Original Article

Nodal signaling activates the Smad2/3 pathway to regulate stem cell-like properties in breast cancer cells

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Abstract: Nodal signaling plays several vital roles in the embryogenesis process. However, its reexpression in breast cancer is correlated with cancer progression, metastasis and poor prognosis. Recently, Nodal has also been reported to regulate self-renewal capacity in pancreatic cancer. This study aimed to explore the role of Nodal in breast cancer stem cells (BCSCs) and the underlying mechanisms. Therefore, the immunohistochemistry staining of Nodal in 135 human breast cancer cases was performed to analyzed the relationship of Nodal signaling, clinical outcomes and BCSC marker. And the results showed that high Nodal expression was positively correlated with poor prognosis and BCSC marker expression in breast cancer samples. We further assessed the effects of Nodal in regulating the BCSC properties in breast cancer cell lines and xenografts. Then, SB431542 was administered in vitro and in vivo to explore the function of the Smad2/3 pathway. And we demonstrated that Nodal signaling up-regulated the expression of ALDH1, CD44, CD133, Sox2, Oct4 and Nanog by activating the Smad2/3 pathway, thereby enhancing the tumorigenicity and sphere-forming ability of breast cancer cells. Furthermore, treatment with SB431542 could inhibit the properties of BCSCs in vitro and in vivo. In conclusion, these findings indicate that Nodal signaling may play a vital role in maintaining the BCSC phenotype in breast cancer and serve as a potential target to explore BCSC-specific therapies.

Keywords: Nodal, Smad2/3 pathway, cancer stem cell, breast cancer

Introduction

Breast cancer, the most frequently diagnosed cancer, is the main cause of cancer-related deaths for women around the world [1]. This heterogeneous disease presents different metastatic potentials even in the same pathological subtypes. Cancer stem cells (CSCs) are a minor subpopulation that present a self-renewal capacity and tumorigenic potential [2, 3]. CSCs are considered to be responsible for tumor progression, metastasis and drug resistance in many solid tumors, including breast cancer [4, 5]. In addition, breast cancer stem cells (BCSCs) may play a significant role in tumor propagation and treatment failure [6-8]. Therefore, it is crucial to identify and eliminate BCSCs, thereby avoiding relapse and improving prognosis in patients.

Nodal protein belongs to the TGF-β superfamily. It plays vital roles in differentiation of the endoderm and mesoderm during embryogenesis [9-11]. This embryonic morphogen is necessary for supporting the undifferentiated state and pluripotent property of stem cells [12]. Nodal expression is commonly absent in differentiated tissues [13]. However, recent studies have suggested that the reexpression of Nodal signaling in malignant tumors correlates with cancer progression, metastasis and poor prognosis [14-17]. Nodal signal activates ALK4/7 via binding to activin-like kinase (ALK) type I and type II receptors, thereby promoting intracellular phosphorylation of Smad2/3, followed by translocation to the nucleus and regulating downstream gene transcription [12]. A recent study found that, similar to the function of Nodal signaling in embryogenesis, Nodal signaling was involved in promoting stem cell-like phenotypes in pancreatic cancer [18]. Moreover, there also reports that Nodal regulates progression, angiogenesis and metastasis in breast
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Consequently, we suggest that Nodal may play an important role in promoting BCSC phenotype.

In this study, we identified the function and signaling pathway of Nodal in breast cancer stem cells. Nodal overexpression was correlated with the expression of cancer stem cell markers in human breast cancer. We also clarified Nodal signaling down-stream targets that regulated properties of BCSCs. Furthermore, the application of a Nodal receptor inhibitor inverted the BCSC phenotype in vitro and in vivo. Accordingly, we demonstrated that Nodal could be a BCSC hallmark and therapeutic target.

Methods

Chemicals and reagents

The following primary antibodies were used: Nodal (sc-81953), Smad (sc-8332), p-Smad (sc-11769) and Oct4 (Sc-8629) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), ALDH1 (Ab23375) and CD44 (Ab119863) from Abcam, Inc. (Cambridge, MA, USA), CD133 (Orb9913) from Biorbyt Ltd (Biobyt, CA, USA), Sox2 (GTX101507) from GeneTex Inc. (GeneTex, CA, USA), Nanog (NBP1-04320) from Novus Biologicals (Novus, CO, USA) and β-actin (P30002) from Abmart (Abmart, NJ, USA). The concentrations of primary antibodies used in this study are listed in the supplementary material. The secondary antibodies were obtained from Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). SB431542 (S4317) was purchased from Sigma-Aldrich (St. Louis, MO).

Patient samples

Specimens from 135 patients with primary breast cancer were surgically removed between 1997 to 2005 at the Tianjin Cancer Hospital. All patients, whose ages ranged from 27 to 74 years of age, were diagnosed from histological findings (median age, 49 years). The follow-up time ranged from 12 to 150 months (median, 80 months). Clinicopathological features were collected for all samples. All slides were defined according to the histological types and grades by two pathologists. The Tianjin General Hospital Ethics Committee approved the study.

Immunohistochemistry

The stained slides were assessed by two pathologists blinded to the clinical pathology data of the samples. Staining was graded using the staining index (SI), defined as follows. Five random fields at 400× magnification were reviewed, and we analyzed one hundred tumor cells in each field. Nodal staining was concentrated in the cytoplasm, and CD44 was present on the cytomembrane. Then, they were scored based on four classes: (0) no appreciable staining; (1) weak staining; (2) intermediate staining; and (3) strong staining. The average percentage of the five fields was categorized into four classes: (0) negative cells, (1) ≤25%; (2) 25-50%; or (3) ≥50%. The sum of the intensity and percentage scores (SI) was used to evaluate the results. An SI score ≥3 was defined as high expression, while a score <3 was defined as low expression [21].

Cell culture and treatment

The human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from the ATCC in 2012 and validated by Genewiz Inc. The short tandem repeat (STR) analysis confirmed that the samples matched the reference cell lines. These cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (Invitrogen, CA, USA) and 1% penicillin-streptomycin in an incubator at 37°C with 5% CO₂. Lentiviral vector expressing Nodal cDNA (catalog no. EX-T9592-Lv201) or a control (EX-NEG-Lv201) and four lentiviral vectors expressing Nodal shRNA (HSH011861-HIVU6) or an shRNA control (CSHCTR001-HIVU6) were obtained as described previously [22]. To establish the stable Nodal-knockdown and Nodal-overexpression cells, MDA-MB-231 and MCF-7 cells were infected with the shNodal and Nodal cDNA vector, respectively, and selected by puromycin. To obtain stable control cell lines, these cells were also transfected with the empty lentiviral vectors.

SB431542 was administered to block the Smad2/3 pathway. MCF-7-Con, MCF-7-Nodal and MDA-MB-231-shCon cells were treated with SB431542 (10 μM) for 48 h. Then, the cells were collected for the following experiments.

Western blotting

The cells in indicated groups were harvested and lysed with RIPA buffer. Equivalent amounts

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of protein were loaded and separated by a 10% SDS-PAGE gel and then electroblotted onto a PVDF membrane (Millipore, MA, USA). After blocking in 5% non-fat milk in TBST buffer, the membrane was incubated overnight with primary antibodies, followed by washing with TBS-Tween three times. Then, the membrane was incubated with secondary antibodies at room temperature (RT) for 2 h. An enhanced chemiluminescence detection kit was used to visualize the immunoreactive bands, and a C-DiGit Blot Scanner (LI-COR) was used to scan and analyze these bands, followed by analysis of the relative density of each target protein by using Image-Pro Plus.

RT-PCR

Total cellular RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. QuantScript RT Kit (Tiangen Biotech) was used to synthesize cDNA. And GAPDH was used as loading control.

Primer sequences: Nodal: Forward: 5'-GCGG-TAGGCGGTAGGTG-3', Reverse: 5'-CTGGCAAAAGGC-3'; GAPDH: Forward: 5'-CTGGCG-CAAGGTGTCATCGAC-3', Reverse: 5'-TGTCATA-CCAGGAAATGAGCTTG-3'.

Immunofluorescence staining

The details are provided in the Supplementary Materials.

Colony formation assay

Three hundred cells from each group were plated in a 6-well plate with complete DMEM medium. After 12 h, all wells were changed with serum-free DMEM. The cells were then incubated with or without SB431542 (10 µM) at 37°C and 5% CO₂ for 2 weeks. The colonies were fixed with methanol and stained with 0.5% crystal violet for 10 min. Colonies >50 µm size were counted in 10 random fields using an inverted microscope.

Tumor sphere formation assay

The cells in each group were seeded as single cells in ultralow attachment 6-well plates (1000 cells per well) (Corning Life Sciences, Acton, MA). The cell concentration kept the cells were individual. Spheres were grown in 2 ml of DMEM/F12 medium with B27, 10 ng/ml human recombinant epidermal growth factor (EGF) (Invitrogen, Carlsbad, CA, USA), and 10 ng/ml human recombinant basic fibroblast growth factor (bFGF) (Invitrogen, Carlsbad, CA, USA) with or without SB431542. Every after 5-7 days, the spheres were digested and passaged 2-3 times to avoid the aggregated cells fusing together. And the number of spheres in each well was counted using light microscopy.

Xenografts and treatments

Forty-two female 3-week-old BALB/c nude mice were purchased from HFK Bioscience Co., Ltd. and raised at the Animal Center of Tianjin Medical University (Tianjin, China). Six mice per group were used in each experimental group, and 5×10⁶ cells were subcutaneously injected into the upper right flank region of each mouse. Three days after inoculation, the SB solution (10 mg/kg/mouse) or buffer solution was injected intraperitoneally into the treatment group or control groups on alternate days. Four weeks later, the mice were sacrificed, and the xenograft tumors were removed. Then, the weight and volume of the tumors were recorded. The immunohistochemical analysis was performed.

Statistical analysis

Statistical analysis was performed using SPSS 21.0. All of the data were presented as the mean ± standard deviation (SD). The differences between the experimental group and the control group were assessed using Student’s t test. The χ² test was used to compare the pathological and clinical characteristics in different groups of breast cancer cases. Survival curves were created using Kaplan-Meier analysis, and log-rank test was used to assess the significance of the differences. Statistical significance was defined as P<0.05.

Results

Nodal expression, clinicopathological features, stem cell marker expression and overall survival in breast cancer

To assess the expression of Nodal in breast cancer tissue, we investigated 135 primary breast cancer samples by IHC assay. In the breast tumor cells, Nodal staining was predomi-
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Figure 1. High Nodal expression correlates with expression of the stem cell marker CD44 and poor outcome in human breast cancer. A: A total of 73 (54.1%) specimens were positive for Nodal staining. Representative pictures of high Nodal expression staining predominantly localized in the cytoplasm are presented (magnification, 200× and 400×). B: A representative picture of the 62 (45.9%) specimens negative for Nodal expression are presented (magnification, 200×). C: Positive cytomembrane CD44 expression staining in breast cancer specimens (magnification, 200×). D: Negative CD44 expression in breast cancer (magnification, 200×). E: Kaplan-Meier analysis showed that compared with Nodal-negative samples, Nodal-positive patients displayed poorer outcomes (P=0.001, log-rank test). F: Compared to patients with low CD44 expression, patients with high CD44 expression had worse overall survival (P=0.022, log-rank test).

nantly located in the cytoplasm (Figure 1A) and was evaluated by SI criteria [21]. Meanwhile, negative Nodal expression is shown in Figure 1B. The results showed that 54.1% (73/135) of the cases were positive for Nodal expression (Nodal\textsuperscript{high}) and 45.9% (62/135) cases showed negative Nodal expression (Nodal\textsuperscript{low}). As shown in Table 1, the comparison of clinico-pathological features between the Nodal\textsuperscript{high} group and Nodal\textsuperscript{low} group revealed that expression of Nodal was positively correlated with lymph node metastasis (P=0.004), higher histological grade (P=0.001) and higher clinical stage (P=0.013), but it was negatively correlated with age (P=0.57) and tumor size (P=0.314).

To explore the correlation between Nodal and BCSCs, the expression of the BCSC marker, CD44, was also assessed, and the results showed that 49.6% (67/135) of the cases were positive for CD44 cytomembrane expression (Figure 1C); Figure 1D shows the negative expression for comparison. Further statistical analysis showed that 64.4% (47/73) of the Nodal\textsuperscript{high} group and 32.3% (20/62) of the Nodal\textsuperscript{low} group expressed CD44. We found that Nodal signaling was significantly correlated with high CD44 expression (P=0.000). Moreover, Kaplan-Meier survival analysis demonstrated that high Nodal expression predicted significantly worse survival than cases with low
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Nodal expression (P=0.001) (Figure 1E). Similarly, a higher CD44 expression level predicted a worse prognosis (P<0.022) (Figure 1F). These results indicated that Nodal expression correlated with BCSC phenotype and might act as a hallmark to predict poor prognosis in breast cancer.

**Nodal is necessary to maintain stemness in breast cancer cell lines**

To investigate the function of Nodal signaling and its mechanisms in BCSCs, we selected human breast cancer cell lines MDA-MB-231 and MCF-7 for further research. First, the Nodal expression in these cell lines was evaluated by western blot, and the results are shown in Figure 2A. MCF-7 cells displayed low Nodal levels, whereas MDA-MB-231 cells had strong Nodal expression. Therefore, we overexpressed Nodal expression in MCF-7 cells using a lentivirus plasmid. In addition, the ShNodal plasmid, which had previously been proven to be effective and showed no off-target effects [22], was used to knock down Nodal expression in MDA-MB-231 cells. Stable cell lines MCF-7-Control (MCF-7-Con), MCF-7-Nodal, MDA-MB-231-shControl (231-shCon) and MDA-MB-231-shNodal (231-shNodal) were established and were confirmed by western blot and RT-PCR analysis (Figure 2B and 2C). To further explore the function of Nodal signaling in the regulation of properties of BCSCs, the expression levels of stem cell markers were detected. Western blot analysis revealed that the expression of ALDH1, CD44, and CD133 were significantly lower in the 231-shNodal cells than in the 231-shCon cells (Figure 2D). Similarly, the results showed that knockdown of Nodal also dramatically down-regulated the expression of pluripotency-associated genes Sox2, Oct4 and Nanog (Figure 2D). At the same time, overexpression of Nodal levels promoted the up-regulation of CSC-associated markers and factors ALDH1, CD44, CD133, Sox2, Oct4 and Nanog (Figure 2E). Taken together, these findings suggested that Nodal signaling was a positive regulator of the BCSC phenotype.

### Table 1. Correlation between Nodal expression, clinicopathologic parameters and CD44 expression in breast cancer

<table>
<thead>
<tr>
<th>Factors</th>
<th>Nodal expression</th>
<th>X²</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>- (%), + (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
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<td></td>
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<tr>
<td>&lt;50</td>
<td>30 (48.4), 43 (58.9)</td>
<td>37.793</td>
<td>0.570</td>
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<tr>
<td>≥50</td>
<td>32 (51.6), 30 (41.1)</td>
<td></td>
<td></td>
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<tr>
<td>Tumor size (diameter)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>D≤2</td>
<td>18 (37.1), 17 (23.3)</td>
<td>18.166</td>
<td>0.314</td>
</tr>
<tr>
<td>2&lt;D≤5</td>
<td>36 (58.1), 50 (68.5)</td>
<td></td>
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<tr>
<td>D&gt;5</td>
<td>3 (4.8), 6 (8.2)</td>
<td></td>
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<tr>
<td>Nodal status</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Negative</td>
<td>41 (66.1), 30 (41.1)</td>
<td>8.426</td>
<td>0.004*</td>
</tr>
<tr>
<td>Positive</td>
<td>21 (33.9), 43 (58.9)</td>
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<tr>
<td>Differentiation grade</td>
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<td></td>
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<tr>
<td>I/II</td>
<td>55 (88.7), 47 (64.4)</td>
<td>10.742</td>
<td>0.001*</td>
</tr>
<tr>
<td>III</td>
<td>7 (11.3), 26 (35.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>14 (22.6), 6 (8.2)</td>
<td>10.83</td>
<td>0.013*</td>
</tr>
<tr>
<td>II</td>
<td>41 (66.1), 46 (63)</td>
<td></td>
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<tr>
<td>III</td>
<td>7 (11.3), 17 (23.3)</td>
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<tr>
<td>IV</td>
<td>0, 4 (5.5)</td>
<td></td>
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<tr>
<td>CD44</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>-</td>
<td>42 (67.7), 26 (35.6)</td>
<td>14.687</td>
<td>0.000*</td>
</tr>
<tr>
<td>+</td>
<td>20 (32.3), 47 (64.4)</td>
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*Significantly different.

Nodal signaling via the Smad2/3 pathway regulates cancer stem cell-like properties in breast cancer

In an attempt to investigate the underlying mechanisms in Nodal regulating breast cancer stem cell properties, we analyzed the canonical TGFβ pathway and applied the Alk4/7 inhibitor SB431542. As an embryonic protein, Nodal exerts its function by binding to activin-like kinase receptors ALK4/7, leading to phosphorylation of Smad2/3, followed by the regulation of target genes. Whole-cell extracts from 231-shCon, 231-shNodal, MCF-7-Con and MCF-7-Nodal cells with or without application of SB431542 (SB) were collected and subjected to western blot analysis. The results showed that knockdown of Nodal in MDA-MB-231 cells or those treated with SB decreased Smad2/3 phosphorylation (Figure 3A). Meanwhile, CSC-associated markers were significantly diminished to different extents. Interestingly, we found that the Smad2/3 pathway might play
Nodal signaling regulates stem cell-like properties in breast cancer

D

E

ALDH1
CD44
CD133
Sox2
Oct4
Nanog
β-actin

Expression Relative to Control

ALDH1
CD44
CD133
Sox2
Oct4
Nanog
β-actin

Expression Relative to Control
more important roles in regulating CD44, CD133 and Nanog. Furthermore, overexpression of Nodal significantly up-regulated the p-Smad2/3 level (Figure 3B). As shown in Figure 3B, the effects of Nodal on the expression of ALDH1, CD44, CD133, Sox2, Oct4 and Nanog in MCF-7-Nodal cells could also be neutralized greatly by the application of SB. Consistently, the immunofluorescence results revealed that down-regulation of Nodal signal strikingly decreased the expression of ALDH1, CD44 and CD133 in MDA-MB-231 (Figure 3C), whereas overexpression of Nodal up-regulated these CSC markers in MCF-7 cells (Figure 3D). In addition, treatment with SB inhibited the expression of stem cell markers in 231-shCon and MCF-7-Nodal cells (Figure 3C, 3D).

Nodal enhances the colony and sphere formation abilities of breast cancer cells

The sphere formation and colony formation assays evaluated the capacity of self-renewal properties in cancer cells, which had been used to illustrate the CSC phenotype [23]. We first investigated whether Nodal signaling affects colony formation of breast cancer cells. The results indicated that knockdown of Nodal expression in MDA-MB-231 cells apparently reduced colony formation (Figure 4A) (P<0.05). Simultaneously, Nodal overexpression significantly increased the size and count of the colonies in MCF-7 cells compared with the control group (Figure 4B) (P<0.05). In addition, when the MDA-MB-231 and MCF-7-Nodal cells were treated with SB, colony formation was blocked (Figure 4A, 4B) (P<0.05). To further characterize the effects of Nodal on BCSCs, the sphere formation assay was performed. Similarly, up-regulated Nodal expression promoted the formation of spheres in MCF-7 cells in ultra-low attachment culture, whereas after exposure to SB, this capacity was abrogated (Figure 4D) (P<0.05). Moreover, the sphere forming rate was obviously diminished in MDA-MB-231 cells when the Nodal expression of the cells was knocked down or the cells were treated with SB (Figure 4D) (P<0.05). These results further corroborated the crucial effect of Nodal signaling in maintaining the properties of BCSCs and illustrated the importance of the Smad2/3 pathway in this process.

Nodal signaling contributes to tumorigenicity and BCSC phenotype in vivo, and its effects were abrogated by the application of SB431542

For the subsequent investigation of the function of Nodal signaling in promoting tumorigenicity and for validating the feasibility of SB431542 application to reduce BCSC phenotype in vivo, identical numbers of 231-shCon, 231-shNodal, MCF-7-Con and MCF-7-Nodal cells were subcutaneously injected into BALB/c nude mice. The SB solution (10 mg/kg/mouse) was subjected to intraperitoneal injection in the treatment groups every other day starting with the third day; in the meantime, the control groups were subjected to a placebo. Four weeks after inoculation, the xenograft tumors were surgically removed, and the weight was measured. We found that tumorigenicity in the 231-shNodal group was strikingly lower than in the 231 shCon group (Figure 5A) (P<0.05). Meanwhile, Nodal overexpression contributed to larger breast tumors in the MCF-7-Nodal group than in the MCF-Con group (Figure 5B) (P<0.01). Consistent with SB treatment in vitro, the 231 shCon+SB, MCF-7+SB and MCF-7-Nodal+SB groups had smaller tumors than the respective control groups (Figure 5A, 5B) (P<0.05). To further elucidate the BCSC phenotype in xenografts, immunohistochemical staining was performed. In accordance with the western bolt results, the expression levels of p-Smad2/3 and CD44 were diminished by SB treatment or knock-down of Nodal signaling (Figure 5C). Analogously, up-regulated Nodal expression significantly increased the expression of p-Smad2/3 and CD44; however, application of SB neutralized its effects (Figure 5D).
Nodal signaling regulates stem cell-like properties in breast cancer

A. MDA-MB-231

- Smad2/3
- p-Smad2/3
- ALDH1
- CD44
- CD133
- Sox2
- Oct4
- Nanog

β-actin

Expression Relative to Control

B. MCF-7

- Smad
- p-Smad2/3
- ALDH1
- CD44
- CD133
- Sox2
- Oct4
- Nanog

β-actin

Expression Relative to Control

C. 231-shCon 231-shCon+SB 231-shNodal

Nodal

D. MCF-7-Con MCF-7-Con+SB MCF-7-Nodal MCF-7-Nodal+SB

ALDH1

Expression Relative to Control
Nodal signaling regulates stem cell-like properties in breast cancer

Figure 3. Effect of the Smad2/3 pathway in Nodal-mediated BCSC phenotype in breast cancer cell lines. A, B: The expression of Smad, p-Smad, ALDH1, CD44, CD133, Sox2, Oct-4 and Nanog proteins were estimated using western blot analysis in the indicated cells. In addition, SB (10 µM) treatment effectively decreased Nodal inducing CSC-associated marker expression. C, D: Immunofluorescence staining was performed to validate the expression of Nodal, ALDH1, CD44 and CD133 in the indicated cells. Assays were performed in triplicate. The data are presented as the mean ± standard deviation (SD). (*P<0.05), scale bar =50 µm.
Nodal signaling regulates stem cell-like properties in breast cancer

A 231-shCon  231-shCon+SB  231-shNodal  B  MCF-7-Con  MCF-7-Con+SB  MCF-7-Nodal  MCF-7-Nodal+SB

C 231-shCon  231-shCon+SB  231-shNodal  D  MCF-7-Con  MCF-7-Con+SB  MCF-7-Nodal  MCF-7-Nodal+SB

Colony Number

231shCon  231shCon+SB  231shNodal

Colony Number

MCF7-Con  MCF7-Con+SB  MCF7-Nodal  MCF7-Nodal+SB
Figure 4. Nodal enhances the colony and sphere formation abilities of breast cancer cells. A, B: Clonogenic assays were performed in the indicated groups, and the number of colonies was calculated. The SB-treated group showed a significant reduction in the colony formation ability compared with the control group. Representative images are shown. The data are presented as the mean ± standard deviation (SD). (**P<0.01), scale bar =200 μm. C, D: The self-renewal capacity was evaluated by serum-free culture sphere formation. The percentage of spheres formed was calculated and plotted for the indicated groups. Compared with the untreated group, application of SB (10 μM) significantly reduced sphere formation efficiency. Assays were performed in triplicate. The data are presented as the mean ± standard deviation (SD). (**P<0.01), scale bar =50 μm.
Discussion

In this study, we provided evidence of a vital role for Nodal signaling in maintaining the BCSC phenotype. We showed that Nodal signaling upregulated the expression of cancer stem cell markers by activating the Smad2/3 pathway, thereby enhancing tumorigenicity and sphere formation. Furthermore, the application of SB431542 could inhibit the properties of BCSCs in vitro and in vivo. In addition, we demonstrated that high Nodal expression is correlated with poor prognosis and increased mortality in 135 samples from breast cancer patients. Accordingly, these data indicate that Nodal signaling regulates BCSC phenotype and may serve as a potential target to explore BCSC-specific therapies.

A number of studies have shown that cancer stem cells (CSCs) take the responsibility to cause the progression, metastasis and recurrence of cancers [4, 24-26]. BCSCs have been reported to be involved in the early dissemina-
tion of cancer cells in bone marrow [27]. Meanwhile, BCSCs were proven to be highly tumorigenic and could not be eliminated by conventional therapies in breast cancer [2, 28, 29]. Previous studies have shown that Nodal re-expression in tumors increased cancer cell aggressiveness and tumorigenicity [30, 31]. Recently, Nodal signaling has been proven to correlate with regulating self-renewal of pancreatic cancer stem cells [18]. Here, we demonstrated that Nodal expression was correlated with the stemness properties of BCSCs in human breast cancer. Consistent with previous studies, we found that Nodal expression in breast cancer samples was strikingly associated with tumor metastasis, differentiation grade and TNM stage. Moreover, high expression levels of Nodal and CD44 increased mortality in breast cancer patients. Importantly, the results displayed that Nodal expression was strongly correlated with CD44 expression. Consequently, we demonstrated that Nodal could serve as a marker of BCSCs and might contribute to the prediction of poor prognosis in breast cancer.

Nodal plays essential roles in the establishment of anterior-posterior axis patterning and left-right asymmetry and maintains the pluripotent properties of stem cells during embryogenesis [11, 12]. A previous study demonstrated that Nodal RNA exogenously expressed in zebrafish embryos could induce a secondary notochord and an ectopic outgrowth [32, 33]. Similarly, its role in increasing cancer cell aggressiveness, tumorigenicity and metastasis in melanoma, breast cancer and pancreatic cancer has been reported [34-36]. Furthermore, Lonardo et al. reported that recombinant Nodal applied to CSCs resulted in increasing spheroid formation, size, and invasion and demonstrated the robust function of Nodal during tumor progression [18]. In the present study, to further explore the effect of Nodal in promoting the BCSC phenotype and its underlying mechanism, we established the stable breast cancer cell lines 231-shCon, 231-shNodal, MCF-7-Con and MCF-7-Nodal. In accordance with previous studies, Nodal overexpression increased the number and size of CSC-like spheres and promoted colony formation. In addition, western blot analysis revealed that high Nodal expression resulted in increased expression of ALDH1, CD44, CD133, Sox2, Oct4 and Nanog. In contrast, Nodal silencing reversed the properties of breast cancer stem cells in regards to the expression of CSC markers and cell function. These results revealed that Nodal signaling was necessary for regulating BCSC features. In addition, previous studies have shown that Nodal is crucial for breast cancer microcirculation, including vasculogenic mimicry formation and endothelial vessel formation [22, 37]. Thus, we infer that, on the one hand, Nodal signaling promotes tumor microenvironment formation thereby providing escape routes for metastatic cells and, on the other hand, positively regulates the properties of BCSCs leading to increasing tumorigenicity of metastatic cells.

Nodal maintained the undifferentiated state and pluripotency in human embryonic stem cells [38, 39]. It was reported that Nodal expression activated Nanog transcription through the SMAD2/3 pathway, thereby promoting pluripotency in human embryonic stem cells [40]. Meanwhile, it was also reported that inhibition of the SMAD2/3 pathway in human embryonic stem cells by SB431542 led to the specification of the neuroectoderm [41]. In the current study, to identify the effect of the SMAD2/3 pathway in the BCSCs, we applied SB431542 in breast cancer cell lines and in engrafted tumor models. Our results showed that the effects of Nodal were abrogated by SB431542 in the functional experiments and the expression of the CSC markers. Interestingly, the SMAD2/3 pathway might play a more significant role in regulating the expression of CD44, CD133 and Nanog. We inferred that the promotion of the BCSC properties by Nodal might involve a complex pathway, but the Smad2/3 pathway played a significant role. Further studies should concentrate on the function of non-Smad-associated pathways in BCSCs. Furthermore, treatment with SB431542 successfully inhibited the promotion of tumorigenicity in human breast cancer xenografts by Nodal signaling. Moreover, consistent with the in vitro results, the SB431542-treated groups had lower CD44 expression. Taken together, we demonstrated that Nodal regulated the BCSC phenotype predominantly via the Smad2/3 pathway.

In conclusion, our results indicated that Nodal plays a significant role in maintaining the self-renewal capacity and tumorigenic potential of
Nodal signaling regulates stem cell-like properties in breast cancer

BCSCs and demonstrated the potential for inhibiting the Nodal signaling pathway using SB431542, thereby shedding light on the molecular pathways that might act as potential therapy targets for BCSCs.

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Disclosure of conflict of interest

None.

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Supplementary Materials

Immunohistochemistry

IHC sections were deparaffinized and rehydrated in xylene and gradient alcohol, sequentially. Then, 3% hydrogen peroxide was used to block endogenous peroxidase activity for 20 min. Epitope retrieval was performed in a microwave oven for 10 min at 95°C. Then, the sections were blocked with normal goat serum for 30 min at room temperature, followed by incubation with primary antibodies for 16 h at 4°C. After 3 washes, the slides were incubated with secondary antibodies for 40 min. After further washing, the staining was developed using DAB for 5-10 min. The slides were counterstained with hematoxylin. Negative controls were incubated with PBS instead of primary antibodies.

Immunofluorescence staining

All the cells were briefly trypsinized and counted, and then the cells in each group were plated in one cell dish, on which several sterile coverslips of the same size had been placed before. These cells were cultured until reaching 50% to 60% confluence. We chose the coverslips with similar cell densities in the same dish, washed them once with PBS, fixed them in absolute methanol for 20 min at -20°C, (permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature), and blocked them for 30 min with 3% BSA in PBS. Then, the cells were incubated with primary antibodies overnight at 4°C, followed by incubation with TRITC-conjugated secondary antibodies for 2 h. After three washes, the coverslips were stained by DAPI (Sigma). Finally, the cells were examined using a fluorescence microscope.

Table S1. Antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>IHC Concentration/WB</th>
<th>Product Number</th>
<th>Manufacture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodal Mouse</td>
<td>1:50 (IHC)</td>
<td>sc-81953</td>
<td>Santa Cruz</td>
<td></td>
</tr>
<tr>
<td>Nodal Rabbit</td>
<td>1:1000 (WB)</td>
<td>ab109317</td>
<td>Abcam</td>
<td></td>
</tr>
<tr>
<td>Smad2/3 Rabbit</td>
<td>1:400 (WB)</td>
<td>sc-8332</td>
<td>Santa Cruz</td>
<td></td>
</tr>
<tr>
<td>p-Smad2/3 Goat</td>
<td>1:200 (WB)/1:200 (IHC)</td>
<td>sc-11769</td>
<td>Santa Cruz</td>
<td></td>
</tr>
<tr>
<td>Oct4 Goat</td>
<td>1:200 (WB)</td>
<td>Sc-8629</td>
<td>Santa Cruz</td>
<td></td>
</tr>
<tr>
<td>ALDH1 Rabbit</td>
<td>1:400 (WB)</td>
<td>Ab23375</td>
<td>Abcam</td>
<td></td>
</tr>
<tr>
<td>CD44 Rabbit</td>
<td>1:200 (WB)/1:100 (IHC)</td>
<td>Ab119863</td>
<td>Abcam</td>
<td></td>
</tr>
<tr>
<td>CD133 Rabbit</td>
<td>1:400 (WB)</td>
<td>Orb9913</td>
<td>Biorbyt</td>
<td></td>
</tr>
<tr>
<td>Sox2 Rabbit</td>
<td>1:400 (WB)</td>
<td>GTX101507</td>
<td>GeneTex</td>
<td></td>
</tr>
<tr>
<td>Nanog Rabbit</td>
<td>1:200 (WB)</td>
<td>NBP1-04320</td>
<td>Novus Biologicals</td>
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</tr>
<tr>
<td>β-actin Rabbit</td>
<td>1:2000 (WB)</td>
<td>P30002</td>
<td>Abmart</td>
<td></td>
</tr>
</tbody>
</table>

Note: IHC: Immunohistochemistry; WB: Western blot.