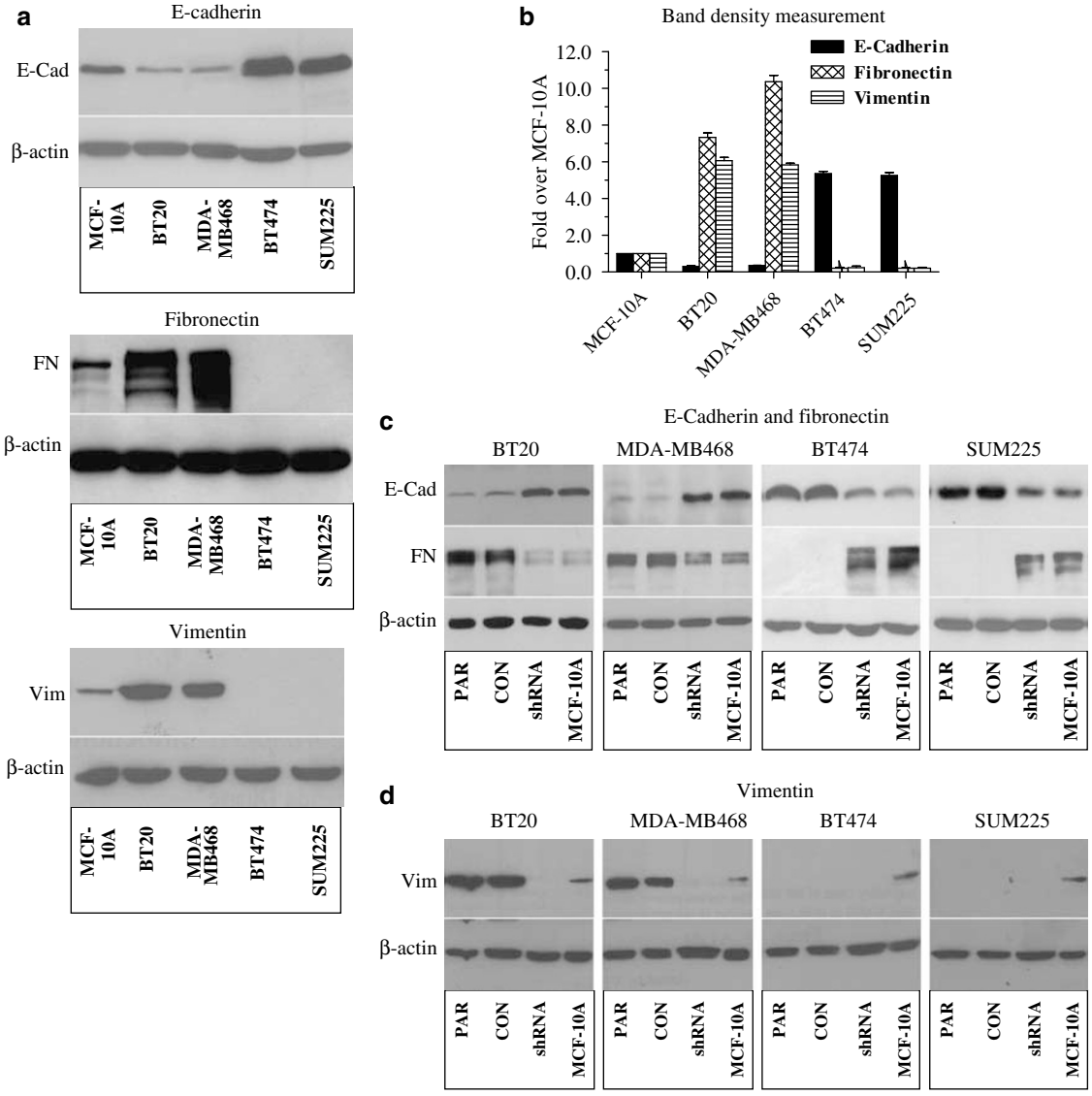

Figure 5 (**a**) Immunoblotting analysis of E-cadherin, the epithelial marker, and fibronectin and vimentin, the mesenchymal markers, in the four breast cancer cells used in this study. (**b**) Band-density measurement of E-cadherin, fibronectin and vimentin levels from three independent experiments. (**c**) Inhibition of Src Homology Phosphotyrosyl phosphatase 2 (SHP2) equilibrates E-cadherin and fibronectin levels to an amount found in MCF-10A cells. (**d**) Inhibition of SHP2 abrogates vimentin expression by the BT20 and the MDA-MB468 cells. Because the BT474 and the SUM225 cells do not normally express vimentin, the effect of SHP2 inhibition could not be determined

and associated leukemia.[4] And finally, the SHP2 protein is overexpressed in approximately 70% of infiltrating ductal carcinoma of the human breast.[16] On the basis of these premises, we sought to investigate the role of SHP2 in breast cancer cell transformation. In this report, we show that SHP2 is essential for breast cancer cell transformation and that its inhibition leads to reversion to a normal breast epithelial phenotype sometimes referred to as MET.

Inhibition of SHP2 suppresses EGF-induced ERK1/2 and Akt activation in breast cancer cell lines, suggesting that SHP2 is important for mitogenic and cell survival signaling not only in normal, but also in cancer cells. Furthermore, inhibition of SHP2 reverses breast cancer cell transformation as evidenced by acquisition of epithelial morphology, loss of anchorage-independent growth (Figure 2) and differentiation in 3D LRBM matrigel (Figure 3). Thus, SHP2 might play a pivotal role in breast cancer.

Remarkably, the highly fibroblastic BT20 and MDA-MB468 breast cancer cells lost their migratory property upon SHP2 inhibition. These results are consistent with previous reports that showed that SHP2 plays a positive role in fibroblast motility and migration.[23–26] Thus, interference with SHP2 function might provide a means to inhibit cancer cell metastasis. Unexpectedly, we discovered that EGF can induce translocation of β-catenin to the nucleus in SHP2-dependent manner. Although the mechanism is not clear, previous work has shown that tyrosine phosphorylation of β-catenin induces disengagement from adherens junctions, inhibits its degradation and promotes nuclear localization.[31] It is possible that EGF induces β-catenin tyrosine phosphorylation,

whereas at the same time activates SHP2 to dephosphorylate α-catenin,[3] the concerted action of which leads to β-catenin nuclear localization and transcriptional activation. Thus, β-catenin can be activated not only by the Wnt, but also by the EGFRR/HER2 signaling pathway.

The HER2-overexpressing BT474 and SUM225 cells are peculiar in their growth behavior; they grow as aggregated colonies with strong adherens junction-mediated intercellular adhesion. Upon EGF stimulation, the colonies of these cells shrink and tend to move as a group in SHP2-dependent manner. The strong adherens junction in these cells might explain why they grow as aggregated colonies. In this connection, it is intriguing to note that enhanced cell–cell adhesion can also contribute to cell transformation as these cells are highly tumorigenic in nude mice. Inhibition of SHP2 in these cells led to an epithelial morphology with vertical (cell–ECM) and lateral (cell–cell) interactions approximating those found in MCF-10A cells. Thus, SHP2 positively modulates divergent breast cancer cell phenotypes and its inhibition leads to reversion to a normal phenotype.

A large body of literature shows that downregulation of E-cadherin and upregulation of fibronectin and vimentin are associated with aggressive tumor growth, local invasion and metastasis to distant organs in many cancer types including breast cancer.[32,33] However, our findings in four breast cancer cell lines, all isolated from infiltrating ductal carcinoma, exhibited divergent expression levels. The BT20 and the MDA-MB468 cells that overexpress EGFR have low E-cadherin and elevated fibronectin and vimentin, whereas the *vice versa* is true for the HER2 overexpressing BT474 and SUM225 cells; they have very low to undetectable fibronectin and vimentin (Figures 5a and b). Consistent with previous reports, elevated expression of E-cadherin is inversely correlated to increased expression of fibronectin and vimentin. However, the occurrence of both events in a cell line-specific manner is somewhat unexpected. Given the reported tumor suppressor role of E-cadherin, its elevated expression in HER2-overexpressing breast cancer cells is puzzling as these cells are highly transformed, grow in soft agar and form tumors in nude mice. So far, there are no reports on elevated E-cadherin expression in breast and other tumors. Similarly, given the highly tumorigenic phenotype of the BT474 and the SUM225 breast cancer cell lines, absence of vimentin expression is another unexpected observation; it indicates that vimentin is a marker for mesenchymal state, but not for cell transformation. It will be interesting to address the significance of an elevated E-cadherin and downregulated vimentin in cancer in future studies.

The most striking finding was that inhibition of SHP2-induced equilibration in the expression levels of E-cadherin and fibronectin (Figure 5c). Inhibition of SHP2 led to an increase in E-cadherin in the BT20 and the MDA-MB468 cells that had low levels and a decrease in the BT474 and SUM225 cells that had elevated levels, ultimately balancing to an amount found in the MCF-10A cells. Similarly, inhibition of SHP2 led to a reduction in fibronectin in the BT20 and the MDA-MB468 cells that had elevated levels and an acquisition by the BT474 and the SUM225 cells that had undetectable levels. With regard to vimentin, inhibition of SHP2 led to loss of vimentin expression by the BT20 and the MDA-MB468 cells, but did not lead to derepression in the BT474 and the SUM225 cells (Figure 5d), suggesting that vimentin is not required for normal epithelial homeostasis. Several conclusions can be drawn from these findings. First, physiological levels of both fibronectin and E-cadherin are important for maintenance of normal breast epithelial phenotype. Second, not only down-regulation and mutational inactivation of E-cadherin, but also its elevated expression might contribute to cell transformation and tumor growth. And third, elevated expression of vimentin predicts the mesenchymal, but not its transformation state. Thus, one of the mechanisms by which SHP2 promotes breast cancer cells transformation is by modulating the expression of E-cadherin, fibronectin and vimentin.

The molecular mechanism of SHP2 in regulating diverse signaling pathways and cellular processes is just beginning to be elucidated. We have previously demonstrated that SHP2 promotes Ras activation by blocking RasGAP recruitment to the plasma membrane.[9] Others have shown that SHP2 promotes Ras and ERK1/2 activation by facilitating RTK-induced activation of the SFKs.[12] SHP2 also promotes β1-integrin-induced SFK activation in a similar way, leading to Ras and ERK1/2 activation.[34] The Gab1-SHP2 signaling axis downstream of RTKs has also been shown to be critical for growth factor-induced signaling.[35] The discovery of Gab2[36] and SHP2[16] overexpression in primary breast tumors and their cooperation in promoting HER2-induced mammary tumorigenesis in transgenic mice[37] further highlight the critical role SHP2 plays in breast cancer. In conclusion, promotion of cell transformation by SHP2 is a multifaceted process that involves, at least in part, augmentation of cell proliferation and survival and suppression of adherens junction-mediated cell–cell interaction. Therefore, inhibition of SHP2 might provide a means for treating breast cancer as it could lead to MET.

Materials and Methods

Cells, cell culture and reagents. The breast cancer cell lines used in this study were the BT20, MDA-MB468, BT474 and SUM225. The MCF-10A, the immortalized breast epithelial cell line, was used as a control. The breast cancer cell lines were purchased from ATCC and Asterand Inc., whereas the MCF-10A cells were obtained from Dr. Daniel Flynn (Marry Babb Randolph Cancer Center, West Virginia University). The BT474 cells were grown in RPMI 1640, the SUM225 in F12, whereas the BT20 and the MDA-MB468 were in Dulbecco's modified Eagle's medium (DMEM) all supplemented with 10% fetal calf serum. The MCF-10A were grown in DMEM supplemented with 5% horse serum, 10 μg/ml recombinant human insulin, 0.5 μg/ml hydrocortisone, 20 ng/ml EGF (PeproTech) and 100 ng/ml cholera toxin (Sigma), as previously described.[21] The MDA-MB468 cells were maintained at 37°C in the absence of CO_2, whereas the rest were maintained at the same temperature, but with 5% CO_2. The anti-β-catenin, anti-E-cadherin and anti-fibronectin antibodies were from Santa Cruz, anti-β-actin antibody was from Sigma, and anti-PTP1D (SHP2) and anti-vimentin antibodies were from Pharmagen. The anti-phospho-ERK1/2 and anti-phospho-Akt antibodies were from Cell Signaling. Horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham, whereas Alexa fluor 488 and rhodamine-labeled secondary antibodies were from Molecular Probes.

shRNA construction and production of stable cell lines. shRNA-mediated ablation of the expression of the SHP2 protein was accomplished by retroviral transduction (BD Biosciences, Palo Alto, CA, USA). A short double-stranded DNA oligonucleotide that codes for anti-SHP2 shRNA was custom synthesized (Integrated DNA Technologies) and ligated into the *Bam*HI and *Eco*RI sites of the retroviral vector termed pSIREN-RetroQ-TetP. The targeting oligonucleotide sequence was 5′-GAAATGGAGCTGTCACCCA-3′. Although the pSIREN-RetroQ-TetP vector can be used as a tetracycline-inducible system in

conjunction with pQC-tTS-IN that express the Tet-responsive repressor protein, it can also be used alone to direct constitutive expression of inserted shRNA. We chose the latter for our experiments as an approximately 70% reduction in SHP2 protein does not lead to cell death, but to differentiation. The SHP2 shRNA construct was transfected into appropriate packaging cells and, after 48 h, transient supernatants were used for infecting the four breast cancer cell lines mentioned above. , Cells were treated with puromycin to remove noninfected cells 48 h post-infection. Because puromycin-resistant populations exhibited heterogeneity in cellular response, cells that showed maximum SHP2 inhibition, as determined by anti-SHP2 immunoblotting, were obtained by cloning.

Preparation of cell lysates and immunoblotting analysis. Cells were lyzed in lysis buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 10% glycerol, 1% triton X-100, 1 mM Na orthovanadate and a protease inhibitor cocktail. All lysates were briefly sonicated to break chromosomal DNA that could interfere with gel separation of proteins. After clearing by centrifugation, the lysates were analyzed as described in the respective experiments. For electrophoresis gel separation of proteins, the cell lysates were denatured by boiling with Laemmli sample buffer and run on an 8% denaturing polyacrylamide gel. After transfer onto a nitrocellulose membrane and blocking with 3% bovine serum albumin, the membranes were stained with the primary antibody either overnight at 4°C or for 2 h at room temperature. Staining with the secondary antibody was for 1 h at room temperature. We used the chemiluminescence detection method for all immunoblotting experiments.

Anchorage-independent growth in agar. The ability of cells to form colonies in soft agar was performed as previously described.[38] Briefly, 6 cm cell culture plates were overlaid with 0.3% agar in the growth medium and allowed to solidify. Cells were suspended in 3 ml of growth medium and mixed with melted agar to a final concentration of 0.3% and immediately poured onto the agar overlay. After 5 min of incubation at room temperature for the agar to solidify, the cells were transferred to a 37°C and 5% CO_2 incubator for 7 days. During this time, the cells were fed with soft agar medium every 3 days. Colony formation was visualized under a microscope and phase contrast pictures were taken using an Olympus IX71 microscope equipped with Olympus DP30BW digital camera.

Cell differentiation in LRBM cultures. LRBM cultures were performed as previously described.[21] Briefly, 80 μl of growth factor-reduced LRBM medium (BD Biosciences) was overlaid onto four-well chamber slides (Falcon) and allowed to solidify for 15 min at 37°C in a cell culture incubator. Approximately 10^3 cells resuspended in 250 μl of assay medium (DMEM supplemented with 2% horse serum, 10 μg/ml recombinant human insulin, 0.5 μg/ml hydrocortisone, 5 ng/ml EGF, 100 ng/ml cholera toxin and 1 $\times$ penicillin/streptomycin) were plated on a solidified LRBM and cultured at 37°C in 7% CO_2 incubator. The cells were refed with assay medium every 4 days and phase contrast pictures were taken after 15 days. The same cultures were processed for confocal IF microscopy as previously described.[21,39] A brief description of IF protocol used for both 2D and 3D cultures is given below.

Monolayer wound-healing assay. Cells were grown to confluency, wounded by scratching with a pipette tip, and then incubated for a total of 48 h. Phase contrast pictures were taken at 0, 24, 36 and 48 h time points following wounding to see if inhibition of SHP2 interferes with the ability of cells to migrate and fill the newly created space.

Immunofluorescence microscopy. Cell in both LRBM chambers and 2D cultures (grown on coverslips) were fixed with 4% paraformaldehyde for 20 min, washed three times with PBS (5 min each), permeablized with 0.2% triton X-100 in PBS for 30 min and blocked with 3% bovine serum albumin in PBS for 1 h. The samples were then stained with primary antibodies at 4°C overnight, washed three times with PBS containing 0.2% triton X-100 and incubated with fluorescent-labeled secondary antibodies for 1 h at room temperature. After washing three times, the coverslips were mounted onto microscopic slides, whereas the LRBM slides were covered with appropriate size coverslips after removal of chambers. Finally, slides were scanned with laser scanning microscope (LSM 510, Zeiss) and pictures collected.

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