RESEARCH ARTICLE

Baicalein inhibits breast cancer growth via activating a novel isoform of the long noncoding RNA PAX8-AS1-N

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Abstract
Baicalein, a natural flavonoid, has fascinating anti-cancer properties in breast cancer. Long noncoding RNAs (lncRNAs), a class of transcripts with no protein-coding potential, also exhibit critical roles in breast cancer. However, the molecular mechanisms mediating the anti-cancer properties of baicalein and whether lncRNAs are involved in the anti-cancer effects are still unclear. In this study, we identified a novel isoform of lncRNA PAX8-AS1 (PAX8-AS1-N), which is activated by baicalein in a dose- and time-dependent manner. Functional assays showed that PAX8-AS1-N reduced cell viability, inhibited cell-cycle progression, and induced apoptosis of breast cancer cells in vitro. Depletion of PAX8-AS1-N promoted breast xenograft tumor growth in vivo. Furthermore, depletion of PAX8-AS1-N attenuated the suppressive roles of baicalein on cell viability, the apoptosis induced by baicalein, and also the suppressive roles of baicalein on tumor growth in vivo. Mechanistically, PAX8-AS1-N bound to miR-17-5p, and up-regulated miR-17-5p targets, such as PTEN, CDKN1A, and ZBTB4. In addition, PAX8-AS1-N was down-regulated in breast cancer and reduced expression of PAX8-AS1-N indicated poor survival of breast cancer patients. In conclusion, our results demonstrated that PAX8-AS1-N activation mediated the anti-cancer effects of baicalein via regulating miR-17-5p, and suggested that baicalein and enhancing PAX8-AS1-N would be potential therapeutic strategies against breast cancer.

KEYWORDS
baicalein, breast cancer, long noncoding RNA, microRNA, progression

INTRODUCTION

Breast cancer is the most frequently diagnosed malignancy and the leading cause of cancer-related death in females worldwide, with 1 676 600 estimated new cases and 521 900 estimated deaths per year in the world.1 With the great progression of surgical resection, chemotherapy, and endocrine therapy, most breast cancer patients have relatively good prognoses.2 However, about a third of breast cancer patients may suffer relapse, and become resistant to chemotherapy and endocrine therapy.3,4 Therefore, further revealing the molecular mechanisms underlying the initiation and development of breast cancer, and searching more...
efficient therapeutic agents and strategies have important clinical values.5,6

Traditional herbal medicines have long been used to treat various diseases.7 Some critical components of medical herbs have been identified and show attractive anti-cancer effects in many cancers, including breast cancer.8 Baicalein (5,6,7-trihydroxyflavone) is a flavonoid derived from the root of Scutellaria baicalensis Georgi and has defined chemical structure.9 Several previous reports, including ours, have documented that baicalein has anti-cancer effects in various cancers such as cervical, breast, liver, and colon cancer.10–13 However, the molecular mechanisms mediating the anti-cancer effects of baicalein in breast cancer are largely unknown.

With great advances of next-generation sequencing technologies, tens of thousands of long noncoding RNAs (lncRNAs) have been identified.14 LncRNAs are RNA molecules with more than 200 nucleotides in length and share many characteristics with messenger RNAs (mRNAs).15,16 Compared to mRNAs, lncRNAs have limited protein coding potential, and therefore exert their biological roles as RNAs, rather than as templates for protein synthesis.17–19 Accumulating evidences have revealed that lncRNAs are implicated in a variety of pathophysiological processes.20–23 Many lncRNAs have been documented to regulate various cancer hallmarks, such as proliferation, cell cycle, apoptosis, metastasis, metabolism, and drug-resistance.24–26 Furthermore, many lncRNAs are dysregulated in various cancers.27,28 However, until now only a small fraction of lncRNAs are functionally characterized, relative to the identified tens of thousands of lncRNAs. Whether lncRNAs are involved in the anti-cancer effects of baicalein are also unclear.

MicroRNAs (miRNAs) are another type of RNA molecules with 19-25 nucleotides in length.29,30 Through directly binding to target mRNAs, miRNAs inhibit translation and/or induce degradation of target mRNAs.31–33 Therefore, miRNAs also play important roles in tumor initiation and progression via regulating oncogenes or tumor suppressors.34,35 Whether miRNAs mediate the anti-cancer effects of baicalein are consistently unclear.

In a previous report, Zhang et al have found that baicalein regulated many lncRNAs in vascular smooth muscle cells via lncRNA microarray analyzing.36 Consistently, we treated breast cancer cells with baicalein, and measured the expression of lncRNAs regulated by baicalein in vascular smooth muscle cells. Among these lncRNAs reported by Zhang et al, we noted that lncRNA AK126431 was activated by baicalein in breast cancer cells. AK126431 is located at chromosome 2q14.1. In this study, we identified AK126431 as a novel different isoform of the lncRNA PAX8-AS1 (Supplementary Figure S1A), and named AK126431 as PAX8-AS1-N. We further investigated the expression, roles, and action mechanisms of PAX8-AS1-N in the anti-cancer processes of baicalein.

2 | MATERIALS AND METHODS

2.1 | Cell culture and treatment

The breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from China Center for Type Culture Collection and Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MDA-MB-231 cells were cultured in L-15 Medium (Gibco, Invitrogen, Carlsbad, CA). MCF-7 cells were cultured in Eagle’s Minimum Essential Medium (Gibco). Both cells were maintained in medium supplemented with 10% fetal bovine serum (FBS) (Gibco) at 37°C in a humidified atmosphere with 5% CO2. Baicalein was obtained from Selleck (Houston, TX) and dissolved in DMSO. Where indicated, cells or animals were treated with indicated concentration of baicalein for indicated time.

2.2 | RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were extracted from cells or tissues with the TRIzol Reagent (Invitrogen) in accordance with the manufacturer’s protocol. After being treated with DNase I (Invitrogen) to remove genomic DNA, the extracted RNA was used to perform reverse transcription to generate first-strand cDNA with PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) in accordance with the manufacturer’s protocol. qRT-PCR was performed with SYBR® Premix Ex Taq™ II (Takara) on the ABI 7500 Real-time PCR system (Applied Biosystems, Foster City, CA) in accordance with the manufacturer’s protocols. β-actin was used as an endogenous control for the quantification of the expression of lncRNAs and mRNAs. The primers sequences were as follows: for PAX8-AS1-N, 5′-GGATGTGTTGTGTGTGTGACAG-3′ (sense) and 5′-AGAGTCGCTGAAGTTCTG-3′ (antisense); for PAX8-AS1, 5′-AGACGGTCAGACCAG-3′ (antisense); for PTEN, 5′-GGCTCTTTGCTGAGATTA TCTGG-3′ (sense) and 5′-CATGAACTTGTCTTCCCCG-3′ (antisense); for CDKN1A, 5′-TGTATTAGCAGCGGAA CAAG-3′ (sense) and 5′-AACAGTACCAGGCGCATATG-3′ (antisense); for ZBTB4, 5′-ACGAAGCATGAAGTGTGGC-3′ (sense) and 5′-GGAGAGGTGGAGAAG-3′ (antisense); and for β-actin, 5′-GGGAAATCGTGCGTGACATTA AG-3′ (sense) and 5′-TGTGGTGGCTGCTAGAG-3′ (antisense). For miRNAs quantification, qRT-PCR was performed as above, using TaqMan microRNA assays (Applied Biosystems) in accordance with the manufacturer’s protocol. The expression of RNAs was calculated using 2−ΔΔCt method.
2.3 Plasmids construction and transfection

PAX8-AS1-N full-length sequence was PCR-amplified with the Platinum® Pfx DNA Polymerase (Invitrogen) in accordance with the manufacturer's protocol and subcloned into the Kpn I and BamHI I sites of pcDNA3.1 plasmid (Invitrogen) or pSPT19 plasmid (Roche, Mannheim, Germany), named as pcDNA3.1-PAX8-AS1-N or pSPT19-PAX8-AS1-N, respectively. The primers sequences were: 5′-CCCAAGCTTCATTTTAGATGCCTCCTGGC-3′ (forward) and 5′-CGGGATCCAAACGTTAAACATCGTTTTTATTCC-3′ (reverse). Two independent oligonucleotides for shRNAs specifically targeting PAX8-AS1-N were designed and synthesized by GenePharma (Shanghai, China), and inserted into the shRNA expression plasmid pGPH1/Neo (GenePharma). The two PAX8-AS1-N shRNAs target sites were: 5′-GCTGTATGACTTCTAGCTATG-3′ and 5′-GCAAAGATCCACAGCACTTTC-3′. A scrambled nonsilencing shRNA was used as negative control for PAX8-AS1-N specific shRNAs. The transfections of plasmids were performed using Lipofectamine 3000 (Invitrogen) in accordance with the manufacturer's protocol.

2.4 Stable cell lines construction

To obtain PAX8-AS1-N stably overexpressed MDA-MB-231 and MCF-7 cells, PAX8-AS1-N expressing plasmid pcDNA3.1-PAX8-AS1-N was transfected into MDA-MB-231 and MCF-7 cells. To obtain PAX8-AS1-N stably depleted MDA-MB-231 and MCF-7 cells, PAX8-AS1-N specific shRNAs were transfected into MDA-MB-231 and MCF-7 cells. Forty-eight hours after transfection, the cells were selected with 800 µg/mL neomycin for 4 weeks.

2.5 Glo cell viability assays

Three thousands indicated breast cancer cells were plated per well in 96-well plates. After incubation for 48 h, cell viability was detected with the CeliTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI) in accordance with the manufacturer's protocol.

2.6 Ethynyl deoxyuridine (EdU) incorporation assays

To evaluate in vitro cell proliferation, indicated breast cancer cells were plated to carry out EdU incorporation assays using the EdU Kit (RiboBio, Guangzhou, China) in accordance with the manufacturer's protocol. The results were acquired using Zeiss photomicroscope (Carl Zeiss, Oberkochen, Germany) and quantified using Image-Pro plus 6.0 software via counting at least five random fields.

2.7 Cell cycle analyses

Cell cycles of indicated breast cancer cells were measured by fluorescence-activated cell sorting (FACS) analyses of propidium-iodide stained cells with the Cell Cycle Analysis Kit (Beyotime Co., Jiangsu, China) on a LARII flow cytometer (BD Biosciences, San Jose, CA) in accordance with the manufacturer's protocols. WinCycle software (Phoenix Flow Systems, San Diego, CA) was used to quantify the percentages of cells at G1, S, and G2 phases.

2.8 TdT-mediated dUTP nick end labeling (TUNEL) assays

To evaluate in vitro cell apoptosis, TUNEL assays were carried out using the Dead End™ Fluorometric TUNEL System (Promega) in accordance with the manufacturer's protocol. The results were acquired using Zeiss photomicroscope (Carl Zeiss, Oberkochen, Germany) and quantified using Image-Pro plus 6.0 software via counting at least five random fields.

2.9 In vivo tumor growth assays

3.0 × 10⁶ indicated breast cancer cells were subcutaneously injected into the flanks of female athymic BALB/c nude mice. After tumor inoculation, the mice were intraperitoneally administrated daily with 0.1 mL DMSO (0.25%) or baicalein (10 mg/kg/day). Tumor volumes were detected using caliper every 7 days and calculated using the following formula: V = 0.5 × L × W² (L, length; W, width). Tumor weights were detected at the 28th day after injection. The Ethical Committee of Southwest Medical University reviewed and approved the in vivo tumor growth assay.

2.10 Ki67 and cleaved caspase-3 immunohistochemistry staining

Subcutaneously tumors were immunohistochemistry stained for Ki67 and cleaved caspase-3. Paraffin embedded tissue sections were deparaffinized, rehydrated, followed by antigen retrieval. After being incubated with primary antibodies specific for Ki67 (Cell Signaling Technology, Boston, MA) or cleaved caspase-3 (Cell Signaling Technology) and then a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology), the proteins in situ were finally visualized with 3, 3-diaminobenzidine. The results were acquired using Zeiss photomicroscope (Carl Zeiss, Oberkochen, Germany) and quantified using Image-Pro plus 6.0 software via counting at least five random fields.

2.11 Cytoplasmic and nuclear RNA isolation

Cytoplasmic and nuclear RNA isolation was carried out using the Cytoplasmic & Nuclear RNA Purification Kit (Norgen,
Belmont, CA) following the manufacturer's protocol. The isolated RNA was detected using qRT-PCR as above described.

### 2.12 RNA pull-down assay

PAX8-AS1-N was in vitro transcribed from pSPT19-PAX8-AS1-N and biotin-labeled using T7 RNA polymerase (Roche) and Biotin RNA Labeling Mix (Roche) following the manufacturer's protocols. After being treated with RNase-free DNase I (Roche), the in vitro transcribed biotinylated PAX8-AS1-N was purified using RNeasy Mini Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's protocols. A total of 50 pmol of purified biotinylated PAX8-AS1-N was incubated with 1 mg of whole-cell lysates from MDA-MB-231 cells for 1 h at 25°C. Then the complexes were incubated with Dynabeads Myone Streptavidin T1 beads (Invitrogen) for an additional 1 h and washed using PBS. The RNA present in the pull-down material was measured by qRT-PCR as above described.

### 2.13 Western blot

Total proteins were extracted from indicated cells using RIPA Lysis Buffer (Beyotime Co., Jiangsu, China) in accordance with the manufacturer's protocol. Identical quantities of extracted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by being transferred onto nitrocellulose filter membranes. After being blocked with bovine serum albumin, the membranes were incubated with PTEN (Abcam, Hong Kong, China), CDKN1A (Abcam), ZBTB4 (Abcam), or β-actin (Proteintech, Rosemont, IL) specific primary antibodies. After being washed, the membranes were further incubated with IRDye 800CW goat anti-rabbit IgG or IRDye 700CW goat anti-mouse IgG (Li-Cor, Lincoln, NE), and measured using an Odyssey infrared scanner (Li-Cor).

### 2.14 Tissue specimens

Seventy-six pairs of breast cancer tissues and adjacent normal breast tissues were acquired with informed consent from breast cancer patients who underwent surgical resection at Southwest Medical University (Luzhou, China). Freshly resected tissue specimens were immediately frozen in liquid nitrogen and stored at −80°C until use. All the tissue specimens were histopathological confirmed by pathological examination. The Ethical Committee of Southwest Medical University reviewed and approved the use of clinical tissue specimens.

### 2.15 Statistical analyses

GraphPad Prism Software was used to perform statistical analyses. Student’s t-test, Mann-Whitney test, Wilcoxon signed-rank test, or Log-rank test was used to perform statistical comparisons as indicated. P < 0.05 was considered as statistically significant.

### 3 RESULTS

#### 3.1 Baicalein activated PAX8-AS1-N expression in breast cancer cells

To investigate the roles of baicalein on the expression of PAX8-AS1 in breast cancer, we treated MDA-MB-231 and MCF-7 cells with different doses of baicalein for 48 h. The results revealed that baicalein significantly up-regulated PAX8-AS1-N expression in both cells as low as 50 µM and caused more marked upregulation in higher doses (Figures 1A and 1B). However, baicalein did not regulate PAX8-AS1 expression in both cells in a dose of 200 µM (Supplementary Figure S1B and 1C). These data suggested that PAX8-AS1-N is a different transcript from the routine transcripts of PAX8-AS1. Furthermore, we carried out time course evaluations of PAX8-AS1-N expression in both MDA-MB-231 and MCF-7 cells. The results revealed that 200 µM baicalein treatment up-regulated PAX8-AS1-N expression as early as 24 h and gradually increased PAX8-AS1-N expression over time in both cells (Figures 1C and 1D). These results demonstrated that baicalein activated PAX8-AS1-N expression in breast cancer cells.

#### 3.2 PAX8-AS1-N decreased cell viability of breast cancer cells

Then, we investigated the biological roles of PAX8-AS1-N in breast cancer. PAX8-AS1-N stably overexpressed MDA-MB-231 and MCF-7 cells were constructed through the transfection of PAX8-AS1-N overexpression plasmids (Figures 2A and 2B). Glo cell viability assays revealed that enhanced PAX8-AS1-N expression significantly decreased cell viabilities of both MDA-MB-231 and MCF-7 cells (Figures 2C and 2D). In addition, EdU incorporation assays also revealed that enhanced PAX8-AS1-N expression significantly inhibited cell proliferation of both MDA-MB-231 and MCF-7 cells (Figure 2E). Cell cycle analyses revealed that enhanced PAX8-AS1-N expression increased the percentages of cells in the G1 phases, and decreased the percentages of cells in the S and G2 phases in both MDA-MB-231 and MCF-7 cells (Figures 2F and 2G). These data suggested that enhanced PAX8-AS1-N expression induced G1 arrest and inhibited cell-cycle progression. TUNEL assays revealed that enhanced PAX8-AS1-N expression significantly induced apoptosis of both MDA-MB-231 and MCF-7 cells (Figure 2H). Collectively, these results demonstrated that PAX8-AS1-N decreased cell viability, inhibited cell-cycle progression, and induced cell apoptosis of breast cancer cells.
3.3 | Depletion of PAX8-AS1-N increased cell viability of breast cancer cells

To completely investigate the biological roles of PAX8-AS1-N in breast cancer, PAX8-AS1-N stably depleted MDA-MB-231 cells were constructed through the transfection of two independent PAX8-AS1-N specific shRNAs (Figure 3A). Glo cell viability assays revealed that depletion of PAX8-AS1-N significantly increased cell viability of MDA-MB-231 cells (Figure 3B). EdU incorporation assays also revealed that depletion of PAX8-AS1-N significantly increased cell viability of MDA-MB-231 cells (Figure 3C). Cell cycle analyses revealed that depletion of PAX8-AS1-N decreased the percentages of cells in the G1 phases, and increased the percentages of cells in the S and G2 phases (Figure 3D). These data suggested that depletion of PAX8-AS1-N significantly inhibited apoptosis of MDA-MB-231 cells (Figure 3E). Collectively, these results demonstrated that depletion of PAX8-AS1-N increased cell viability, promoted cell-cycle progression, and inhibited cell apoptosis of breast cancer cells.

3.4 | PAX8-AS1-N knockdown attenuated the anti-cancer effects of baicalein in breast cancer cells in vitro

To investigate whether PAX8-AS1-N is involved in the anti-cancer effects of baicalein in breast cancer, PAX8-AS1-N stably depleted and control MDA-MB-231 and MCF-7 cells were treated with 200 µM baicalein for 48 h (Figures 4A and 4B). Consistent with previous reports,44 Glo cell viability assays revealed that baicalein significantly reduced cell viabilities of both MDA-MB-231 and MCF-7 cells (Figures 4C and 4D). Furthermore, Glo cell viability assays
FIGURE 2 Enhanced PAX8-AS1-N expression decreased cell viability, inhibited cell-cycle progression, and induced apoptosis of breast cancer cells. A and B, PAX8-AS1-N expression in PAX8-AS1-N stably overexpressed and control MDA-MB-231 (A) or MCF-7 (B) cells was measured by qRT-PCR and normalized to β-actin. C and D, Cell viabilities of PAX8-AS1-N stably overexpressed and control MDA-MB-231 (C) or MCF-7 (D) cells were measured by Glo cell viability assays. E, Cell proliferation of PAX8-AS1-N stably overexpressed and control MDA-MB-231 or MCF-7 cells was measured by EdU incorporation assays. The red color represents EdU-positive and proliferative cells. Scale bars, 100 μm. F and G, Cell cycles of PAX8-AS1-N stably overexpressed and control MDA-MB-231 (F) or MCF-7 (G) cells were measured by fluorescence-activated cell sorting (FACS) analyses of propidium-iodide stained cells. H, Cell apoptosis of PAX8-AS1-N stably overexpressed and control MDA-MB-231 or MCF-7 cells was measured by TUNEL assays. The green color represents TUNEL-positive and apoptotic cells. Scale bars, 100 μm. Results are shown as mean ± SD from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t-test.
also revealed that PAX8-AS1-N knockdown significantly attenuated the cell viability depression caused by baicalein in both cells (Figures 4C and 4D). EdU incorporation assays revealed that baicalein inhibited cell proliferation of both MDA-MB-231 and MCF-7 cells, PAX8-AS1-N knockdown promoted cell proliferation of both cells, and the cell proliferation suppression caused by baicalein was significantly attenuated by PAX8-AS1-N knockdown in both cells (Figures 4E and 4F). TUNEL assays revealed that baicalein induced cell apoptosis of both MDA-MB-231 and MCF-7 cells, PAX8-AS1-N knockdown inhibited cell apoptosis of both cells, and the cell apoptosis induced by baicalein was significantly attenuated by PAX8-AS1-N knockdown in both cells (Figures 4G and 4H). Collectively, these results demonstrated that baicalein repressed cell viability and induced apoptosis of breast cancer cells, which was attenuated by PAX8-AS1-N knockdown.

### 3.5 | PAX8-AS1-N knockdown attenuated the anti-cancer effects of baicalein in breast cancer cells in vivo

To completely investigate the involvement of PAX8-AS1-N in the anti-cancer effects of baicalein in breast cancer, PAX8-AS1-N stably depleted and control MDA-MB-231 cells were subcutaneously injected into the flanks of nude mice. Then the mice were treated with 0.1 mL DMSO (0.25%; i.p.) or baicalein (10 mg/kg/day; i.p.). The xenograft tumor volume was examined every 7 days, and the tumor weight was examined at 28th day after injection. The results revealed that...
FIGURE 4  PAX8-AS1-N knockdown attenuated the cell viability suppression and apoptosis induction caused by baicalein. A and B, After treatment with 200 µM baicalein for 48 h, PAX8-AS1-N expression in PAX8-AS1-N stably depleted and control MDA-MB-231 (A) or MCF-7 (B) cells was measured by qRT-PCR and normalized to β-actin. C and D, After treatment with 200 µM baicalein for 48 h, cell viabilities of PAX8-AS1-N stably depleted and control MDA-MB-231 (C) or MCF-7 (D) cells were measured by Glo cell viability assays. E and F, After treatment with 200 µM baicalein for 48 h, cell proliferation of PAX8-AS1-N stably depleted and control MDA-MB-231 (E) or MCF-7 (F) cells was measured by EdU incorporation assays. The red color represents EdU-positive and proliferative cells. Scale bars, 100 µm. G and H, After treatment with 200 µM baicalein for 48 h, cell apoptosis of PAX8-AS1-N stably depleted and control MDA-MB-231 (G) or MCF-7 (H) cells was measured by TUNEL assays. Results are shown as mean ± SD from three independent experiments. *P < 0.05, ***P < 0.001 by Student's t-test.
baicalein significantly repressed breast cancer xenograft tumor growth in vivo, PAX8-AS1-N knockdown promoted breast cancer xenograft tumor growth, and the tumor growth suppression caused by baicalein was significantly attenuated by PAX8-AS1-N knockdown (Figure 5A-D). Proliferation marker Ki67 immunohistochemistry staining revealed that xenograft from mice injected with baicalein had significantly decreased Ki67 versus the xenograft from mice injected with carrier control (DMSO) (Figure 5E). Xenograft derived from PAX8-AS1-N stably depleted MDA-MB-231 cells had significantly increased Ki67 versus that derived from control cells (Figure 5E). The decrease of Ki67 caused by baicalein was significantly attenuated by PAX8-AS1-N knockdown (Figure 5E). Apoptosis marker cleaved caspase-3 immunohistochemistry staining revealed that xenograft from mice injected with baicalein had significantly increased cleaved caspase-3 versus the xenograft from mice injected with carrier control (DMSO) (Figure 5F). Xenograft derived from PAX8-AS1-N stably depleted MDA-MB-231 cells had significantly decreased cleaved caspase-3 versus that derived from control cells (Figure 5F). The increase of cleaved caspase-3 caused by baicalein was significantly attenuated by PAX8-AS1-N knockdown (Figure 5F). Collectively, these results demonstrated that PAX8-AS1-N was a critical mediator of the anti-cancer effects of baicalein both in vitro and in vivo.

3.6 | PAX8-AS1-N up-regulated PTEN, CDKN1A, and ZBTB4 expression via interacting with miR-17-5p

To elucidate the molecular mechanisms underlying the biological roles of PAX8-AS1-N, we first measured the subcellular distribution of PAX8-AS1-N via cytoplasmic and nuclear RNA isolation. As shown in Figure 6A, PAX8-AS1-N was mainly located in the cytoplasm. Many cytoplasmic lncRNAs have been reported to act as competing endogenous RNAs (ceRNA) via competitively binding common miRNAs. Using the TargetScan algorithm, we predicted two miR-17-5p binding sites on PAX8-AS1-N in a relatively short span (Figure 6B). miR-17-5p was frequently reported to function as an oncogenic miRNA in many cancers, including breast cancer. To investigate whether PAX8-AS1-N genuinely binds to miR-17-5p, we carried out RNA pull down assays using in vitro transcribed biotin-labeled PAX8-AS1-N. As shown in Figure 6C, PAX8-AS1-N specifically bound to miR-17-5p, but not miR-200b which did not have predicted binding site on PAX8-AS1-N. The well-known tumor suppressors PTEN, CDKN1A (p21), and ZBTB4 were reported to be critical targets of miR-17-5p. Therefore, we further investigated the effects of PAX8-AS1-N on the expression of PTEN, CDKN1A, and ZBTB4. qRT-PCR assays revealed that enhanced PAX8-AS1-N expression up-regulated PTEN, CDKN1A, and ZBTB4 mRNA levels (Figure 6D). Depletion of PAX8-AS1-N down-regulated PTEN, CDKN1A, and ZBTB4 mRNA levels (Figure 6E). Western blot assays revealed that enhanced PAX8-AS1-N expression up-regulated PTEN, CDKN1A, and ZBTB4 protein levels (Figure 6F). Depletion of PAX8-AS1-N down-regulated PTEN, CDKN1A, and ZBTB4 protein levels (Figure 6G). Furthermore, consistent with enhanced expression of PAX8-AS1-N, treatment with 200 µM baicalein for 48 h also up-regulated PTEN, CDKN1A, and ZBTB4 mRNA and protein levels (Figures 6H and 6I). Collectively, these results demonstrated that PAX8-AS1-N bound to miR-17-5p, up-regulated PTEN, CDKN1A, and ZBTB4 expression, which were also regulated by baicalein.

3.7 | PAX8-AS1-N was down-regulated in breast cancer and associated with prognosis of breast cancer patients

To investigate the clinical significances of PAX8-AS1-N in breast cancer, PAX8-AS1-N expression in 76 pairs of breast cancer tissues and adjacent normal breast tissues was measured by qRT-PCR. As shown in Figure 7A, PAX8-AS1-N was significantly down-regulated in breast cancer tissues compared with that in normal breast tissues. Furthermore, we analyzed PAX8-AS1-N expression in ER negative and positive, and Her2 negative and positive breast cancer. The results showed that PAX8-AS1-N was also down-regulated in ER positive, ER negative, Her2 negative, Her2 positive breast cancer tissues compared with that in paired normal breast tissues (Figure 7B-E). Kaplan-Meier survival analysis revealed that decreased PAX8-AS1-N expression indicated worse overall survival of breast cancer patients (Figure 7F). Collectively, these results demonstrated that PAX8-AS1-N was down-regulated in breast cancer and decreased expression of PAX8-AS1-N indicated poor outcome of breast cancer patients.

4 | DISCUSSION

In this study, we identified a novel lncRNA PAX8-AS1-N, which is down-regulated in breast cancer and could be activated by baicalein. Decreased expression of PAX8-AS1-N indicated poor prognosis of breast cancer patients. Functionally, we found that PAX8-AS1-N reduced cell viability, inhibited cell-cycle progression, and induced apoptosis of breast cancer cells in vitro. Depletion of PAX8-AS1-N promoted breast xenograft tumor growth in vivo. Furthermore, we found that depletion of PAX8-AS1-N attenuated the cell viability suppression and apoptosis
FIGURE 5 PAX8-AS1-N knockdown attenuated the tumor growth inhibition caused by baicalein in vivo. A, PAX8-AS1-N stably depleted and control MDA-MB-231 cells were subcutaneously injected into the flanks of nude mice. The mice were intraperitoneally injected with 0.1 mL 0.25% DMSO (carrier) or baicalein (10 mg/kg/day). Tumor volume was measured every 7 days with caliper. B, Tumor volume at 28th day after injection was measured with caliper, and normalized to carrier control (DMSO) groups, respectively. C, The tumors were excised and weighed at 28th day after injection. D, Tumor weight was measured and normalized to carrier control (DMSO) groups, respectively. E, The subcutaneous tumors were used to carry out Ki67 immunohistochemistry staining. Scale bars, 50 μm. F, The subcutaneous tumors were used to carry out cleaved caspase-3 immunohistochemistry staining. Scale bars, 50 μm. Results are shown as mean ± SD from six mice in each group. *P < 0.05, **P < 0.01 by Mann-Whitney test.
induction caused by baicalein in vitro, and also the tumor growth suppression caused by baicalein in vivo. We could conclude that depletion of PAX8-AS1-N attenuated the anti-cancer effects of baicalein in breast cancer. Thus, our results suggested that PAX8-AS1-N functioned as a tumor suppressor in breast cancer, and that baicalein exerted its anti-cancer effects at least partially through activating PAX8-AS1-N.
Wang et al. have reported that baicalein inhibited breast cancer growth via up-regulating DDIT4 expression.\textsuperscript{48} Nguyen \textit{et al} have reported that baicalein inhibited epithelial to mesenchymal transition, migration, and invasion of breast cancer cells via down-regulating Cyr61 and LOXL-2.\textsuperscript{49} Zhou \textit{et al}\textsuperscript{50} reported that baicalein induced apoptosis of breast cancer cells via the ERK/p38 MAPK pathway. Other reports also revealed the various anti-cancer effects of baicalein in breast cancer.\textsuperscript{7} However, to our knowledge, this is the first report identifying an lncRNA mediating the anti-cancer effects of baicalein in breast cancer. The concentrations of baicalein used to inhibit cancer are relative high, which may be disadvantage for clinical application. Therefore, elucidating the mechanisms mediating the anti-cancer effects of baicalein and improving the sensitivity of cancer cells to baicalein may promote clinical application of baicalein. Consideration of the important roles of PAX8-AS1-N in the anti-cancer processes of baicalein, the combination of baicalein with PAX8-AS1-N would be efficiently therapeutic strategy for breast cancer. Due to the large number and various functions of lncRNAs, we speculate that other lncRNAs may be also involved in the anti-cancer processes of baicalein, which needs further investigation.

Interestingly, in this study, we found that baicalein activated PAX8-AS1-N expression, but not PAX8-AS1. PAX8-AS1-N has different promoter compared to other isoforms of PAX8-AS1. Thus, we speculate that the different promoters may contribute to the different responses to baicalein. The function of PAX8-AS1 in breast cancer has not been reported. Whether PAX8-AS1 has similar or different roles with PAX8-AS1-N in breast cancer need further study.

Presently, the precise action mechanisms of lncRNAs remain largely unclear.\textsuperscript{51} A mainly proposed action model is that lncRNAs bind to other proteins, DNAs, miRNAs, and/or mRNAs, and further change the expression, location, and/or functions of the interaction partners.\textsuperscript{52,53} In this study, we found

FIGURE 7  PAX8-AS1-N was down-regulated in breast cancer and decreased expression of PAX8-AS1-N indicated poor outcome of breast cancer patients. A, PAX8-AS1-N expression in 76 pairs of breast cancer tissues and adjacent normal breast tissues was measured by qRT-PCR and normalized to $\beta$-actin. $P < 0.001$ by Wilcoxon signed-rank test. B, PAX8-AS1-N expression in 52 pairs of ER positive breast cancer tissues and adjacent normal breast tissues was measured by qRT-PCR and normalized to $\beta$-actin. $P < 0.001$ by Wilcoxon signed-rank test. C, PAX8-AS1-N expression in 24 pairs of ER negative breast cancer tissues and adjacent normal breast tissues was measured by qRT-PCR and normalized to $\beta$-actin. $P < 0.001$ by Wilcoxon signed-rank test. D, PAX8-AS1-N expression in 59 pairs of Her2 negative breast cancer tissues and adjacent normal breast tissues was measured by qRT-PCR and normalized to $\beta$-actin. $P = 0.002$ by Wilcoxon signed-rank test. E, PAX8-AS1-N expression in 17 pairs of Her2 positive breast cancer tissues and adjacent normal breast tissues was measured by qRT-PCR and normalized to $\beta$-actin. $P < 0.001$ by Wilcoxon signed-rank test. For A-E, results are shown as median with interquartile range. F, Kaplan-Meier survival analyses of the association between PAX8-AS1-N expression and overall survival of these 76 breast cancer patients. The median PAX8-AS1-N expression level was used as the cutoff. $P = 0.007$ by Log-rank test.
that PAX8-AS1-N mainly localized in cytoplasm. Many cytoplasmic lncRNAs function as ceRNAs via binding common miRNAs.39 Intriguingly, we predicted two miR-17-5p binding sites on PAX8-AS1-N in a relatively short span and further confirmed the interaction between PAX8-AS1-N and miR-17-5p using RNA pull-down assays. miR-17-5p was regarded as onco-miR-1, which is commonly regarded as the first identified oncogenic miRNA.54–57 Increased expression and oncogenic roles of miR-17-5p have been reported in breast cancer, pancreatic cancer, B-cell lymphoma, liver cancer, and so on.42,45,58,59 In this study, we further demonstrated that via physically binding to miR-17-5p, PAX8-AS1-N up-regulated miR-17-5p targets, such as PTEN, CDKN1A (p21), ZBTB4, which are critical tumor suppressors in many cancers. Through activating PAX8-AS1-N, baicalein up-regulated PTEN, CDKN1A, and ZBTB4. Thus, our results suggested that baicalein/PAX8-AS1-N/miR-17-5p/PTEN, CDKN1A, ZBTB4 regulation axis exits in breast cancer.

In summary, we identified a novel lncRNA PAX8-AS1-N which is activated by baicalein in breast cancer. PAX8-AS1-N is down-regulated in breast cancer, and reduced expression of PAX8-AS1-N indicates poor prognosis of breast cancer patients. PAX8-AS1-N functions as a tumor suppressor in breast cancer. Baicalein exerts its anti-cancer effects through activating PAX8-AS1-N. Mechanistically, we found that PAX8-AS1-N physically bound to miR-17-5p, and up-regulated miR-17-5p targets PTEN, CDKN1A, and ZBTB4. Therefore, our results provided new insights into the anti-cancer mechanism of baicalein, and suggested that baicalein and enhancing PAX8-AS1-N would be potential therapeutic strategies against breast cancer.

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CONFLICTS OF INTEREST

The authors declare that no competing interest exists.

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SUPPORTING INFORMATION
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