Abstract. Background/Aim: Although axis inhibition protein 2 (Axin2) has been reported to act as a tumour suppressor, recent findings suggest that it exhibits oncogenic effects by mediating Snail1-induced epithelial-mesenchymal transition (EMT) in breast cancer cells. EMT is a crucial biological process involved in the initiation of metastasis in cancer progression. This study elucidated the biological significance and mechanism of Axin2 in breast cancer using transcriptomic and molecular techniques. Materials and Methods: The expression of Axin2 and Snail1 in MDA-MB-231 breast cancer cells was determined by western blotting analysis, and the role of Axin2 in breast cancer tumorigenesis was investigated in xenograft mouse models constructed using pLKO-Tet-shAxin2-transfected triple negative (TN) breast cancer cells. Additionally, the expression levels of EMT markers were determined using qRT-PCR, and clinical data were analysed using Kaplan-Meier (KM) plotter and The Cancer Genome Atlas (TCGA). Results: Axin2 knockdown significantly decreased (p<0.001) the proliferation of MDA-MB-231 cells in vitro and attenuated (p<0.05) the tumorigenic potential of the cells in vivo. Moreover, Axin2 knockdown significantly increased the relative mRNA levels of epithelial markers but decreased the expression of mesenchymal markers in MDA-MB-231 cells. Conclusion: Axin2 may be involved in the progression of breast cancer, particularly triple-negative breast cancer, through the regulation of Snail1-induced EMT, making it a potential therapeutic target.

Breast cancer is the most common type of cancer among women (1), and the second leading cause of cancer-related death in women worldwide, with approximately more than one million new cases recorded yearly (2). Triple-negative (TN) breast cancer is a subtype of cancer that does not express the oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). TN breast cancer is characterized by high metastatic potential, invasiveness, relapse tendency, and poor prognosis. TN breast cancer is insensitive to endocrine therapy or HER2 treatment owing to the lack of ER, PR, and HER2 expression (3). Currently available breast cancer treatments remain unsatisfactory, indicating the need for further studies on potential therapeutic targets for effective treatment of the disease.

There are 19 Wnt genes in mammalian genomes, and the canonical Wnt signalling pathway plays a critical role in mammary gland morphogenesis via the regulation of epithelial-mesenchymal cell interactions (4). Moreover, aberrant activation of the Wnt signalling pathway is a common occurrence in breast cancer and is known to play a critical role in breast cancer progression (5, 6). Wnt signalling activation is induced by the binding of a Wnt ligand to its co-receptors, seven-transmembrane-domain Frizzled receptors, and one of the low-density lipoprotein (LDL) receptor-related proteins (LRPs, LRP5/LRP6). Moreover, β-catenin is a major player in the Wnt signalling pathway. Axis inhibition protein 2 (Axin2) is a negative regulator of the canonical Wnt signalling pathway and suppresses signalling activity in the absence of a Wnt ligand. Axin2 forms part of a β-catenin destruction complex

Implications of Axis Inhibition Protein 2 in Breast Cancer Progression

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Key Words: Axin2, Snail1, EMT, triple negative breast cancer.

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with adenomatous polyposis coli (APC), glycogen synthase kinase 3 beta (GSKβ), and casein kinase 1 (CK1), which contributes to the regulation of β-catenin degradation (7). This destruction complex suppresses the expression of downstream β-catenin target genes that modulate various cellular functions, such as apoptosis, differentiation, proliferation, migration, and tumorigenesis. Therefore, Axin2 was initially categorized as a potential tumour suppressor gene.

However, the activation of the Wnt signalling pathway induces Dishevelled (Dsh) phosphorylation, facilitating the subsequent translocation of Axin proteins by activated Dsh to the membrane to bind to the LRP5 receptor (8, 9). This sequence of activities following the activation of the Wnt signalling pathway leads to the inactivation of the β-catenin destruction complex. β-catenin is therefore released from the degradation machinery and accumulates in the cytoplasm where it is further translocated to the nucleus. In the nucleus, β-catenin is involved in the transcription of Wnt target genes via interaction with the transcription factors T-cell factor/lymphoid enhancer-binding factor (Tcf/Lef) (9).

As one of the Wnt target genes, Axin2 is frequently over-expressed in various cancers, including hepatoblastoma and colorectal, lung, cervical, and breast cancers, owing to aberrant activation of the Wnt signalling pathway (10-12). Specifically, Axin2 over-expression markedly increases the activity of Snail1 in cancer cells owing to increased nuclear export of GSKβ, a dominant kinase that modulates the stability of Snail1. The increase in Snail1 activity down-regulates the expression of E-cadherin, a key transmembrane cell adhesion protein, by binding to E-boxes in the E-cadherin promoter, leading to epithelial-mesenchymal transition (EMT), which is the first step in cancer cell invasion and metastasis (10). These findings suggest that Axin2 serves as an oncogene rather than a tumour suppressor through its mediation of Snail1-induced EMT (10, 13). Additionally, some findings have shown that Axin proteins are involved in various non-canonical signalling pathways, including the Ras/ERK and SAPK/JNK pathways (14-16).

Overall, it could be speculated that Axin2 may be a multifunctional protein involved in several aspects of the molecular mechanisms underlying cancer. Here, we elucidated the mechanism of Axin2 in breast cancer, using human breast cancer cells and xenograft mouse models.

### Materials and Methods

**Cell culture and establishment of Axin2 knock-down MDA-MB-231 cells.** A human TN breast cancer cell line, MDA-MB-231 (17), and 293T cells were purchased from the Korean Cell Line Bank (Seoul, Republic of Korea), and the cells were maintained in 10% foetal bovine serum (FBS; Invitrogen, Waltham, MA, USA) containing Roswell Park Memorial Institute (RPMI) 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and Dulbecco’s Modified Eagle’s medium (DMEM; Gibco BRL, Grand Island, NY, USA), respectively. The pLKO-Tet-On vector (Addgene, Cambridge, MA, USA) was used to express short hairpin RNA (shRNA) targeted against Axin2 (pLKO-Tet-shAxin2). Axin2 oligos were inserted into the Age1/EcoR1 restriction sites of the vector to generate inducible shRNA targeted against Axin2. The Axin2 oligo sequence was as follows: 5’-ACCACCACATACCATCCACCA-3’.

<table>
<thead>
<tr>
<th>Target primers</th>
<th>Primer sequences</th>
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<tbody>
<tr>
<td>Axin2</td>
<td>Forward; 5’-AACGACTACCGGCAACGAC-3’</td>
</tr>
<tr>
<td>Snail1 (SNAIL)</td>
<td>Forward; 5’-TTGCTGAGAAGCAGCAGACGAC-3’</td>
</tr>
<tr>
<td>β-Actin (ACTB)</td>
<td>Forward; 5’-GGATACGATTGTCGTCGACGAC-3’</td>
</tr>
<tr>
<td>PCNA</td>
<td>Forward; 5’-GAGGGATGTCGTCGACGACGAC-3’</td>
</tr>
<tr>
<td>K67</td>
<td>Forward; 5’-GCCGATGTCGTCGACGACGAC-3’</td>
</tr>
<tr>
<td>Claudin1 (CLDN1)</td>
<td>Forward; 5’-GAGGGATGTCGTCGACGACGAC-3’</td>
</tr>
<tr>
<td>Occludin (OCLN)</td>
<td>Forward; 5’-GAGGGATGTCGTCGACGACGAC-3’</td>
</tr>
<tr>
<td>E-cadherin (CDH1)</td>
<td>Forward; 5’-GAGGGATGTCGTCGACGACGAC-3’</td>
</tr>
<tr>
<td>N-cadherin (CDH2)</td>
<td>Forward; 5’-GAGGGATGTCGTCGACGACGAC-3’</td>
</tr>
<tr>
<td>β-Catenin (CTNNB1)</td>
<td>Forward; 5’-GAGGGATGTCGTCGACGACGAC-3’</td>
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**Table I. Primer sequences.**
To generate lentiviruses, 293T cells were co-transfected with 1.8 μg of Axin2 shRNA-encoding plasmid, 0.6 μg of pMD2.G (Addgene), and 1.2 μg of psPAX2 (Addgene) plasmids using Lipofectamine 2000 (Invitrogen). Lentiviral supernatant was collected every 24 h for 3 days and used to infect MDA-MB-231 cells at a multiplicity of infection (MOI) of one. Transduced cells were then selected using a medium containing 1 μg/ml of puromycin (Sigma-Aldrich, St. Louis, MO, USA). MDA-MB-231 cells stably expressing Axin2 shRNA were grown in RPMI1640 supplemented with 10% Tet-approved FBS (HyClone Laboratories, Inc., Logan, UT, USA) and 1 μg/ml of puromycin. Axin2 shRNA expression was induced by culturing cells in the presence of 5 μg/ml of doxycycline (Dox; Sigma-Aldrich).

Cell proliferation assay. A cell proliferation assay was performed to determine whether Axin2 expression affected the viability of MDA-MB-231 cells. The assay was performed at three time points of 24, 48, and 72 h. Briefly, MDA-MB-231 cells were plated in 96-well plates at a density of 5×10^3 cells per well (Thermo Fisher, Waltham, MA, USA), followed by the addition of Cell Counting Kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan) diluted with serum-free RPMI-1640 at a ratio of 1:10. The plates were incubated in the dark at 37°C for 1 h under a 5% CO₂ atmosphere, and absorbance was measured at 450 nm using a microplate reader with SoftMax® Pro Software (Molecular Devices, San Jose, CA, USA).

Tumour growth in mice. Six female 6-week-old BALB/c-nu/nu mice (BALB/c Slc-nu/nu; Japan SLC, Inc., Hamamatsu, Japan) were randomized into two groups (n=3 per group). A total of 5×10^5 of pLKO-Tet-shAxin2-transfected MDA-MB-231 cells were suspended in 100 μl of phosphate-buffered saline (PBS) and subcutaneously injected into both sides of the mammary fat pad. For the experimental group, the cells were pre-treated with 5 μg/ml of Dox for 48 h before injection. After cell injection, mice in the experimental group were administered Dox at 10 mg/kg body weight (MPK) by intraperitoneal injection for five consecutive days per week for 21 days. Mice in the control group were administered PBS according to the same schedule as those in the experimental group. Tumour volume was determined every 3 days by measuring the length and width of the tumour nodules (18) and calculated using the following formula: V=(L×W²)/2 (L, length; W, width). At the end of the study period, the mice were sacrificed, and the tumour nodules removed. Tissue lysate was isolated from tumour cryosections using Pro-prep protein extraction solution (Intron, Republic of Korea), and the expression of Axin2 and Snail1 proteins was quantified using western blot analysis.

Figure 1. Axin2 knockdown suppressed Snail1 expression in MDA-MB-231 cells. (A) The protein expression of Axin2 and Snail1 in MDA-MB-231 cells transfected with pLKO-Tet-shAxin2 in the presence or absence of doxycycline (Dox) treatment was determined by western blotting. (B) The mRNA expression of Axin2 and Snail1 in MDA-MB-231 cells transfected with pLKO-Tet-shAxin2 in the presence or absence of Dox treatment was determined using qRT-PCR. Significant differences between groups were determined using the two-tailed Student’s t-test [**p<0.01 and ***p<0.001 compared with Dox (-)]. All experiments were conducted in triplicate.
Western blotting. Western blot analysis was performed for Dox-treated or untreated pLKO-Tet-shAxin2-transfected MDA-MB-231 cells and tumour nodules obtained from the animal study. Total protein extraction was performed using lysis buffer (RIPA buffer; Cell Signaling Technology, Inc., Danvers, MA, USA) and then the protein was resolved on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Thereafter, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA), blocked using 5% non-fat milk, and then incubated with antibodies against Axin2 (working dilution 1/1,000; Abcam), Snail1 (working dilution 1/1,000, Cell Signaling Technology), or tubulin (working dilution 1/5,000; Cell Signaling Technology) at 25˚C for 2 h. Antibody reaction was detected using an enhanced chemiluminescence (ECL) detection system (Pierce Biotechnology, Rockford, IL, USA).

Quantitative RT-PCR (qRT-PCR). Total RNA was extracted from MDA-MB-231 cells using TRIzolTM reagent (Invitrogen, Waltham, MA, USA), and cDNA was synthesized using AccuPower RT PreMix (Bioneer, Daejeon, Republic of Korea) according to the manufacturer’s protocol. qRT-PCR was performed using the StepOnePlus system with TB Green Premix Ex Taq II (Takara, Tokyo, Japan) and specific primers (Table I). The relative expression of the genes was normalized to that of β-actin (ACTB; internal control) and calculated using the 2-ΔΔCT method (19).

Bioinformatic analysis. The effect of Axin2 expression in patients with breast cancer was analysed using Kaplan-Meier (KM) Plotter and the Human Genome Atlas for cancer genomics. Information on TN breast cancer was obtained from The Cancer Genome Atlas (TCGA). Data of Axin2 mRNA expression and SNAI1 protein

Figure 2. Axin2 knockdown suppressed the proliferation of MDA-MB-231 cells. (A) The viability of doxycycline (Dox)-treated cells was significantly lower than that of untreated MDA-MB-231 cells at the indicated time points. (B) The mRNA expression levels of Ki67 and PCNA in Dox-treated cells were significantly lower than those of the untreated cells. The results were analysed using the two-tailed Student’s t-test [***p<0.001 compared with Dox (-)]. All experiments were conducted in triplicate.
Figure 3. Axin2 knockdown attenuated the tumorigenic potential of MDA-MB-231 cells in vivo. (A) The tumorigenic ability of doxycycline (Dox)-treated MDA-MB-231 cells was significantly attenuated compared with the untreated cells. (B) Tumour weight was markedly reduced in the Dox-treated group compared with the untreated group. (C) Body weight. (D) There was a decrease in Axin2 and Snail1 expression levels in tumour nodules obtained from mice injected with Dox-treated MDA-MB-231 cells. Tumour tissues were collected and the protein expression of Axin2 and Snail1 in the tumour lysates was determined using western blot analysis. (E) Axin2 and Snail1 protein abundances from tumour lysates. The band intensity on each blot was normalized to the loading control and the relative protein abundance is shown as a ratio to that of the untreated control. The results were analysed using the two-tailed Student’s t-test [*p<0.05, **p<0.01, and ***p<0.001 compared with Dox (-)]. All experiments were conducted in triplicate.
Figure 4. Axin2 knockdown alleviated Snail1-mediated EMT in MDA-MB-231 cells. (A) The mRNA expression levels of Claudin1, Occludin, and E-cadherin were markedly higher in Dox-treated cells than in untreated cells. (B) The mRNA expression of N-cadherin and β-catenin was markedly lower in Dox-treated cells than in untreated MDA-MB-231 cells. (C) The mRNA expression of Snail1 was significantly lower in Axin2-knockdown cells than in untreated cells. The results were analysed using the two-tailed Student’s t-test [**p<0.01 and ***p<0.001 compared with Dox (-)]. All experiments were conducted in triplicate.
expression in breast tissues were selected. KM plotter was used to analyse the survival rate of patients with TN breast cancer depending on Axin2 expression.

**Statistical analysis.** All statistical analyses were performed using the SPSS 26 statistical package (SPSS, Inc., Chicago, IL, USA), and data were expressed as the mean±standard deviation (SD) of values from at least three independent experiments. Statistical significance was determined using two-tailed Student’s *t*-tests. Data were expressed as the mean±standard error of mean (SEM) for tumour volume. The difference between groups’ volume of tumour nodules was assessed using the Mann-Whitney *U*-test. Mean values were considered statistically significant at *p*<0.05.

**Ethics approval and consent to participate.** All animal experiments were approved by the Seoul Metropolitan Government Seoul National University Boramae Medical Center Institutional Animal Care and Use Committee (IACUC No. 2022-0002).

**Results**

**Axin2 knockdown inhibited Axin2 and Snail1 expression in MDA-MB-231 cells.** The effect of Axin2 knockdown on Axin2 and Snail1 expression was examined at the protein and mRNA levels, using western blot analysis and qRT-PCR, respectively. Axin2 knockdown followed by Dox treatment significantly decreased the protein expression of Axin2 and Snail1 in MDA-MB-231 cells (Figure 1A). Similarly, qRT-PCR showed that Dox treatment significantly decreased the mRNA levels of Snail1 (*p*<0.001) and Axin2 (*p*<0.01) compared with those of the untreated group (Figure 1B). Overall, these results suggest that Snail1 expression in breast cancer cells is regulated by Axin2.

**Axin2 knockdown suppressed the proliferation of MDA-MB-231 cells.** The effect of Axin2 knockdown on the proliferation of MDA-MB-231 cells was examined. Axin2 knockdown followed by Dox treatment significantly decreased (*p*<0.001) the proliferation of MDA-MB-231 cells after 24-72 h compared with the untreated group (Figure 2A). Moreover, the Dox-treated group showed significantly lower (*p*<0.001) mRNA levels of Ki67 and PCNA compared with the untreated group (Figure 2B). Overall, these results indicate that Axin2 exhibits a tumorigenic potential in MDA-MB-231 cells *in vitro*.

**Axin2 knockdown attenuated the tumorigenic potential of MDA-MB-231 cells in vivo.** Furthermore, the effect of Axin2 knockdown on tumorigenesis was examined using tumour xenograft models. Axin2 knockdown significantly attenuated tumour growth *in vivo* (*p*<0.05) and decreased the relative tumour weight (*p*<0.001; Figure 3A and B) but did not affect the body weight of the mice (Figure 3C). The expression of
In the present study, it was observed that high Axin2 expression may play a crucial role in breast cancer progression. Previous in vitro studies have shown that Axin2 silencing can suppress oncogenic activity by reducing the viability and invasive capability of cancer cells (22, 24). Similarly, Axin2 expression was significantly related to breast cancer cell proliferation, migration, and invasion in the present study. Moreover, Axin2 over-expression has been reported to increase the expression of Snail1, a well-known zinc-finger transcription factor, by regulating the nuclear export function of GSK3 in breast cancer cell lines (10). Previous studies have shown that Snail1 directly binds to the E-box sequences located in the E-cadherin (epithelial marker) promoter region and suppresses its transcription (25). Snail1 is regarded as a potent inducer of the EMT in both normal and neoplastic cells. Several studies have shown that Snail1 is over-expressed in various human malignancies, including bladder (26), cervical (27), colorectal (28), and breast (29) cancers. In breast cancer, Snail1 expression is related to cancer cell invasion, metastasis, poor prognosis, and recurrence (29, 30). Previous studies have shown that the cellular localization and stability of Snail1 are related to its phosphorylation status. Generally, Snail1 is localized in the nucleus of cells, but Snail1 phosphorylation leads to its translocation from the nucleus to the cytoplasm, where inactivation and subsequent proteasomal degradation occurs (31, 32). Therefore, down-regulation of Snail1 target genes occurs only in cell lines with pure cytoplasmic expression of Snail1 (32).

Considering that the prognosis of breast cancer patients is largely dependent on the subtype of breast cancer, there is a need to assess Axin2 protein expression according to the breast cancer subtype. TN breast cancer is a subtype of breast cancer that is insensitive to endocrine therapy or HER2 treatment owing to the lack of ER, PR, and HER2 expression (3, 33). Moreover, TN breast cancer is characterized by high metastatic potential, invasiveness, relapse tendency, and poor prognosis, and therefore necessitates further studies.

The regulatory effect of Axin2 on Snail1-mediated EMT may be a crucial mechanism in the pathogenesis of TN breast cancer. aberrant activation of the Wnt signalling pathway frequently occurs in TN breast cancer cell lines, and inhibition of the Wnt signalling pathway can induce apoptosis and inhibit cell proliferation and migration in TN breast cancer cell lines (34). In the present study, knockdown of Axin2 in the MDA-MB-231 TN breast cancer cells attenuated the tumorigenic potential of these cells in vivo.

TN breast cancer has the worst prognosis among all breast cancer subtypes because there are fewer treatment modalities for affected patients (35-37). TN breast cancer is unique in its molecular basis and treatment response (38, 39). Accordingly, the identification of potential therapeutic molecular targets for TN breast cancer may enable the
selection of appropriate treatment modalities to improve the outcome of patients. *Axin2* may serve as an oncogene in breast cancer by mediating *Snail1*-induced EMT. Overall, the results of the present study suggest that *Axin2* may have implications in the pathogenesis of breast cancer and could be a therapeutic target for TN breast cancer treatment.

**Conflicts of Interest**

All the Authors declare no conflicts of interest in relation to this study.

**Authors’ Contributions**

Conceptualization: Ahn SY; Data curation: Ahn SY; Funding acquisition: Jo CH; Investigation: Ahn SY; Methodology: Ahn SY; Supervision: Jo CH; Writing – original draft: Ahn SY; Writing – review & editing: Ahn SY, Jo CH.

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**References**


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