SND1 Acts Downstream of TGFβ1 and Upstream of Smurf1 to Promote Breast Cancer Metastasis

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Abstract

SND1 is an AEG-1/MTDH/LYRIC-binding protein that is upregulated in numerous human cancers, where it has been assigned multiple functional roles. In this study, we report its association with the TGFβ1 signaling pathway, which promotes epithelial–mesenchymal transition (EMT) in breast cancer. SND1 was upregulated in breast cancer tissues, in particular in primary invasive ductal carcinomas. Transcriptional activation of the SND1 gene was controlled by the TGFβ1/Smad pathway, specifically by activation of the Smad2/Smad3 complex. The SND1 promoter region contained several Smad-specific recognition domains (RD motifs), which were recognized and bound by the Smad complex that enhanced the transcriptional activation of SND1. We found that SND1 promoted expression of the E3 ubiquitin ligase Smurf1, leading to RhoA ubiquitination and degradation. RhoA degradation in breast cancer cells disrupted F-actin cytoskeletal organization, reduced cell adhesion, increased cell migration and invasion, and promoted metastasis. Overall, our results define a novel role for SND1 in regulating breast tumorigenesis and metastasis. Cancer Res; 75(7); 1275–86. ©2015 AACR.

Introduction

Breast cancer is the most commonly diagnosed cancer in women and the leading cause of female cancer death worldwide (1). Distant metastasis and invasion of advanced cancer are responsible for more than 90% of cancer-related deaths (2). However, the molecular mechanisms underlying the breast cancer tumorigenesis and metastasis are largely unknown.

The TGFβ signaling pathway is a key player in the epithelial–mesenchymal transition (EMT) in breast cancer. The misregulation of TGFβ signaling may promote tumor invasion and migration, leading to detrimental consequences for prognosis in patients with breast cancer (3–5). The TGFβ signaling pathway contains different R-Smads (Smad1, Smad2, Smad3, and Smad5) and co-Smad (Smad4) transcription factors (6–8). Smad1, Smad5, and Smad8 are specifically activated by the bone morphogenetic protein (BMP) receptors, whereas Smad2 and Smad3 are specifically activated by TGFβ-related receptors (9). In the activated pathway, the phosphorylated R-Smads enter the nucleus to form complex with co-Smad Smad4 and then combine with specific Smad recognition domains (RD) in the promoter region of target genes (10).

Staphylococcal nuclease domain-containing 1 (SND1), also known as Tudor staphylococcal nuclease (Tudor-SN) or p100 protein, ubiquitously exists in human and many other species. Proteomic analysis of clinical breast cancer samples shows a close correlation of elevated SND1 expression with cancer metastasis (11). It was also reported that SND1 could interact with astrocyte elevated gene-1 (AEG-1) in breast cancer and strongly promote lung metastasis (12). In our earlier work using chromatin immunoprecipitation guided ligation and selection (ChIP-GLAS) assays, we have demonstrated that SND1 is potentially involved in the TGFβ signaling pathway in breast cancer cells (13). However, it remains unclear how SND1 is upregulated in breast cancer and how SND1 participates in breast cancer metastasis.

In the present study, we demonstrate for the first time that SND1 is a novel target of the Smad complex. The gene transcriptional activation of SND1 could be regulated by the TGFβ/Smad pathway. In addition, SND1 mediates the gene transcriptional activation of Smurf1, which is an E3 ubiquitin ligase, promoting RhoA ubiquitination, causing F-actin rearrangement, and leading to increased invasiveness and metastasis of breast cancer.

Materials and Methods

Cell lines, siRNA, and cell transfection

The cell lines MCF-7, T47D, SK-BR-3, MDA-MB-231, and BT549 were obtained from the ATCC. MDA-MB-231 was

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maintained in L-15 medium with 10% FBS, BT549 was cultured in RPMI1640 with 10% FBS, and MCF-7, T47D, and SK-BR-3 cells were cultured in DMEM with 10% FBS. All the siRNAs used in the present study were designed according to previous report. siRNAs of Smad2, Smad3, Smad4, and Smurf1 (14, 15) were purchased from Santa Cruz Biotechnology. Smad5 (16) siRNA was purchased from Abnova. Smad1 (17) and scramble control (18) siRNAs were purchased from Cell Signaling Technology. The cells were transfected with Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol.

Plasmids, lentivirus, and stable cell construction

The pGL3 series of plasmids were purchased from Promega. The pSG5-SND1 plasmid containing full-length SND1 and pSil-SND1-sh plasmid containing shRNA targeting SND1 were constructed as described previously (19).

Lentiviruses containing either the shRNA targeting SND1 (SND1-sh, antisense sequence: 5'-GTTACCATCCTTCATC-CAAAT-3') or the negative control shRNA (scramble control, sequence: 5'-TCTCCGAACGTGTCACGT-CAAG-3') were constructed and packaged by GenePharma. The titers of the virus solutions were 1.0 to 1.2 × 10^9/mL.

The breast cancer cell lines MCF-7 and MDA-MB-231 were infected with 30 μL of lentivirus solution per 6-cm dishes. Forty-eight hours after infection, the cells were treated with 4 μg/mL puromycin for 14 days to select stable cell lines. The stable cell lines were named as MCF-7-SND1-sh, MCF-7-scramble, MDA-MB-231-SND1-sh, and MDA-MB-231-scramble.

IHC

The malignant and normal breast tissues with histopathology reports were requested from the Cancer Hospital of Tianjin Medical University. Informed consent was obtained from each patient. Use of the tissue samples in this study was approved by the Institutional Review Board. The fresh tissues were fixed in 4% formaldehyde and embedded in paraffin. After deparaffinization and rehydration, five-micrometer sections were incubated with a primary antibody directed against SND1 (1:75 dilution; Abcam) at room temperature for 2 hours. A biotinylated secondary antibody was added for 30 minutes at 37°C, followed by immunohistochemical staining using a DAB kit (Zhongshan).

Nude mice and tumor implantation

All nude mouse procedures were approved by the committee on the use and care of animals of Tianjin Medical University. Six-week-old female BALB/c nude mice were used for the animal studies. The mice were fed with a standard rodent diet in an aseptic room with 60% to 70% humidity at 25°C. The mice were then observed daily for diet consumption, stools, and mental state. The tumor sizes were measured every 5 days. In 25 days, the formed tumors were surgically removed, and the weight of tumor was measured.

Transwell and wound-healing assay

MCF-7 and MDA-MB-231 cells were seeded in transwell inserts with an 8-μm pore size (BD Biosciences) at 5 × 10^5 cells per well. After 24 hours, the cells migrated through the filter were fixed stained with 10% crystal violet. For each chamber, the mean of migrated cell number from five randomly fields was calculated and displayed in the plot.

MCF-7 and MDA-MB-231 cells were seeded at 90% confluent in 6-well plates. Three vertical wounds were scratched per well. The relative migration was assessed at ×100 magnification using inverted microscope at the designated time (0 and 24 hours), then calculated by following formula: (0 h wound width − 24 h wound width)/0 h wound width.

RNA extraction and real-time RT-PCR

Total RNA was isolated from cells using TRIzol (Invitrogen). Primers were synthesized by Sangon Biotechnology Co. Ltd. (Supplementary Table S1). The RevertAid First Strand cDNA Synthesis Kit (Fermentas) was used to synthesize cDNA. The PCR reactions were performed with FastStart Universal SYBR Green Master Mix (Roche) on a StepOne Real-Time PCR System (Applied Biosystems). The PCR conditions were 95°C for 2 minutes and 40 cycles of 95°C for 30 seconds and 60°C for 1 minute. GAPDH or β-actin (Supplementary Fig. S1) was used as reference genes, and the mRNA data were presented as the ratio of target gene against reference gene.

Western blotting and antibodies

Total cell lysates (40 μg) were subjected to 12% SDS-PAGE gel electrophoresis and were blotted with series of antibodies: rabbit monoclonal antibodies against RhoA, Cdc42, phosphorylated Smad3, and phosphorylated Smad5 (1:1,000; CST); mouse monoclonal antibodies against SND1, Rac1, Smad1, Smad2, Smad3, Smad4, Smad5, and Smurf1 (1:1,000; Abcam); and a mouse monoclonal antibody against β-actin (1:6,000 dilution; Sigma).

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChiP) assay was performed using the EZ-Magna-ChiP-Chromatin Immunoprecipitation Kit (Millipore) according to the manufacturer's protocol. Immunoprecipitation was performed with the anti-Smad3 antibody (8 μg; Abcam) or anti-IgG antibody (8 μg; Sigma). The purified immunoprecipitated DNA samples were used in qPCR reactions.

Luciferase assay

MCF-7 cells were cotransfected with both pGL3-SND1-pro reporter construct and Renilla construct. After 24 hours, the MCF-7 cells were starved overnight and stimulated with 15 ng/mL TGFβ1 (CST) or vehicle control for 6 hours. The luciferase reporter assay was performed using the Dual-Luciferase Reporter Assay Kit according to the manufacturer's protocol (Promega).

Immunoprecipitation

MCF-7 cells were cotransfected with pSG5-SND1 and Smurf1 siRNA or scramble siRNA. After 36 hours, the total cell lysates were incubated with a rabbit monoclonal anti-RhoA antibody (8 μg/mg; CST) or a rabbit polyclonal IgG antibody as a control (8 μg/mg; Santa Cruz Biotechnology). The incubation was performed overnight at 4°C. Then, the proteins were precipitated by protein A/G Sepharose (Sigma). The bound proteins were blotted with a mouse monoclonal anti-RhoA antibody (1:1,000; Sigma).
Abcam) and a mouse monoclonal anti-ubiquitin antibody (1:1,000 dilution; CST).

**Immunofluorescence**

Cells were seeded on poly-D-lysine-coated coverslips and grown until 70% confluent. Then, the coverslips were fixed and blocked with 3% BSA/PBS. F-actin was detected using Alexa 488–labeled phalloidin (1:600; Invitrogen), and RhoA was detected using a rabbit monoclonal antibody (1:200; CST), followed by the Alexa 594–conjugated secondary antibody (1:600; Invitrogen). Images were captured by confocal fluorescent microscope (FV1200, Olympus).

**Statistical analysis**

Numerical data are presented as the mean ± SD from at least three independent experiments and compared by the Student t test or one-way ANOVA using SPSS 16.0 software. A P value < 0.05 was considered as statistical significance.

**Results**

**SND1 expression is upregulated in breast cancer tissues and positively correlates with the metastatic ability of breast cancer cells**

To investigate the relevance of SND1 expression to breast cancer development and metastasis, we used immunohistochemical approach to detect SND1 in clinical samples of normal breast epithelium, ductal carcinoma in situ, and primary invasive ductal carcinoma (IDC; Fig. 1A). For the normal breast epithelium tissues, SND1 expression was negative in all 12 of the studied cases (100%). Among the 28 cases with DC in situ tissues, SND1 expression was highly positive in 6 cases (21.43%), weakly positive in 19 cases (67.86%), and negative in three cases (10.71%). The expression of SND1 in the IDC samples was significantly higher than the DC in situ samples (**, P < 0.01 vs. DC in situ; Fig 1B and Supplementary Table S2). SND1 expression was positive in all 23 studied cases: 18 were highly positive (78.26%) and 5 were weakly positive (21.74%). The survival time of the IDC cases was analyzed by the Kaplan–Meier assay, and the result was presented in Fig. 1C. Median survival time of high SND1 staining samples (n = 18, 57 months) was significantly shorter than low SND1 staining samples (n = 5, >107 months, P = 0.00306). According to the pathologic data of IDC tissues, there was no significant correlation with SND1 expression and estrogen receptor (ER), progesterone receptor (PR), or HER2 level, thus the involvement of SND1 in the tumorigenesis of breast cancer is likely to be independent with ER, PR, and HER2 (Supplementary Table S3).

We then examined SND1 expression in different established breast cancer cell lines (20). SND1 was highly expressed at both mRNA (Fig. 1D) and protein level (Fig. 1E) in the metastatic breast cancer cell line (MDA-MB-231 and BT549), comparing with the nonmetastatic (MCF-7, T47D, and SK-BR-3). These data further demonstrate the correlation of SND1 expression with the metastasis of breast cancer cells.

To investigate the involvement of SND1 in tumorigenesis in vivo, the effect of the loss-of-function of SND1 on tumorigenesis of MDA-MB-231-SND1-sh cells was assessed in nude mice by orthotopic implantation. The results showed that knockdown of SND1 slowed down the growth of the tumors in situ than scramble control (Fig. 1F). After 25 days, the tumors were surgically removed. The average diameter (Fig. 1G) and weight (Fig. 1H) of the implanted tumors with knockdown of SND1 were significantly less than those of the tumors from the MDA-MB-231 scramble cells. These data further verify the involvement of SND1 in breast cancer tumorigenesis.

**SND1 promotes the metastasis phenomenon of breast cancer cells**

The effect of gain-of-function or loss-of-function of SND1 was performed in MCF-7 and MDA-MB-231 cells to investigate the involvement of SND1 on metastatic ability. qRT-PCR and Western blot analysis revealed that both mRNA (Fig. 2A) and protein level (Fig. 2B) of SND1 were efficiently overexpressed or knocked down by transfecting of pSG5-SND1 plasmid or psi-SND1-sh plasmid, respectively.

The wound-healing assay was performed to detect the migration activity of different breast cancer cells (Fig. 2C). The results showed that the migration ability was significantly increased with ectopically overexpression of SND1 (SND1) in both MCF-7 (left panel) and MDA-MB-231 (right panel), comparing with parental control or scramble control cells. In contrast, knockdown of endogenous SND1 protein (SND1-sh) inhibited the migration ability. Similarly, the transwell invasion assay (Fig. 2D) demonstrated that the invasion ability was increased in SND1 overexpression cells (SND1), but decreased in SND1 knockdown cells (SND1-sh). Furthermore, we performed SND1 rescue experiment in MCF-7-SND1-sh and MDA-MB-231-SND1-sh stable cells to confirm the effect of SND1 in breast cancer metastasis. The wound-healing assays and the transwell invasion assays demonstrated that comparing with the basal control level, the migration and invasion ability was increased in cells with overexpression of SND1, but decreased in untreated SND1-sh knockdown cells. Although the migration and invasion ability of SND1-sh knockdown cells with rescue expression of SND1 was increased compared with untreated SND1-sh cells and rehabilitated to the same level as the basal control groups (Supplementary Fig. S1B and S1C), migration and invasion abilities were the key components of the metastatic cascades, and all these data further indicate the effect of SND1 on the metastatic phenotype of breast cancer cells.

**SND1 expression is upregulated by TGFβ1 stimulation**

To investigate the underline mechanisms of upregulation of SND1 in breast cancer cells, we employed bioinformatics analysis and revealed that the promoter region of the SND1 gene contains two Smad recognition domains (RD1 and RD2) and one sp1 recognition domain. Smads are essential transcription factors in the TGFβ signaling pathway, which are overactive in advanced breast cancer (21), thus the hyperexpression of SND1 in breast cancer cells may be regulated by the activated TGFβ pathway. We first detected the expression of SND1 in MCF-7 cells at different time points with TGFβ1 treatment (15 ng/mL). qRT-PCR results (Fig. 3A) showed that with TGFβ1 treatment, the mRNA level of SND1 was gradually increased, and was 2.93-fold higher than the basal level (non TGFβ1 treatment) after TGFβ1 treatment for 12 hours. Western blot analysis indicated that the SND1 protein level was also increased in a time-dependent manner following TGFβ1 treatment (Fig. 3B); for 24 hours treatment, the SND1 protein level was 2.5-fold higher than the nontreated one, and was approximately 6.0-fold higher with treatment for 48 hours. We then performed luciferase reporter...
assay to examine whether TGFβ1 regulates SND1 expression at the gene transcription level. MCF-7 cells were transfected with the luc-reporter plasmids containing full length of the SND1 promoter region, and then treated with or without TGFβ1 (15 ng/mL) for different time points. The results showed that the gene transcriptional activation of SND1 was increased in a time-dependent manner with TGFβ1 treatment. These data demonstrate that TGFβ1 is likely to promote SND1 expression via gene transcriptional activation in a time-sequence manner, the mRNA level of SND1 primarily increases, and the protein level increases consequently.

Activated Smad2 and Smad3 are required for TGFβ1-mediated SND1 expression in breast cancer cells

To identify the specific Smad responsible for regulating gene transcriptional activation of SND1, we used a series of siRNAs to knock down different endogenous Smads, respectively, in breast cancer cells to block the TGFβ signaling pathway. The knockdown efficiency of siRNAs was detected by Western blot (Fig. 4A). The results showed that the mRNA level of SND1 was remarkably enhanced by TGFβ1 treatment in the MCF-7 cells with knockdown of endogenous Smad1 or Smad5 or the scramble control, but no significant enhancement in the
cells with knockdown of endogenous Smad2, Smad3, or Smad4. Correspondingly, Western blot analysis (Fig. 4C) also showed that knockdown of Smad2, Smad3, or Smad4 (lanes 8, 10, and 12), unlike Smad1 or Smad5 (lane 4 or 6), blocked TGFβ1-induced upregulation of SND1 protein levels in the MCF-7 cells. Our current observations suggest that TGFβ1-induced upregulation of SND1 in breast cancer cells is mediated by Smad2/Smad3/Smad4.

When TGFβ signaling is activated, Smad2 and Smad3 can be phosphorylated and form a heterodimer, which then bind to Smad4 to form the Smad complex, being translocated into the nucleus and regulating the target gene transcription (22), while...
Smad1/Smad5/Smad8 maintain unphosphorylated. MCF-7 cells were treated with Smad2/Smad3 phosphorylation inhibitor SB431542 (SB), or Smad1/Smad5/Smad8 phosphorylation inhibitor LDN193189 (LN), to further investigate whether Smad2 and Smad3 are required for TGFβ1-induced SND1 expression. Western blot results showed that (Fig. 4D) the phosphorylation of Smad3 (top, lane 4) was efficiently inhibited by SB, and Smad5 phosphorylation was inhibited by LN (bottom, lane 12). The same amount of Smad3 or Smad5 protein was observed in different samples (middle). The qRT-PCR results (Fig. 4E) showed that comparing with control cells (con+vehi), the mRNA level of SND1 in the MCF-7 cells treated with SB was not enhanced (con+SB), even
after TGFβ1 treatment (TG+SB). However, TGFβ1 significantly increased the SND1 mRNA level in the cells incubated with LN (TG+LN), which was at the same level as parental cells (TG+vehl). Western blot showed similar results (Fig. 4F). Comparing with the control cells without (lane 1) or with (lane 2) TGFβ1 treatment, SB significantly inhibited the enhancement of SND1 protein level induced by TGFβ1 (lane 3 and lane 4), whereas LN did not block TGFβ1-mediated SND1 expression (lane 5 and lane 6). Furthermore, luciferase reporter assays indicated (Fig. 4G) that TGFβ1 treatment significantly enhanced the transcriptional activation of SND1 with/without treatment of LN, but the enhancement of SND1 was completely blocked by SB. Collectively, all these data indicate that TGFβ1-mediated upregulation of SND1 is dependent on the Smad2/Smad3 activation.

SND1 gene is a novel target of the Smad complex

To determine the specific sequence in the SND1 promoter region that could be recognized by the Smad complex, we generated different luc-reporter plasmids containing different sequence of SND1 promoter regions as illustrated in Fig. 5A. MCF-7 cells were transfected with different luc-reporter plasmids, respectively, and luciferase reporter assays were carried out. The results showed that (Fig. 5A) disruption of the upstream Smad recognition domain (RD2), either individually (ΔSmad RD2) or in combination with RD1 (ΔSmad RD1+2), significantly blocked the enhancement of gene transcriptional activation of SND1 induced by TGFβ1. However, the deletion of RD1 motif (ΔSmad RD1) had no significant effect, comparing with the control cells (full-length promoter region). Although TGFβ1 could still increase SND1 transcriptional activation with deletion of sp1 motif in the promoter region (Δsp1), it was not as efficient as the control group. These observations strongly support the idea that RD, especially RD2, in the promoter region of the SND1 gene is essential for TGFβ1-mediated gene transcription of SND1.

To further verify that the Smad complex could interact with the specific promoter region of SND1, we then employed ChIP experiments in MCF-7 cells with control IgG or antibodies against Smad3, with or without TGFβ1 treatment, and then real-time PCR was performed with different primers, as indicated in Fig. 5B, to detect the corresponding regions associated with Smad3 protein. The results showed that, with TGFβ1 treatment, Smad3 protein interacted with the motif within the two major cis-elements of SND1 promoter, the −2100- to −2000-bp region and the −1100- to −1000-bp region, which correspond to RD2 and RD1, respectively (9.31-fold for RD2; 4.93-fold for RD1). Consistently, inhibiting the phosphorylation state of Smad2/Smad3 with SB significantly reduced the association of Smad3 to RD1 and RD2, whereas the treatment of LN did not show obvious effect. These results indicate that TGFβ1 induces Smad2/Smad3 phosphorylation, which could recognize and bind with RD, especially RD2 motif in the promoter region of SND1, and then mediate gene transcriptional activation of SND1.

SND1 promotes Smurf1 expression and RhoA ubiquitination

As SND1 is regulated by the TGFβ1/Smad2/Smad3 pathway, to gain mechanistic insights into the role of SND1 in the breast...
cancer metastasis, we then analyzed the gene expression correlation between SND1 and TGFβ signaling pathway molecules based on 151 subjects with usable gene expression data from The Cancer Genome Atlas (TCGA) breast IDC dataset BRCA (http://cancergenome.nih.gov). Statistical analysis of SND1 and Smurf1 expression found a Pearson correlation (n = 151, \( r = 0.5243, P < 0.00001 \), Fig. 6A, Supplementary Table S4), indicating a strong positive correlation between the expression of SND1 and Smurf1. Furthermore, the network analysis was performed by R software, and the result presented the involvement of SND1 in breast cancer metastasis through promoting Smurf1 expression (Fig. 6B). We then performed qRT-PCR to

![Image of graph](http://cancergenome.nih.gov)
detect the correlation between SND1 and Smurf1 in the stable MCF-7 cells with overexpression of SND1 (SND1), knockdown of endogenous SND1 (SND1-sh), or gain-of-function of SND1 in SND1-sh cells (SND1-sh cells transfected with pSG5-SND1). The result showed that (Fig. 6C) the expression of Smurf1 is positively correlated with SND1 levels.

It has been reported that Smurf1 promotes the ubiquitination and degradation of the small GTP protein RhoA, thereby regulates cell polarity and protrusion (23), which are important events in the TGF-β-induced EMT (24, 25). We therefore detected the effect of SND1 on regulating the protein level of Smurf1 and RhoA in MCF-7 cells. As shown in Fig. 6D, compared with the control MCF-7 cells (lane 1), the ectopic overexpression of SND1 protein (SND1) enhanced the Smurf1 protein level but reduced the RhoA protein level (lane 2), whereas knockdown of endogenous SND1 protein (SND1-sh) decreased the Smurf1 protein level but increased the RhoA protein level (lane 3). In the rescue experiment with the gain-of-function of SND1 protein in SND1-sh cells, the protein level of Smurf1 was increased but RhoA was decreased (lane 4), which further indicates that SND1 could positively regulate the expression of Smurf1, as a result, reducing the protein level of RhoA.

We then determined the RhoA ubiquitination level in the manipulated breast cancer cells with knockdown of endogenous Smurf1, together with or without overexpression of SND1. Correlated with the expression of SND1 and Smurf1 (Fig. 6E), the protein level of RhoA was decreased with overexpression of SND1 (lane 3), but increased with knockdown of Smurf1 (lane 2 and lane 4), comparing with the control cells (lane 1). The same lysates were immunoprecipitated with anti-RhoA antibody, or anti-IgG as negative control, and then blotted with anti-RhoA or anti-ubiquitin antibody. The results showed that (Fig. 6F) the same amount of RhoA was immunoprecipitated from different cell lysates (top, lanes 5-8). RhoA was slightly ubiquitinated in the control MCF-7 cells (bottom, lane 5), but was strongly ubiquitinated in the cells with overexpression of SND1 protein (lane 7). In contrast, there was no detectable ubiquitinated RhoA band in the cells with knockdown of endogenous Smurf1 (lanes 6, 8), even with overexpression of SND1 (lane 8). These data further demonstrate that SND1-induced RhoA degradation is mediated by Smurf1.

**SND1 promotes the metastatic ability of breast cancer cells via Smurf1-induced RhoA degradation**

Proteasome inhibitor MG132 is commonly used to inhibit protein degradation caused by ubiquitination. The stable MCF-7 cells with overexpression of SND1 (SND1 cells) were transfected with Smurf1 siRNA to knockdown endogenous Smurf1 protein or pretreated with MG132 to inhibit the degradation of RhoA protein. The migration (Fig. 7A) and invasion (Fig. 7B) abilities were increased significantly with overexpression of SND1 (SND1+scram-si), but remained at the similar level as control cells together with knockdown of endogenous Smurf1 (SND1+Smurf1-si) or pretreated with MG132 (SND1+MG132). Western blot analysis showed that (Fig. 7C) overexpression of SND1 (lane 2 and lane 3) increased the expression of Smurf1 protein (lane 2 and lane 3), and remarkably reduced the protein level of RhoA (lane 2), but not in the cells pretreated with MG132 (lane 3). Meanwhile, we also detected the protein level of Cdc42 and Rac1, which are the other two members of Rho family GTPase. However, there was no detectable change of the protein level of Cdc42 or Rac1 with overexpression of SND1 (lane 2).

RhoA plays essential roles in maintaining the assembly of focal adhesions and contracile actin-myosin stress fibers (26). The lack of RhoA can lead to F-actin cytoskeleton disorganization. We then performed immunofluorescence assays to examine the cytoskeletal structure in MCF7 cells with different expression levels of SND1 protein. As shown in Fig. 7D, in the control cells, the F-actin was distributed as a net (green, Fig. 7D, 1a) representing of normal cytoskeletal structure, and RhoA resided in both cytoplasm and nucleus (red, Fig. 7D, 2a). Overexpression of SND1 caused the alteration of the F-actin cytoskeleton with extended cellular protrusions (Fig. 7D, 1b), meanwhile the protein level of RhoA was decreased. (red, Fig. 7D, 2b). These data further demonstrate that overexpression of SND1 protein can cause the morphologic change in MCF7 cells and affect the normal function of F-actin through regulating the activity of the Smurf1–RhoA pathway. Thus, the functional correlation of SND1 and Smurf1 expression illustrates that SND1 can promote the metastatic ability of breast cancer cells via Smurf1-mediated RhoA ubiquitination and degradation (Fig. 7E).

**Discussion**

SND1 upregulation has been reported in various malignant tumors, such as hepatocellular carcinoma (27), prostate cancer (28), and colon cancer (29). The molecular mechanisms for the involvement of SND1 in tumorigenesis of different types of cancers appear to be diverse. Through regulating alternative splicing, SND1 promotes prostate cancer cell growth and survival (30). It is reported that overexpression of SND1 increases RNA-induced silencing complex activity and contributes to cutaneous malignant melanoma metastases (31), and SND1 can also promote tumor angiogenesis via miR-221 in hepatocellular carcinoma (32). In human colon cancer, SND1 is upregulated and contributes to cancer development at the early stages via activation of the Wnt signaling pathway (33). Meanwhile, SND1 could also interact with AEG-1 in colon cancer (29). In the present study, we demonstrate that SND1 plays important roles in breast cancer metastasis.

Around 50% of DC in situ will progress to IDC or metastasis breast cancers if untreated (34). However, the molecular mechanisms underlying the progression of DC in situ to invasive DC are largely unknown. ER and HER2 are the two markers currently used in routine assessment of ER-positive DC in situ, but there is no marker for ER-negative DC in situ. Our present study indicates that SND1 could be a potential novel biomarker to predict invasive occurrence for DC in situ independent to ER and HER2 expression.

Several TGFβ/Smad-regulated genes control the metastasis processes (35, 36). Supportively, we identified SND1 as a novel downstream target gene of the TGFβ/Smad pathway: (i) bioinformatics analysis reveals that the SND1 promoter region contains two Smad RDs; (ii) knockdown of endogenous Smad2, Smad3, or Smad4, or inhibition of the phosphorylation of Smad2 and Smad3 could block TGFβ-induced enhancement of SND1 at both mRNA and protein level; (iii) the luciferase reporter assay and ChIP experiments further illustrate that Smad3 could specifically recognize the RD in the SND1 promoter region. Because TGFβ signaling is constitutively activated in breast cancer cells, the
observed upregulation of SND1 expression in breast cancer tissues is likely to be the result of TGFβ-induced activation of the Smad pathway.

The most novel and interesting finding in the study is the promoting effect of SND1 on Smurf1 expression and consequent RhoA degradation. Smurf1 is an E3 ubiquitin ligase, which is involved in the TGFβ-induced EMT (25, 37), could bind to nucleotide-free RhoA, and promote RhoA ubiquitination (38). The present study indicates that SND1 enhances Smurf1 expression, leading to increased RhoA ubiquitination and proteasomal degradation, causes morphologic alterations of the actin cytoskeleton, and enhances cell motility. There is evidence that strongly supports our novel findings: (i) knockdown of endogenous Smurf1 protein blocked the SND1-induced metastatic ability of breast cancer cells; (ii) the invasive phenotype correlated with SND1 hyperexpression was inhibited by blocking the proteasomal degradation of RhoA with proteasome inhibitor. This novel function of SND1 is
similar to the proto-oncogene c-Myc, which is upregulated in many cancers causing cytoskeleton depolymerization and inhibition of the RhoA signaling pathway (39).

In the present study, we provide suggestive evidences to indicate that the TGFβ-Smad pathway could activate SND1 gene transcriptions, in which the Smad complex recognizes and binds to RD motif in the SND1 promoter region, and consequently enhances the transcriptional activation of SND1. Furthermore, SND1 promotes Smurf1 expression, leading to RhoA ubiquitination and degradation. Most importantly, the degradation of RhoA protein in the breast cancer cells could disrupt the distribution of F-actin cytoskeleton, increase cell migration and invasion abilities, thus promoting breast cancer metastasis (Fig. 7E). Our study demonstrates a novel role of SND1 in regulating breast cancer tumorigenesis and metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: L. Yu, X. Yang, Z. Yao, J. Yang

Development of methodology: L. Yu, J. Yang

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Yu, X. Liu, K. Cui, Y. Di, J. Yang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Yu, X. Liu, L. Xin

Writing, review, and/or revision of the manuscript: L. Yu, X. Yang, J. Yang

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Liu, Y. Di, W. Zhang

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