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SHON, a novel secreted protein, regulates epithelial–mesenchymal transition through transforming growth factor-β signaling in human breast cancer cells

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The epithelial–mesenchymal transition (EMT) is one of the main mechanisms contributing to the onset of cancer metastasis, and has proven to be associated with breast cancer progression. SHON is a novel secreted hominoid-specific protein we have previously identified; it is specifically expressed in all human cancer cell lines tested and is oncogenic for human mammary carcinoma cells. Here, we show that ectopic overexpression of *SHON* in immortalized human mammary epithelial cells is sufficient for cells to acquire the mesenchymal traits, as well as the enhanced cell migration and invasion, along with the epithelial stem cell properties characterized by increased $CD44^{high}/CD24^{low}$ subpopulation and mammosphere-forming ability. Moreover, we demonstrate that SHON positively activates the autocrine transforming growth factor-β (TGF-β) pathway to contribute to EMT, while *SHON* itself is induced by TGF-β in mammary epithelial cells. These data are in favor of a SHON-TGFβ-SHON-positive feedback loop that regulates EMT program in breast cancer progression. Finally, examination of the human clinic breast cancer specimens reveals that tumor cells may extracellularly release SHON protein to promote the cancerization of surrounding cells. Together, our findings define an important function of *SHON* in regulation of EMT *via* TGF-β signaling, which is closely associated with the invasive subtypes of human breast cancer.

Key words: SHON, EMT, TGF-β, human breast cancer
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Breast cancer remains the most commonly diagnosed cancer among women both in developed and developing countries.[1] Tumor metastasis is the leading cause of mortality associated with cancer including breast cancer.[2–4] The epithelial–mesenchymal transition (EMT), a developmental process in which epithelial cells lose their polarity and acquire the migratory properties of mesenchymal cells, is known to be the pivotal mechanism contributing to cancer metastasis. EMT is characterized by the dissolution of cell–cell junctions, cytoskeletal rearrangements, increased cell motility and synthesis of extracellular matrix.[4–6] In addition, certain epithelial cells that undergo an EMT acquire stem-like properties.[7,8] In association with these properties, many EMT biomarkers have been identified, some of which have been used to detect EMT in clinical samples.[9,10] Although the phenotypic correlation between EMT and cancer progression is firmly established, the underlying molecular mechanisms remain largely unclear.

The transforming growth factor-β (TGF-β) has emerged as a potent inducer of EMT, and it induces EMT in cultured cells, allowing for dissection of signaling pathways that lead to EMT.[11–15] Moreover, increased production of active TGF-β by tumor cells, which enhances the autocrine signaling of TGF-β, is thought to contribute to EMT in carcinoma cells *in vivo*.[16–18]

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What's new?
The epithelial-mesenchymal transition (EMT) is one of the main mechanisms behind cancer metastasis, the leading cause of mortality associated with breast cancer. SHON is a novel secreted protein that exerts a crucial function in the oncogenic transformation of mammary carcinoma cells and in breast cancer progression. This article highlights the new role of SHON as an EMT inducer through the activation of TGF-*β* signaling in an autocrine and/or paracrine manner. The findings may improve the understanding of SHON and provide useful clues for the assessment of its potential value as a molecular marker for poor prognosis in human breast cancer.

We have recently identified a novel secreted hominoid oncogene, termed *SHON*, in breast cancer, and found that it plays an important role in the development of mammary carcinoma.[19] *SHON* is expressed in most human cancer cell lines, and is oncogenic for human mammary carcinoma cells. *SHON* expression is correlated with tumor grades and can be used as a prognostic biomarker to predict a patient's response to endocrine therapy in breast cancer.[19] *SHON* has also been shown to upregulate many important regulators of EMT, such as *Snail*, *Vimentin* and *Twist*.[19] On the basis of these previous data, we speculate that *SHON* might play a role in breast cancer progression through induction of an oncogenic EMT program.

In this report, we provide evidence that *SHON* is a novel EMT inducer in breast cancer. *SHON* induces an EMT program in epithelial cells and potentiates the stem-like properties, characterized by increased number of $CD44^{high}/CD24^{low}$ population and mammosphere-forming ability. We also show that *SHON* activates the autocrine TGF-β signaling and interacts with *TGF-β1*, resulting in self-autocrine feedback. These data suggest that *SHON* regulates the EMT *via* a SHON-TGFβ-SHON feedback loop in breast cancer progression, and is closely associated with invasive subtypes of human breast cancer.

Material and Methods

Cell culture

MCF10A and MCF7 cell lines were obtained from the American Type Culture Collection (ATCC), where the cell lines were characterized by DNA fingerprinting and isozyme detection. Cells were immediately expanded and frozen such that they could be revived every 3–4 months. MCF10A cells were cultured as previously described[20] in DMEM/F12 supplemented with 5% horse serum, 20 ng/mL EGF, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 mg/mL insulin and pen/strep. MCF7 cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Hyclone, Logan, UT).

Co-culture experiments

Experiments were carried out as described in Supporting Information for Materials and Methods.

Reagents and antibodies

The reagents and antibodies are listed in Supporting Information for Materials and Methods.

Immunoblotting

Experiments were carried out as described in Supporting Information for Materials and Methods.

Immunofluorescence

Immunofluorescence was carried out essentially as previously described.[21] A description of procedures is detailed in Supporting Information for Materials and Methods.

Plasmids and stable cell lines

The details of the *SHON*-expressing plasmid pIRESneo3-SHON and the pIRESneo3 empty vector, the *SHON*-specific siRNA-expressing plasmid pSilencer 2.1-U6-shSHON (shSHON) and the negative siRNA control pSilencer 2.1-U6 (shCtrl) were previously described.[19] The constructions of the SHON-flag fusion expression plasmid and the stable cell lines are detailed in Supporting Information for Materials and Methods.

Transwell migration and invasion assays

Experiments were carried out as described in Supporting Information for Materials and Methods.

Live-cell imaging system

MCF10A cells expressing control or SHON were seeded on coverslips or glass-bottom cell culture dishes overnight. Cells were maintained at 37°C throughout the experiment. The random migration of the cells was recorded with the Live-cell Imaging System, and traces and migration speed of migrating cells were analyzed with MetaMorph.

Mammosphere formation assay

Mammosphere assays were carried out as described elsewhere[22] with minor modifications. A description of procedures is detailed in Supporting Information for Materials and Methods.

Human breast tumor specimens and immunohistochemistry

The use of breast cancer samples in our study was approved by the Institutional Review Board of the Second Hospital of Jilin University, China. Immunohistochemistry was carried out essentially as previously described.[23] A description of

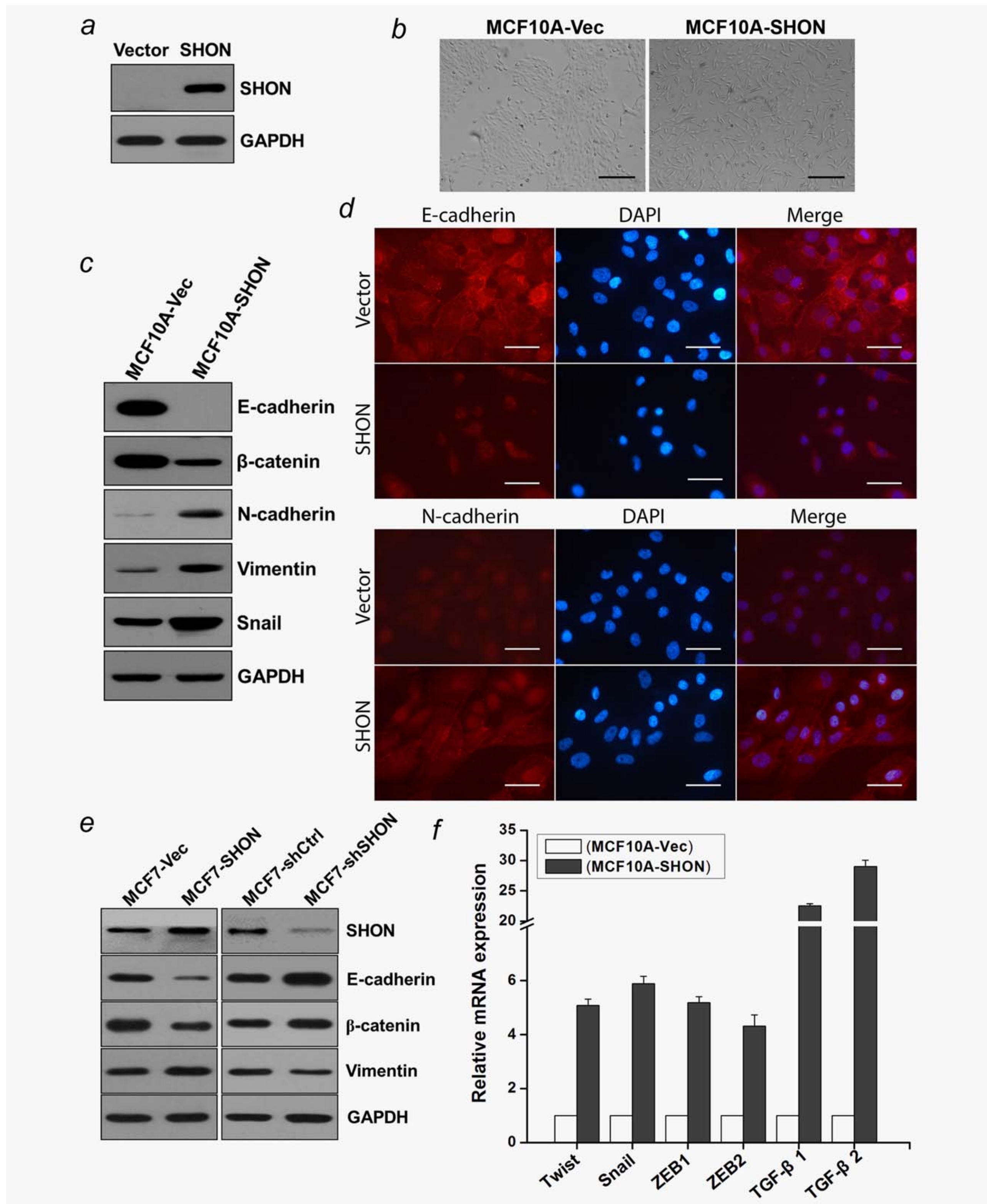

Figure 1. Ectopic expression of SHON induced an EMT program in MCF10A and MCF7 cells. (*a*) Immunoblotting assessment of the ectopic SHON expression in the stable MCF10A cells expressing SHON or empty vector. (*b*) Morphologic changes in SHON-overexpressing MCF10A cells and in control cells. Scale bar = 100 μm. (*c*) Immunoblotting analysis of the expression of the epithelial markers E-cadherin and β-catenin, and the mesenchymal markers Vimentin, N-cadherin and the transcription factor Snail. (*d*) Immunofluorescence staining for the epithelial and mesenchymal markers. Scale bar = 100 μm. (*e*) Immunoblots of the expression of SHON and the epithelial markers E-cadherin and β-catenin, and the mesenchymal marker Vimentin in MCF7 cells either overexpressing or silencing of SHON. (*f*) mRNA expression levels of known EMT inducers were assessed by real-time PCR. Error bars represent the mean ± SD of triplicate experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

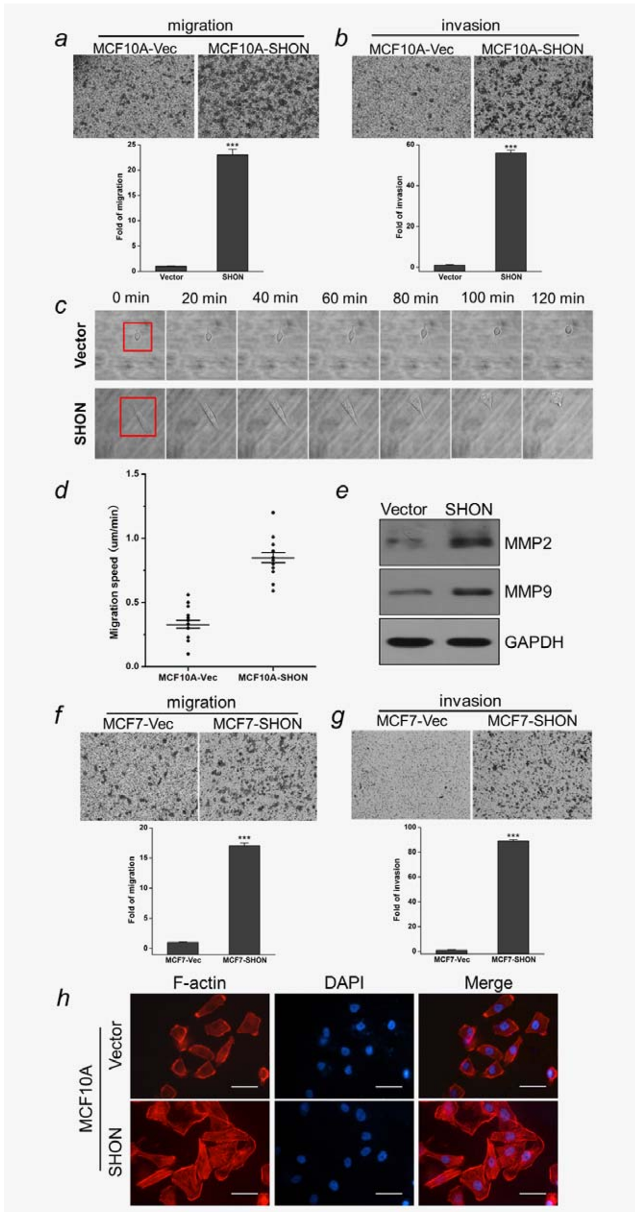

Figure 2. Ectopic expression of SHON promoted cell motility and influenced the cytoskeleton remodeling. (*a* and *b*) Migration (24 hr; *a*) and invasion (48 hr; *b*) assays in stable MCF10A cells. The means were derived from cell counts of five randomly chosen fields, and each experiment was repeated three times (***$p < 0.001$, compared with the control). Representative images of migrated and invaded cells are shown. (*c*) Tracing of migration of single cell recorded on a Live-cell Imaging System. Images were obtained by tracking the movement of cells over the course of 2 hr. (*d*) Scattered dot plot of cell migration speed obtained by cell tracking. Ten vector-treated cells and ten SHON-treated cells were tracked. Horizontal bars represent mean ± SEM ($p < 2 \times 10^{-5}$) as calculated by one-tailed Student's *t*-test. (*e*) Immunoblotting assessment of the expression of MMP2 and MMP9 in MCF10A cells expressing SHON or empty vector. (*f* and *g*) Migration (24 hr; *f*) and invasion (48 hr; *g*) assays in stable MCF7 cells. The means were derived from cell counts of five fields, and each experiment was repeated three times (***$p < 0.001$, compared with the control). Representative images of migrated and invaded cells are shown. (*h*) Immunofluorescence staining for F-actin with phalloidine in MCF10A-Vec and MCF10A-SHON cells. Scale bar = 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

procedures is detailed in Supporting Information for Materials and Methods.

Flow cytometry

Experiments were carried out as described in Supporting Information for Materials and Methods.

RT-PCR and real-time PCR analysis

A description of procedures is detailed in Supporting Information for Materials and Methods.

Statistical analysis

Data are presented as mean ± SD. The Student's *t*-test (two-tailed) was used to determine the statistical significance of differences between groups. The $p < 0.05$ was considered statistically significant. SHON expression level in human breast cancer samples was analyzed by χ^2 test. Statistical analysis was carried out using the SPSS17.0 software.

Results

Ectopic expression of *SHON* induced an EMT program in human mammary epithelial and cancer cells

We previously showed that *SHON* was able to oncogenically transform the immortalized normal human mammary epithelial cell line MCF10A.[19] To determine whether *SHON* is involved in EMT, we first stably overexpressed *SHON* in MCF10A cell line, and the expression was confirmed by immunoblotting (Fig. 1*a*) and real-time PCR (Supporting Information Fig. 1*a*, upper). We observed that ectopic expression of *SHON* converted the predominant epithelial phenotype to an mesenchymal phenotype (Fig. 1*b*), accompanied by a decrease in the expression of epithelial cell markers E-cadherin and β-catenin, and an increase in the expression of mesenchymal cell markers N-cadherin and Vimentin (Fig. 1*c* and Supporting Information Fig. 1*a*, lower). Immunofluorescence microscopy was then applied to compare the immunostaining of E-cadherin and N-cadherin in MCF10A-vector *versus* MCF10A-SHON cells. As can be seen, the SHON-expressing MCF10A cells exhibited a complete loss of E-cadherin at cell–cell junctions; meanwhile, the mesenchymal markers N-cadherin were dramatically upregulated (Fig. 1*d*). To further investigate whether *SHON* plays a similar role in the regulation of EMT in breast cancer cell, we stably overexpressed *SHON* in MCF7 cells, a luminal epithelial breast cancer cell line, and we also detected a significant downregulation of E-cadherin and β-catenin, as well as an upregulation of Vimentin (Fig. 1*e*, left and Supporting Information Fig. 1*b*). Consistently, knockdown of *SHON* in MCF7 cells increased the expression of *E-cadherin* and *β-catenin* and reduced the expression of *Vimentin* (Fig. 1*e*, right and Supporting Information Fig. 1*c*). These results implicated an apparent transition of the SHON-expressing MCF10A and MCF7 cells from an epithelial to mesenchymal phenotype. To elucidate the possible interactions between *SHON* and other EMT-inducing transcription factors, we examined the

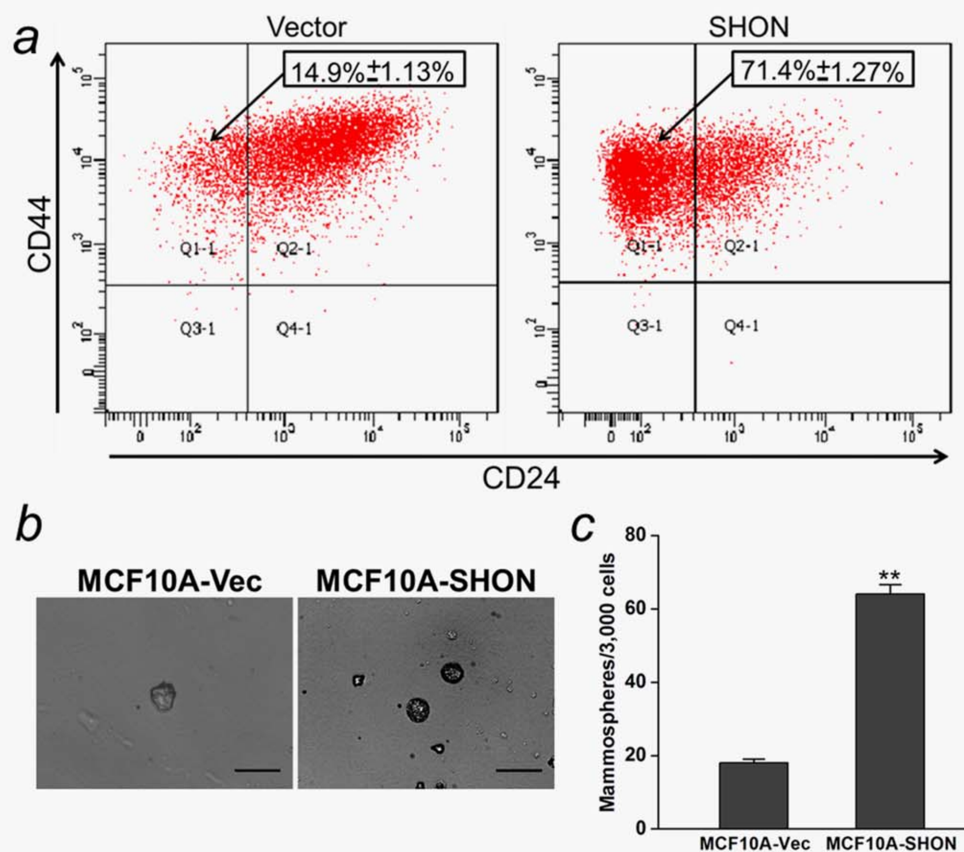

Figure 3. SHON-induced EMT generated stem-like cells. (*a*) FACS analysis of cell surface markers CD44 and CD24 in MCF10A cells expressing SHON or empty vector. Percentages of mean CD44^{high}/CD24^{low} subpopulation ± SD based on triplicate experiments are indicated. (*b*) Phase contrast images of mammospheres formation. Scale bar = 100 μm. (*c*) Quantification of mammosphere numbers formed from three independent experiments (error bar, mean ± SD; **$p < 0.01$, compared with the control). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

expression of other known EMT inducers in MCF10A-SHON cells by using real-time PCR, and we found that the endogenous mRNA levels of *Snail*, *Twist*, *ZEB1*, *ZEB2* and *TGF-β* were significantly enhanced in response to *SHON* overexpression (Fig. 1*f*). Among these transcription factors, TGF-β was the most apparently upregulated. *TGF-β* has been identified as a main inducer of EMT, and is implicated to interact with *Snail*, *Twist*, *ZEB1* and *ZEB2*.[15,24,25] Together, these results indicate that *SHON* is a novel inducer of EMT and its function may be associated with TGF-β signaling.

Ectopic expression of *SHON* promoted cell motility and influenced the cytoskeleton remodeling

Because the EMT phenotype is usually accompanied by the acquisition of such cell traits as greater migration and more invasive ability, we intended to determine whether *SHON* was involved in the migration and invasion in MCF10A cells by using an invasion assay in Transwell chambers. As shown in Figures 2*a* and 2*b*, SHON-expressing MCF10A cells gained dramatically potentiated migratory and invasive behaviors. Live-cell tracking revealed that overexpression of *SHON* in MCF10A epithelial cells increased the migration speed of cells by ∼150% (Figs. 2*c* and 2*d*). Moreover, we detected enhanced protein and mRNA levels of matrix metalloproteinase (MMP)2 and MMP9 in response to *SHON* expression (Fig. 2*e* and Supporting Information Fig. 2); these enzymes are thought to degrade the surrounding matrix to facilitate the invasive behavior following EMT.[26,27] In addition, we also found that ectopic expression of *SHON* promoted the migration and invasion of MCF7 cells (Figs. 2*f* and 2*g*). These data suggest that *SHON* is a strong stimulator of cell motility for both breast epithelial and breast cancer cells.

In line with our findings, reports have shown that the acquisition of cell migration and invasion properties during EMT involves a dramatic reorganization of the actin cytoskeleton, and extensive reorganization of cytoskeleton is a prerequisite for cell motility.[28–31] Thus, we examined the effect of SHON on cytoskeleton reorganization. In an attempt to study whether SHON could stimulate the formation of stress fiber, we monitored the cytoskeleton F-actin assembly, which represents a hallmark of moving cells.[32] As shown in Figure 2*h*, the F-actin polymerization was stimulated in SHON-

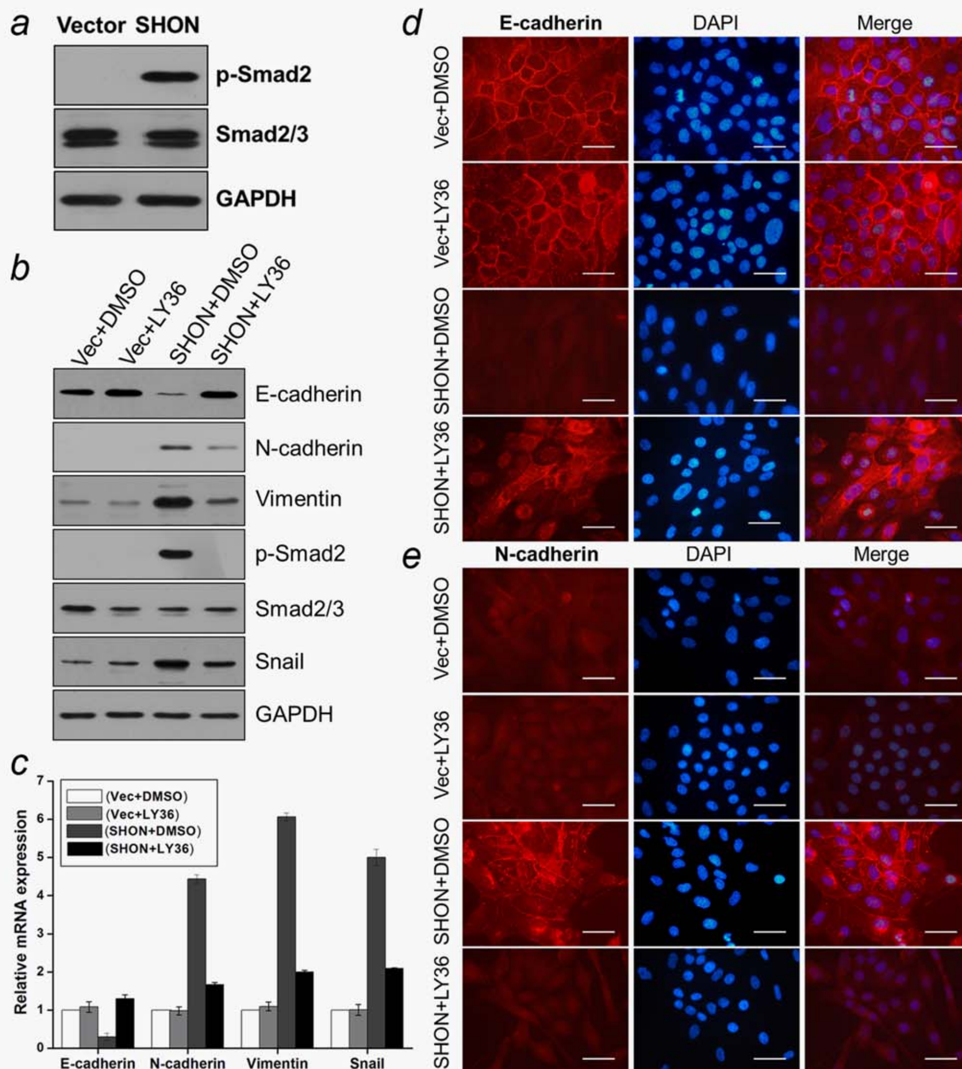

Figure 4. Activation of TGF-β signaling was necessary for SHON-induced EMT. (*a*) Immunoblots of p-Smad2 and total Smad2/3 protein levels in MCF10A cells expressing SHON or empty vector. (*b*–*e*) MCF10A-SHON and MCF10A-Vec cells were treated with 10 μM LY364947 (LY36) for 24 hr, respectively. Immunoblots of p-Smad2, total Smad2/3 protein, E-cadherin, N-cadherin, Vimentin and Snail (*b*); real-time PCR analysis of E-cadherin, N-cadherin, Vimentin and Snail (*c*); immunofluorescence of E-cadherin (*d*) and N-cadherin (*e*), scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

expressing MCF10A cell, indicative of the formation of stress fiber in the cells. These results implicate that *SHON* stimulates F-actin polymerization and promotes cell motility.

The SHON-induced EMT generated stem-cell-like populations

Mammary epithelial cells undergoing an EMT program have been linked to stem cell phenotypes such as an increased CD44^{high}/CD24^{low} population and mammosphere formation ability.[7,33,34] To elucidate whether *SHON* could influence the EMT-associated stem cell properties, we carried out flow cytometry (FACS) to identify CD44^{high}/CD24^{low} populations. We found that ectopic expression of *SHON* in MCF10A cells resulted in a significant increase of the CD44^{high}/CD24^{low} stem cell population compared with MCF10A-vector cells (Fig. 3*a*). Meanwhile, the SHON-expressing MCF10A cells increased the formation of mammospheres both in size and in number compared with that in MCF10A-vector cells (Figs. 3*b* and 3*c*). Clearly, the EMT process induced by *SHON* generated mesenchymal cells with stem cell properties.

Activation of TGF-β signaling was necessary for SHON-induced EMT and acquisition of stem-cell-like properties

We have shown above that *TGF-β*, an important inducer of EMT, was associated with SHON-induced EMT process (Fig. 1*f*). We then intended to identify whether TGF-β signaling was necessary for SHON-induced EMT. Immunoblotting analysis revealed that the level of phosphorylated Smad2 protein, a downstream effector of TGF-β signaling pathway, was significantly increased in SHON-expressing MCF10A cells

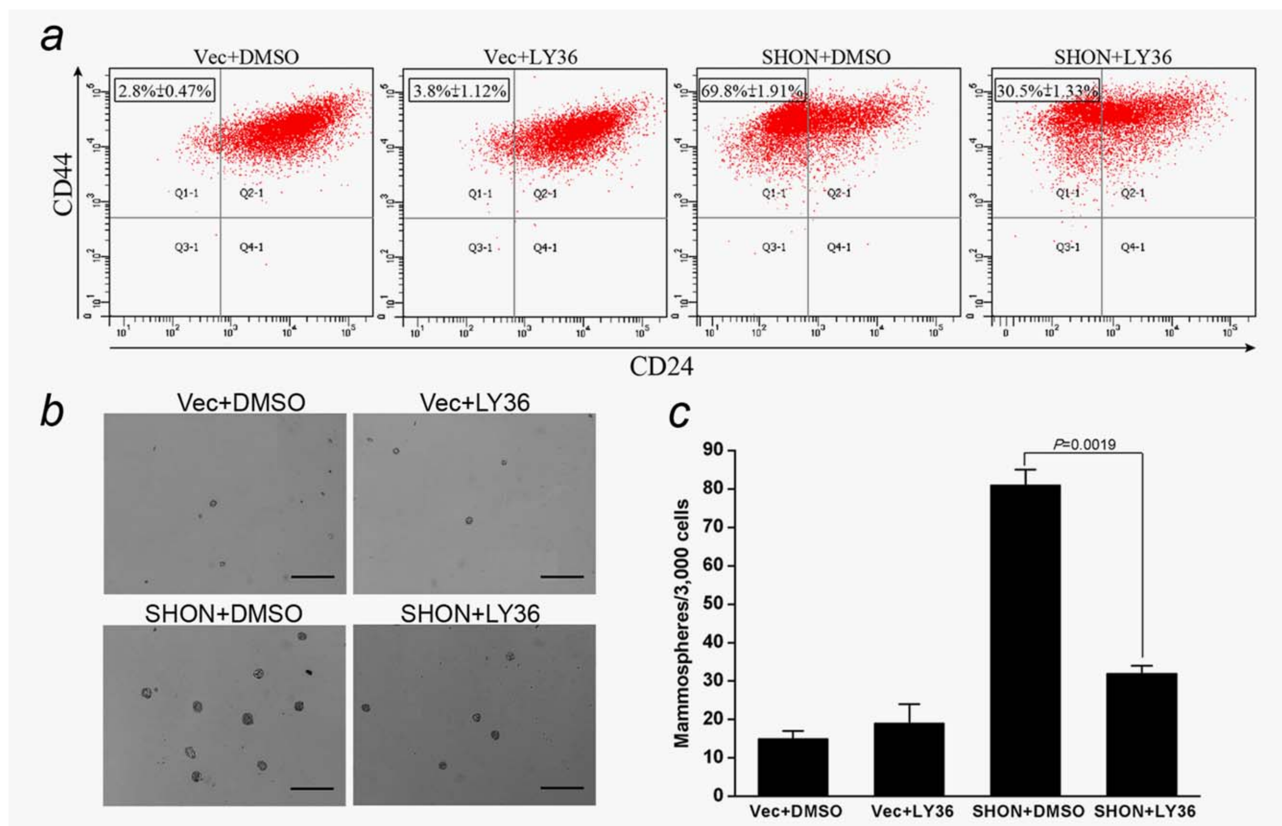

Figure 5. Activation of TGF-β signaling was necessary for SHON-induced stem-cell-like properties. (*a*) MCF10A-SHON and MCF10A-Vec cells were treated with 10 μM LY364947 (LY36) for 24 hr, respectively. FACS analysis of cell surface markers CD44 and CD24 was performed. Percentages of mean $CD44^{high}/CD24^{low}$ subpopulation ± SD based on triplicate experiments are indicated. (*b* and *c*) MCF10A-SHON and MCF10A-Vec cells were treated with 10 μM LY364947 (LY36) for 21 days, respectively. Phase contrast images of mammospheres formation (*b*), and quantification of mammosphere numbers formed from three independent experiments (*c*) (error bar, mean ± SD; $^{**}p < 0.01$, compared with the control), scale bar = 200 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Fig. 4*a*). We next used a specific TGF-β receptor kinase inhibitor LY364947 to block the TGF-β signaling in SHON-expressing MCF10A cells, and we found that the inhibitor counteracted the effects of *SHON* overexpression, as it reduced the levels of Smad2 phosphorylation and mesenchymal cell markers N-cadherin and Vimentin, as well as the transcription factor *Snail*; all of which were increased by *SHON* expression (Fig. 4*b*). The inhibitor LY364947 also significantly restored the level of the epithelial cell marker E-cadherin that was diminished upon *SHON* expression (Fig. 4*b*). Consistent results of the mRNA levels of these markers were obtained by using the real-time PCR assays (Fig. 4*c*). Moreover, our immunofluorescence study further confirmed the changes of *E-cadherin* and *N-cadherin* expression upon LY364947 treatment (Figs. 4*d* and 4*e*). Furthermore, treatment of SHON-expressing MCF10A cells with SB431542, another specific TGF-β receptor kinase inhibitor, gained consistent results; specifically, it decreased Smad2 phosphorylation, as well as N-cadherin, Vimentin and Snail levels, as identified by immunoblotting (Supporting Information Fig. 3*a*), real-time PCR (Supporting Information Fig. 3*b*) and immunofluorescence (Supporting Information Figs. 3*c* and 3*d*). These results suggested that the activation of TGF-β signaling was necessary for SHON-induced EMT.

To further determine whether TGF-β signaling is involved in SHON-induced stem-cell-like properties, we used the inhibitor LY364947 to block the TGF-β signaling in SHON-expressing MCF10A cells, and then we carried out FACS to identify $CD44^{high}/CD24^{low}$ populations. We found that the treatment of SHON-expressing MCF10A cells with LY364947 reduced the number of the $CD44^{high}/CD24^{low}$ stem cell population compared with the cells without treatment (Fig. 5*a*). Meanwhile, the treatment of SHON-expressing MCF10A cells with the inhibitor decreased the formation of mammospheres both in size and in number (Figs. 5*b* and 5*c*). These data provided evidence that *SHON* induced EMT to generate mesenchymal cells with stem cell properties through activation of TGF-β signaling.

SHON and *TGF-β* were mutually regulated to contribute to EMT process

To investigate the relationship between *SHON* and TGF-β signaling, we treated MCF10A with TGF-β1 at different concentrations and for different time periods. Interestingly, we found that although *SHON* was able to upregulate the *TGF-*

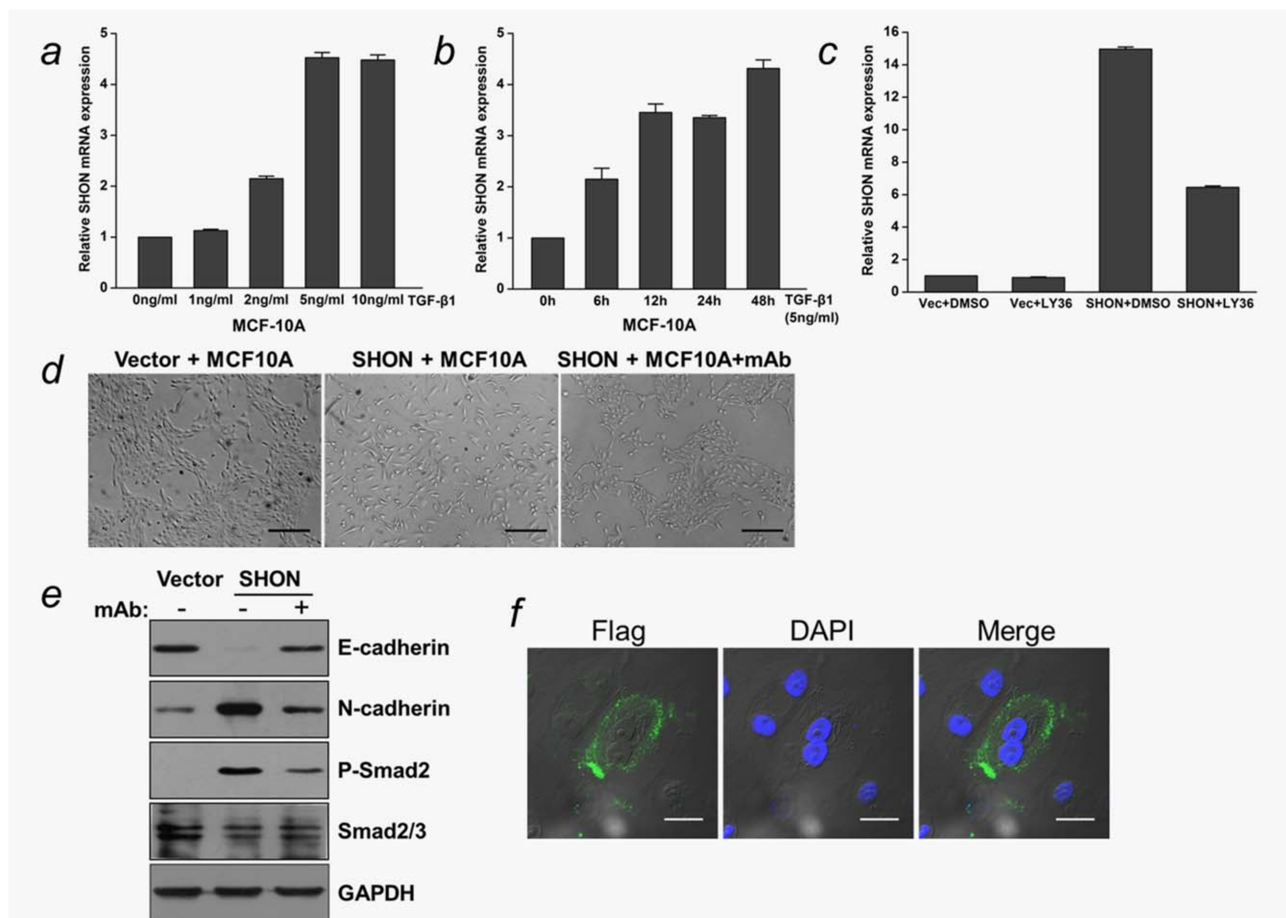

Figure 6. SHON and TGF-β were mutually regulated to initiate an EMT process. (*a* and *b*) SHON mRNA expression levels in MCF10A cells treated with activated TGF-β1 at indicated concentrations (5 ng/ml) (*a*) and time periods (*b*). Error bar represents mean ± SD of triplicate assays. (*c*) Expression of SHON mRNAs was determined by real-time PCR in MCF10A-SHON and MCF10A-Vec cells treated with 10 μM LY364947 (LY36) for 24 hr. (*d*) Morphologic change of MCF10A cells co-cultured for 60 hr with MCF10A-Vec cells (left), MCF10A-SHON cells (middle) and MCF10A-SHON cells with specific anti-SHON antibody in medium (right). Scale bar = 100 μm. (*e*) Immunoblots of p-Smad2, total Smad2/3 protein, E-cadherin and N-cadherin proteins in MCF10A cells co-cultured with MCF10A-Vec, MCF10A-SHON (with or without anti-SHON antibody). (*f*) Immunofluorescence staining for SHON-flag in MCF10A cells co-cultured with MCF10A-SHON-flag cells for 36 hr. Scale bar = 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

β1 expression, as shown in Figure 1*f* above, TGF-β1 could conversely increase the SHON expression (Figs. 6*a* and 6*b*). Consistent with these results, we also showed that treatment of SHON-expressing MCF10A cells with TGF-β signaling inhibitors LY364947 and SB431542 reduced the level of *SHON* expression (Fig. 6*c* and Supporting Information Fig. 4). These data implicated a mutual regulation between *SHON* and *TGF-β*.

As SHON has been identified as a secreted hominoid-specific oncoprotein,[19] we speculate that SHON may activate the TGF-β signaling pathway through an autocrine and/or paracrine approach. To test this, we established an MCF10A/MCF10A-SHON cell co-culture system to study the interaction between *SHON* and TGF-β signaling. As shown in Figures 6*d* and 6*e*, co-culture of MCF10A with SHON-expressing MCF10A cells led to an increase of Smad2 phosphorylation level, as well as a change of cell morphology from epithelial to mesenchymal phenotype, which was accompanied by a decrease of E-cadherin and an increase of N-cadherin. Meanwhile, application of 50 ng/ml anti-SHON neutralizing monoclonal antibody effectively counteracted the effects of *SHON*-expressing MCF10A cells on MCF10A cells in the co-culture (Figs. 6*d* and 6*e*). These results suggested that SHON was able to activate TGF-β signaling in a paracrine manner, though possibility of the involvement of an autocrine approach could not be ruled out. Consequently, the MCF10A-SHON cells in the co-culture initiated an EMT program in MCF10A cells *via* TGF-β signaling.

To further understand the way in which the secreted SHON protein influences adjacent cells, we cloned the sequence of *SHON* with a C-terminal flag epitope tag into the plasmid pIRESneo3-SHON (termed pIRESneo3-SHON-flag), and then assayed the flag expression in MCF10A cells after co-culture with the cells that were transiently transfected

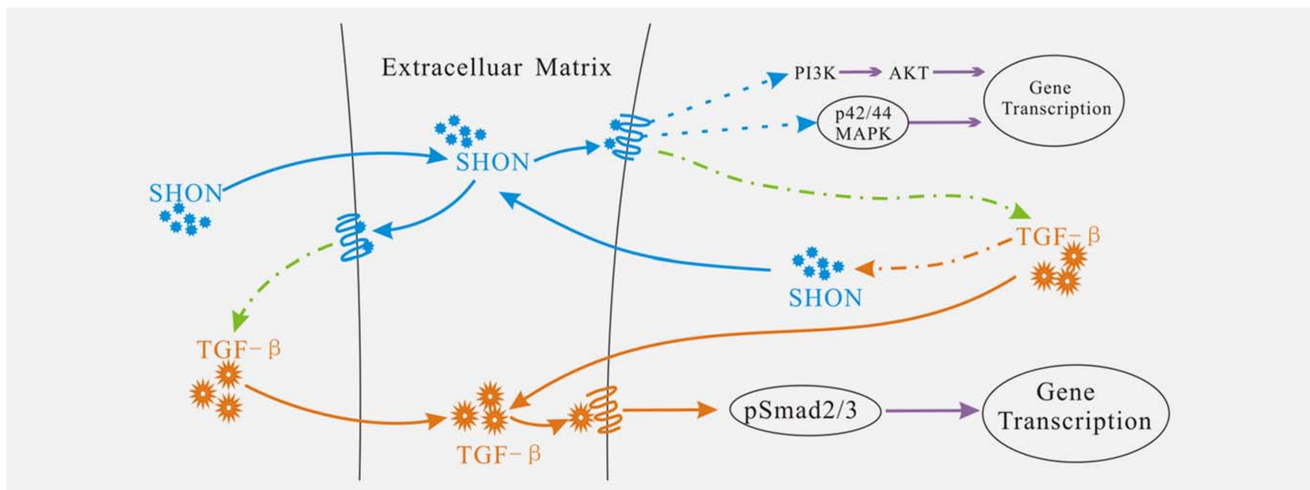

Figure 7. Schematic illustration of a proposed model about the interaction between SHON and TGF-β. The model describes two self-stimulating and cross-communicating signaling loops mediated by SHON and TGF-β. Cell-secreted SHON and TGF-β ligands act upon their corresponding receptors in an autocrine and/or paracrine fashion. The subsequent activation of TGF-β-Smad2/3 drives breast epithelial cell differentiation and endogenous SHON and TGF-β expression, thereby generating self-stimulating autocrine signaling loop and SHON-TGFβ-SHON feedback loop. AKT and MAPK signaling pathways are also involved in the event. Crosstalk between these signaling loops therefore stimulates one another and further boosts breast epithelial cell differentiation. The dotted lines denote the unclear pathways. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with pIRESneo3-SHON-flag plasmid or vector plasmid. Immunofluorescence experiments revealed that the flag-tagged SHON protein was densely located on the surface of the cells (Fig. 6*f*), indicative of the secretory nature of the protein, and the cells transfected with vector plasmid revealed no fluorescence signal of flag on the surface of the cells (data not shown). These experiments provide evidence that the SHON protein secreted by tumor cells can be released to extracellular environment to affect the surrounding cells resulting in cell cancerization.

SHON played a role in breast cancer progression

In a previous study, we identified a positive correlation between *SHON* expression and better survival outcomes of breast cancer patients.[19] To further evaluate the clinical relevance of *SHON* expression in human mammary carcinomas, we carried out IHC staining of SHON in 60 human breast tissues, and found that 68% (41 of 60) of the breast cancer samples exhibited positive SHON immunoreactivity (data not shown). Interestingly, consistent with the results from cell culture, among the 41 SHON-positive tumor samples, nearly all the tissues showed strong extracellular matrix staining (Supporting Information Fig. 6*a*, red), while the paracarcinoma tissues with negative SHON immunoreactivity did not (Supporting Information Fig. 6*a*, blue). Additionally, we also found that E-cadherin was significantly downregulated in tumor tissues formed by MCF7-SHON cells *in vivo* xenograft experiments compared with that formed by MCF7-vector cells (Supporting Information Fig. 6*b*). These results implicate that *SHON* may also downregulate the expression of epithelial marker E-cadherin, and affect the surrounding tissue as a secreted protein in breast carcinoma tissues.

To summarize, we unraveled in our study that the secreted protein *SHON* is an inducer of EMT and it contributes to breast cancer progression. This process is implemented through the activation of TGF-β signaling by *SHON* in an autocrine and/or paracrine manner. In addition, our data implicate that *SHON* expression can be stimulated by TGF-β signaling. On the basis of these results, we propose a working model to depict the functional relationship between *SHON* and *TGF-β*, in which paracrine and/or autocrine stimulation of the *SHON* is sufficient for the acquisition of mesenchymal-like morphology of the cells, resulting in increased motility and invasion ability (Fig. 7).

Discussion

SHON is a novel secreted protein we have previously identified to exert a crucial function in breast cancer progression and in the oncogenic transformation of mammary carcinoma cells *in vitro* and *in vivo*.[19] However, the mechanisms in which *SHON* implements its oncogenic effects during breast cancer progression have not been clearly elucidated. As a continuing insight of the previous results, the aim of our study was to define and characterize the mechanistic actions of *SHON* in breast carcinoma. We demonstrated that *SHON* functioned as a trigger of EMT to contribute to breast cancer progression, a previously unreported role of *SHON* in breast cancer. Specifically, we found that ectopic expression of *SHON* in both epithelial MCF10A and cancerous MCF7 cells repressed the epithelial status and induced the mesenchymal status (Fig. 1), which was accompanied by changes in

expression of the classic EMT marker E-cadherin (Fig. 1 and Supporting Information Fig. 1), a factor that plays a pivotal role in epithelial cell–cell adhesion. Loss of E-cadherin is considered to enable metastasis by disrupting intercellular contacts, which is an early step in metastatic dissemination.[35,36]

The actin cytoskeleton is known to play important roles in metastatic behavior of breast cancer cells.[30,32] We observed in this work that *SHON* influenced the microtubule cytoskeleton remodeling and dramatically increased the migration and invasion abilities of MCF10A and MCF7 cells (Figs. 2*a* and 2*b* and 2*f*−2*h*). Our results suggest that *SHON* may play a role in microtubule cytoskeleton remodeling associated with its potent in induction of an EMT program and in promotion of cancer metastasis.

Moreover, our experiments demonstrated that the ectopic overexpression of *SHON* upregulated the mRNA expression of several EMT-inducing transcription factors, notably the *Snail*, *Twist*, *ZEB* and *TGF-β* (Fig. 1*f*). These results are in accordance with a recent study that discovers that overexpression of one of the EMT inducers upregulates a subset of other EMT-inducing transcription factors, implicating the interactions among these EMT inducers.[37] Induction of Snail expression has been noted in all EMT processes that have been studied.[38,39] *Snail* is closely related to TGF-β signaling during EMT progress,[40] and TGF-β induces *Snail* expression in many human cancers including breast cancer, skin cancer and lung cancer.[41,42] Consistent with these reports, our results showed that *SHON* upregulated the expression of *TGF-β*, accompanied by increased *Snail* expression (Fig. 1*f*), and when the TGF-β signaling pathway was blocked with an inhibitor in MCF10A-SHON cells, the expression of *Snail* was reduced accordingly (Figs. 4*b* and 4*c*).

We have shown in a previous study that *SHON* is involved in cell migration and invasion through MAPK and AKT signaling pathways.[19] In our study, although we detected increased expressions of p-P42/44 and p-AKT in MCF10A-SHON cells (Supporting Information Fig. 5*a*), the most remarkable change was the significantly increased expression of *TGF-β* (Fig. 1*f*). Our results revealed that *SHON* upregulated the *TGF-β* expression and activated the Smad-TGFβ signaling pathway, characterized by the enhanced level of phosphorylated Smad2 protein, which in turn promoted the cell migration and invasion (Figs. 4*a* and 4*b*). To further demonstrate that TGF-β signaling is involved in the MAPK and AKT signaling pathways in SHON-induced EMT progress, we used the inhibitor LY364947 to block the TGF-β signaling in SHON-expressing MCF10A cells, and then we carried out immunoblotting to identify whether the SHON-dependent AKT and MAPK response is blocked by the TGF-β inhibitor. As a result, we found no distinct changes in the phosphorylation of AKT and P42/44 upon the treatment (Supporting Information Fig. 5*b*). This result may indicate that the activation of AKT and MAPK signaling is TGF-β-independent in SHON-induced EMT progress. We also carried out the MCF10A/MCF10A-SHON cell co-culture experiment to verify the manner of SHON in induction of AKT and MAPK signaling. As shown in Supporting Information Figure 5*c*, co-culture of MCF10A with SHON-expressing MCF10A cells enhanced the phosphorylation of AKT and P42/44, while application of 50 ng/ml anti-SHON-neutralizing monoclonal antibody effectively counteracted the increase of SHON-expressing MCF10A cells on MCF10A cells in the co-culture (Supporting Information Fig. 5*c*). This result illustrates the existence of the paracrine approach, although the possibility of the involvement of an autocrine approach could not be ruled out. These data suggest that SHON is able to activate the AKT and MAPK signaling in an autocrine and/or paracrine manner.

More importantly, our data implicated that *SHON* was also involved in TGF-β-induced EMT. Presumably, an interaction between *SHON* and *TGF-β* may be a key event in *SHON*-triggered EMT, considering that both SHON and TGF-β are secreted proteins. This was partly validated by our experimental results that ectopic overexpression of *SHON* activated the TGF-β signaling *via* autocrine/paracrine of the protein (Figs. 6*d* and 6*e*). And in breast epithelial cells, *SHON* increased the expression of TGF-β, while SHON was in turn induced by TGF-β (Figs. 6*a*–6*c*). Also, we observed the concentrated distribution of SHON in the extracellular matrix in breast cancer tissue samples (Supporting Information Fig. 6*a*). In addition, it should be noticed that although TGF-β has a dual role in the progression of human cancer, not all genes that are regulated by TGF-β have such a due function. Overall, our observations support a model of a SHON-TGFβ-SHON-positive feedback loop that functions as a novel signaling pathway in EMT regulation, as well as in breast cancer progression (Fig. 7).

Recent studies have shown that EMT can induce noncancerous stem cells to acquire cancer stem cell (CSC)-like properties in breast cancer cells that exhibit a $CD44^{high}/CD24^{low}$ antigenic phenotype.[7] Furthermore, therapies aimed at eliminating the $CD44^{high}/CD24^{low}$ cells may represent a new approach for the clinical management of basal-like breast cancer, currently the only major breast tumor subtype without effective targeted treatment strategies and with poor prognosis.[43] Our findings that ectopic expression of *SHON* in MCF10A cells increases the $CD44^{high}/CD24^{low}$ subpopulation and enhances the mammosphere-forming ability (Fig. 3) may provide useful clues for the development of this potent clinical therapeutic strategy for highly aggressive and malignant breast cancer.

We have shown in our previous study that SHON-positive cancer patients have better prognosis.[19] We have also demonstrated that *SHON* is an oncogene and plays an important role in cancer progression. It is easy to understand that the expression of an oncogene is linked to a worse outcome. However, that is not always the case. About 75% of breast cancer is estrogen dependent. Estrogen receptor (ER) signaling is the target of endocrine (antiestrogen) therapy. An important conclusion reached from our previous study is that SHON expression predicts a good outcome of endocrine

therapy, which targets the ER signaling. Thus, it is not surprise that the expression of SHON, just like ER itself, can have a good prognosis for ER-positive patients receiving endocrine (antiestrogen) therapy.

Collectively, data arising from this study validate the SHON protein as an effective inducer of EMT program, which endows the breast epithelial cells with CSC-like properties. We further demonstrate that *SHON* interacts with *TGF-β* and functions as a novel signaling pathway in the regulation of breast cancer progression. These results confirm that *SHON* plays an important role in EMT and contributes to breast cancer progression.

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