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Flurbiprofen Axetil Targets SOX9-Mediated Wnt/β-Catenin/c-Myc Signaling to Suppress Tumorigenesis and Stemness in Luminal A Breast Cancer

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1. Abstract

1.1. Background

SOX9, overexpressed in breast cancer and driving Wnt/β-catenin signalling and stemness, is a potential target. The NSAID Flurbiprofen axetil (FA) shows anti-tumour activity and reduces SOX9 in non-cancer contexts, but its role in LABC is unknown.

1.2. Methods

In order to explore the influence of FA on the function of LABC. In vitro (MCF-7/ZR-75-1), we visually evaluated the proliferation (CCK-8/5-Ethynyl-2'-deoxyuridine assay), migration and metastasis (scratch/transwell assay), and stemness (colony/sphere formation assay) of LABC cells treated by FA. Meanwhile, we used flow cytometry and western blot assay to evaluate the effects of FA on apoptosis and stemness of LABC cells at the molecular level. In addition, we used the xenograft model to further verify the effect of FA on LABC in vivo.

1.3. Results

FA treatment suppressed proliferation, migration, invasion, stemness and induced apoptosis in LABC cells. Mechanistically, we found that FA downregulated SOX9 expression, leading to inhibition of the Wnt/β-catenin/c-Myc signaling pathway. These mechanical findings were further validated through experiments involving SOX9 overexpression and Wnt activation rescue. In vivo, FA significantly attenuated tumor growth, reduced stemness marker expression, and promoted apoptosis in xenograft models. These effects were consistently associated with suppressed SOX9 pathway activity, highlighting its central role in mediating FA's anti-tumor activity.

1.4. Conclusion

This study demonstrated that FA targets the SOX9/Wnt/β-catenin/c-Myc axis to inhibit LABC progression. The dual inhibition of tumor growth and stemness highlights potential of FA as a translational therapeutic agent.

2. Introduction

Breast cancer is an epithelial malignant tumor that occurred in the mammary gland. According to a report released by the World Health Organization (WHO) in 2020, a staggering 2.3 million women received a breast cancer diagnosis, resulting in 685,000 fatalities from the global perspective[1]. Despite the common tendency to conceptualize breast cancer as a singular disease entity, which exhibits a diverse nature with four basic subtypes that correlate with mRNA intrinsic subtypes-Luminal A, Luminal B, human epidermal growth factor receptor 2 (HER2)-enriched and Basal-like. The subtypes differ in etiology, treatment options, and prognosis[2]. Compared to other breast cancer subtypes, luminal A breast cancer (LABC) is marked by high expression of ER-related genes, low expression of HER2 and proliferation-related genes, which are fortunately associated with a more favorable clinical outcome of patients with LABC[3]. LABC is estrogen-dependent, and endocrine therapy is one of the most effective treatments for it. Despite the efficacy of endocrine therapy, resistance remains a major clinical hurdle, necessitating

the exploration of novel therapeutic strategies for LABC[4].The sex-determining region Y-box (SOX) genes are a family of high-mobility groups that encode a set of transcriptional factors. These factors have emerged as potent modulators involved in governing embryonic development and cell fate, organogenesis, and stem cell maintenance[5]. A substantial body of fundamental and clinical research has firmly established SOX9, a constituent of the SOX transcription factor family, as a dispensable transcription factor that regulates numerous developmental signalling pathways. Its aberrant expression has been correlated with tumour initiation, proliferation, metastasis, and stem cell maintenance[6]. SOX9 is frequently overexpressed and extensively identified as an oncogene in breast cancer[7-9], and was demonstrated to regulate β-catenin expression in the canonical Want pathway and to influence the downstream effectors Cyclin D1 and c-Myc[10,11]. The Wantsignalling pathway is a highly conserved pathway[12] and was confirmed to be involved in the proliferation[13] and metastasis of breast cancer[14]. This pathway is a potential target for the treatment of breast cancer.Non-steroidal anti-inflammatory drugs (NSAIDs), reversible inhibitors of cyclo-oxygenase (COX), are the most extensively used over-the-counter FDA-approved analgesic medicines for treating the symptomatic relief of pain, whether traumatic, infectious, episodic or rheumatologic[15]. A substantial body of research has demonstrated the efficacy of NSAIDs in the treatment of human malignancies[16]. Flurbiprofen axetil (FA), a NSAID belonging to the propionic acid derivative class, is predominantly prescribed in clinical settings for its anti-inflammatory, analgesic, and antipyretic properties. This effect is achieved by non-selectively inhibiting cyclooxygenase (COX) and impeding the synthesis of prostaglandins[17]. In addition to its incorporation in multimodal postoperative analgesia regimens for cancer patients in clinical practice[18-20], the potential of FA to function as a tumor suppressor in cervical cancer[21], colorectal cancer[17], and thyroid cancer[22] has been proposed. The recent publication has demonstrated that FA can exert anti-metastatic effects in basal-like breast cancer[23]. Intriguingly, FA has been reported to downregulate SOX9 in non-cancer contexts[24], suggesting its potential to disrupt Wnt/β-catenin signaling in LABC. However, whether FA exerts anti-tumor effects in LABC via SOX9-mediated Wnt/β-catenin/c-Myc signaling remains unknown. This study aims to elucidate the therapeutic potential of FA in LABC and its molecular mechanism, offering a repurposing strategy for endocrine-resistant cases.

3. Materials and Methods

3.1. Cell Culture and Treatment

Immortalized non-tumorigenic human breast epithelial MCF-10A cells were cultivated in MEGM Bullet Kit (Lonza, CC-3150), which contained cholera toxin (100 ng/mL). The LABC cell lines, MCF-7 and ZR-75-1, were respectively maintained in two different media: Minimum Essential Medium (MEM) and RPMI-1640 medium. Both media were enriched with 10% fetal bovine serum (FBS, Gbico) and 1% penicillin/streptomycin mixture. The treatment of both MCF-7 and ZR-75-1 cells was conducted using varying concentrations of FA. Cells were infected with the empty vector (Ov-NC) or SOX9-overexpressing (Ov-SOX9) lentivirus (pLV-EF1α-MCS-IRES-Puro vector system) with Lipofectamine 3000 reagent (Invitrogen, L3000015). Lentiviral infection was performed at a multiplicity of infection (MOI) of 20, in the presence of 8 μg/mL polybrene (Sigma-Aldrich, TR-1003), and cells were incubated with viral supernatant for 12–16 hours before replacement with fresh culture medium. For selection of stably infected cells, puromycin was added at a final concentration of 2 μg/mL for MCF-7 and ZR-75-1 cells after 48 hours post-infection, and maintained for 3–5 days or until uninfected control cells were fully eliminated. Drug treatments were initiated with stably infected cells. Alternatively, cells were pretreated with 10 mM of a Want/beta-catenin pathway agonist (LiCl)[25] with treatment of FA.

3.2. Proliferation Assay

After being prepared in the 96-well plates (3~5×103/well), cells were treated with gradient doses of FA ranging from 0 to 200 μM. FA was dissolved in DMSO to prepare stock solutions and diluted with culture medium to the desired concentrations, ensuring the final DMSO concentration did not exceed 0.1%. After 24, 48, and 72 hours of treatment, 10 μL of CCK-8 reagent (Dojindo, CK04) was added to each well and incubated for 2 hours at 37°C. Optical absorbance value was recorded at 450 nm using a plate reader. The drug concentration that inhibited 50% of the growth of control cells (IC50) was computed by plotting the dose-response curves in GraphPad Prism 8.0 software.The MCF-7 and ZR-75-1 cells were prepared in 96-well plates (3×104 cells/well) and subsequently exposed to 50 μM 5-Ethynyl-2’-deoxyuridine (EDU) working buffer (Beyotime, C10310-1) for 2h. Prior to being reacted with Click Addictive Solution, the cells were fixed with 4% paraformaldehyde and permeated with 0.5% Triton X-100 (Sigma-Aldrich, T8787). Following the nuclear identification of cells using DAPI (Beyotime, C1002), microscopic images were captured to assess the proliferation of these cells (Olympus, IX73).

3.3. Migration and Invasion Assays

In order to analyze the migration function of MCF-7 and ZR-75-1 cells, they were prepared in 6-well plates (1×105 cells/well). Upon attaining 95% confluency, the cells were scraped using a vertical cross of constant width in the center of each well. After a rinse with Phosphate Buffered Saline (PBS), the culture medium was replaced with serum-deprived medium. The area occupied by migrated cells in the scratch was evaluated by light microscopy after a 24h incubation. The migration area was quantified using ImageJ software. To observe the invasion of MCF-7 and ZR-75-

1 cells. After being precoated with Matrigel (BD Biosciences, 356234), the upper compartment of the Transwell chambers (contains an 8 μm porous membrane) were loaded with 6 x 10^4 MCF-7 and ZR-75-1 cells per well within serum-deprived medium. The medium blended with 10% FBS was put in the lower compartment. After 24h, cells that had passed the membrane were fixed with 4% formaldehyde (Solarbio, P1110), stained with 0.1% crystal violet (Beyotime, C0121) for 20 minutes, and counted under a light microscope (Leica, DM500).

3.4. Flow Cytometry Assay

After being prepared in the 6-well plates, MCF-7 and ZR-75-1 cells (4×105 cells/well) were rinsed with PBS. After being mixed with 500 μL of 1 × binding buffer, cells were sequentially reacted with 5 μl FITC-labeled Annexin V/propidium iodide (PI) (BD Biosciences, 556547) for 15 min at room temperature in dark. The cell apoptotic rate was eventually analyzed with a flow cytometer (BD, FACSCanto II), and data were processed with **3.5. FlowoodSoftware.**

Colony formation and sphere formation assays

MCF-7 and ZR-75-1 cells were distributed into 6-well plates (500 cells/well) for colony formation assay. When the colonies were obviously visible, the cells were subjected to fixation with 4% formaldehyde and staining with 0.1% crystal violet. The colony numbers were counted manually.For sphere formation assay, MCF-7 and ZR-75-1 cells (4×103 cells/well) were plated in ultra-low attachment 6-well plates (Corning, 3471) composed of serum-deprived medium supplemented with 2% B27 (Gibco, 17504044), 20 ng/ml bFGF (PeproTech, 100-18B) and 20 ng/ml of EGF (PeproTech, AF-100-15). The spheroids were photographed by light microscopy (Nikon, Eclipse TS2) after 2 weeks of culture.

3.6. Immunofluorescence

The fixed tumor tissues were then dehydrated and paraffinized. The 3 μm-thick sections were dewaxed, rehydrated, and heated in citric acid buffer (pH 6.0) (Beyotime, P0081). The cells were fixed with 4% paraformaldehyde and permeated in 0.5% Triton X-100. Sections were then blocked with 3% bovine serum albumin (BSA) (Sigma-Aldrich, A1933) for 30 minutes at room temperature. The sections or cells were probed overnight at 4 °C with primary antibodies recognize Ki67 (Abcam, ab15580, 1μg/ml) and cleaved caspase3 (Cell Signaling Technology (CST), #9661, 1:400), which were later detected with Alexa Fluor 488 (Invitrogen, A-11001, 1:500) and Alexa Fluor 594 (Invitrogen, A-11012, 1:500) in 3% BSA. After DAPI nuclear staining, the intensity was recorded under a fluorescence microscope.

3.7. Real-Time Quantitative PCR

Total RNA was extracted from cells utilizing RNAiso plus reagent (Takara, 9108). The All-In-One cDNA Synthesis Kit (Vazyme, R333-01) was manipulated to reverse-transcribe the RNA. PCR was operated with the SYBR Premix Ex Taq Kit using an ABI PRISM 7900 Real-time System. The $2^{-\Delta\Delta Ct}$ method for relative quantification of the gene expression. The following primers were used for Sox9 amplification:

Forward primer: AGGAAGCTCGCGGACCAGTAC

Reverse primer: GGTGGTCCTTCTTGTGCTGCAC

3.8. Western Blot

After being scraped in RIPA lysis buffer (Beyotime, P0013B), the protein that were isolated from tumor tissues and cells were quantified by means of BCA Protein Assay kit (Thermo Fisher Scientific, 23225). After being separated by 10% SDS-PAGE with a loading amount of 40 μg/lane, proteins were shifted to PVDF membranes (Millipore, IPVH00010). After being placed in 5% skim milk for blocking, the membranes were probed overnight at 4°C with the purpose primary antibodies: BCL2 (1:1000, CST, #4223), Bax (1:1000, CST, #5023), SOX9 (1:1000, Abcam, ab185966), β-catenin (1:1000, CST, #8480), c-Myc (1:1000, CST, #5605), NANOG (1:1000, Abcam, ab109250), CD44 (1:1000, CST, #3570), GAPDH (1:5000, Abcam, ab8245) as loading control. PVDF membranes were incubated with HRP-conjugated secondary antibodies (1:5000) for 1h at room temperature then detected by ECL chemiluminescence. The target proteins were quantified with ImageJ software after visualization with ECL solution.

3.9. Xenograft Model of LABC and Drug Administration

The 5-week-old female nude mice (20±2 g) on the BALB/c background were maintained in an SPF setting (22±2°C and 55±5% humidity) on a 12 h light–dark cycle with food and water supplied ad libitum throughout the experimental period. All experiments involving animals were operated in accordance with national guidelines for the care and use of laboratory animals and obtained the approval from the Committee for Medical Ethics at Guangdong Women and Children Hospital (approval number: 202301029).After being implanted with 1x106 MCF-7 and ZR-75-1 cells via the subcutaneous route, the mice were administrated with 10 mg/kg FA (100 μL) via intraperitoneal injection when the tumor volume reached 100 mm3. Tumor volume was calculated as: V = (length × width2) / 2 every 3 days. On the 18th day, the mice were euthanized by cervical dislocation, and the tumors were dissected for further analysis.

3.10. Statistical Analysis

All statistical calculations were performed using GraphPad Prism 8.0 software (GraphPad software, Inc., San Diego, CA, USA). Two-way ANOVA was used to compare tumor growth in the mouse tumor model. The single variable among groups were analyzed using t-test or one-way ANOVA followed by Tukey's post hoc test. All numerical results are depicted as a mean ± standard deviation and statistical significance was established as $P< 0.05$.

4. Results

4.1. FA inhibits Proliferation and Induces Apoptosis in LABC Cells

To explore the anti-tumor effects of FA on LABC progression, we assessed its impact on cell viability and apoptosis. CCK-8 assays demonstrated that FA treatment suppressed the proliferation of MCF-7 and ZR-75-1 cells in a concentration-dependent manner (0–200 μM) (Figure S1A). The calculated IC50 values were 20.47 μM for MCF-7 and 30.99 μM for ZR-75-1 cells (Figure S1B), indicating differential sensitivity between the two cell lines. According to the IC50 values, we used 3 different doses to treat cell lines then evaluate their variation of proliferation via CCK-8 assay. It showed a concentration-dependent inhibition of FA on LABC cell lines (Figure S1D). Further validation using EDU assays revealed a significant reduction in DNA synthesis of LABC cell lines upon FA treatment (Figure 1A, C). The results of immunofluorescence staining showed that FA treatment significantly reduced the positive signal of the proliferation marker Ki67 in MCF-7 and ZR-75-1 cells (Figure 1D), further supporting the inhibitory effect of FA on the proliferation of LABC cells.We examined the apoptotic signaling of MCF-7 and ZR-75-1 using flow cytometry in order to elucidate the effect of FA on the viability of LABC cells. FA treatment increased the apoptotic rate of both cell lines in a dose-dependent manner (Figure 1A, S2). Western blot analysis corroborated these findings, showing decreased expression of the anti-apoptotic protein BCL2 and increased expression of the pro-apoptotic protein Bax (Figure 1B). Notably, the BCL2/Bax ratio was markedly reduced in FA-treated groups, further confirming apoptosis induction. These results demonstrated that FA exerts dual anti-tumor effects by suppressing proliferation and promoting apoptosis in LABC cells.

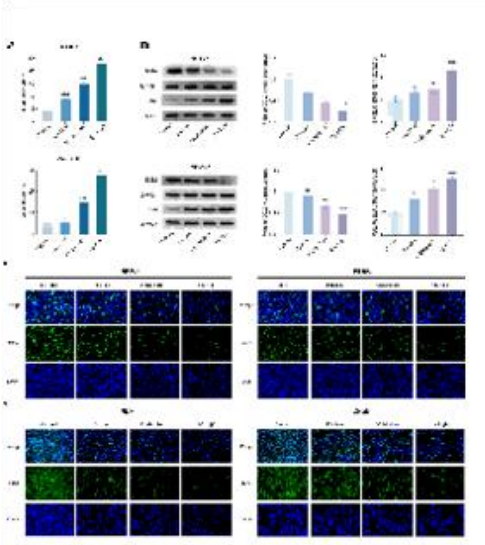

Figure 1: FA impairs the viability of LABC cell lines.

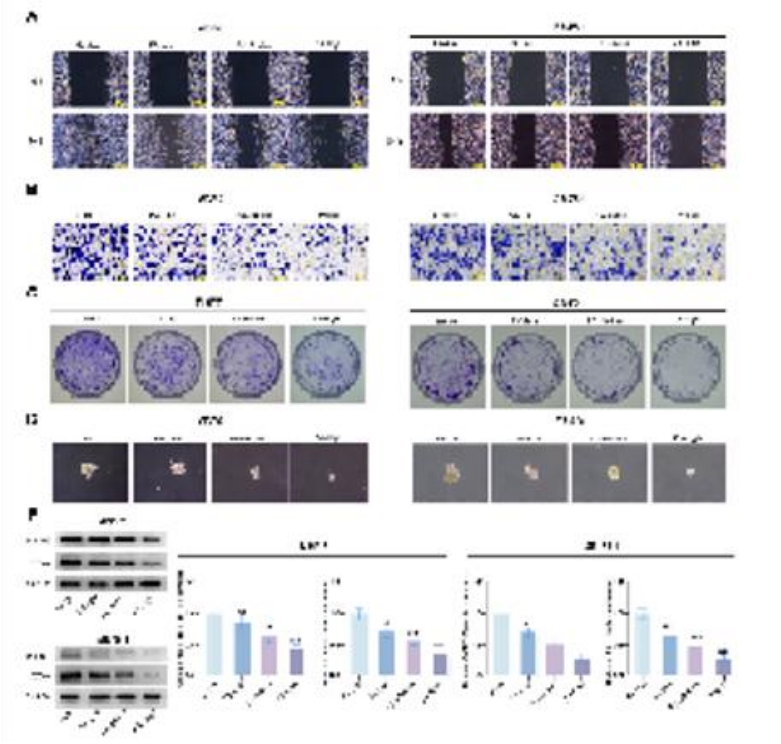

Figure 2: FA inhibits metastatic potential and stemness in LABC cell lines.

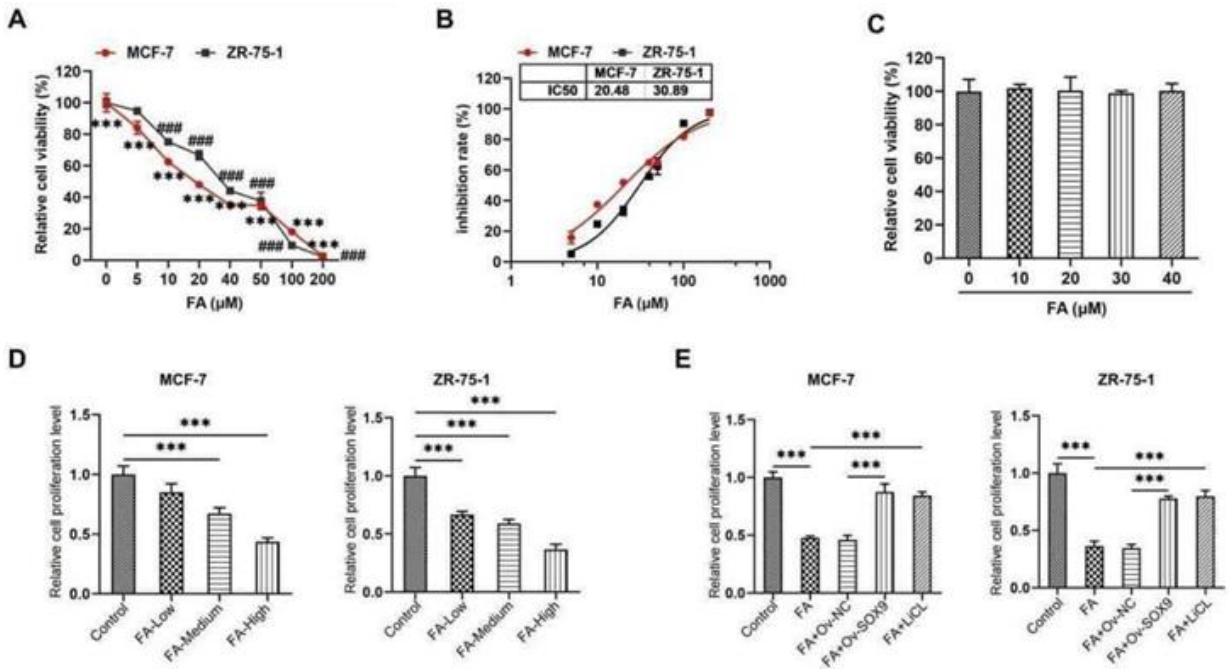

Figure S1. A: CCK-8 assay was used to detected the IC50 of FA on MCF-7 (red) and ZR-75-1 (black). B. Dose-response curve of FA on MCF-7 (red) and ZR-75-1 (black). C. The viability of MCF-10A cells was detected using a CCK-8 assay following treatment with FA. D. The cell viability of MCF-7 and ZR-75-1 was detected using CCK-8 after treatment with varying concentrations of FA. E. The cell viability of MCF-7 and ZR-75-1 cells, which overexpressed SOX9 or were treated with LiCl, was detected using a CCK-8 assay following treatment with FA. *** and ### is P<0.001.

4.2. FA Suppresses Metastatic Potential and Stemness of LABC Cells

We conducted functional assays targeting migration, invasion, and self-renewal capabilities to investigate the impact of FA on metastatic behavior and stemness maintenance in LABC. Scratch assays revealed that FA treatment markedly reduced the migration of MCF-7 and ZR-75-1 cells, with the wound closure area significantly diminished compared to controls (Figure 2A). Similarly, Transwell invasion assays demonstrated that FA suppressed the invasive capacity of both cell lines, as evidenced by reduced cell penetration through Matrigel-coated membranes (Figure 2B). These findings indicate that FA robustly inhibits the metastatic potential of LABC cells.Furthermore, FA treatment profoundly impaired the stemness of LABC cells. Colony formation assays showed a concentration-dependent reduction in the number and size of colonies formed by MCF-7 and ZR-75-1 cells (Figure 2C). In addition, sphere formation assays revealed that FA-treated cells generated smaller tumor spheroids under non-adherent conditions (Figure 2D). At the molecular level, western blot analysis confirmed a significant downregulation of stemness-associated markers Homeobox Transcription Factor Nanog (NANOG) and CD44 in FA-treated cells (Figure 2E). These results collectively demonstrated that FA not only restricts the metastatic behavior of LABC cells but also disrupts their self-renewal capacity by targeting stemness-related pathways.

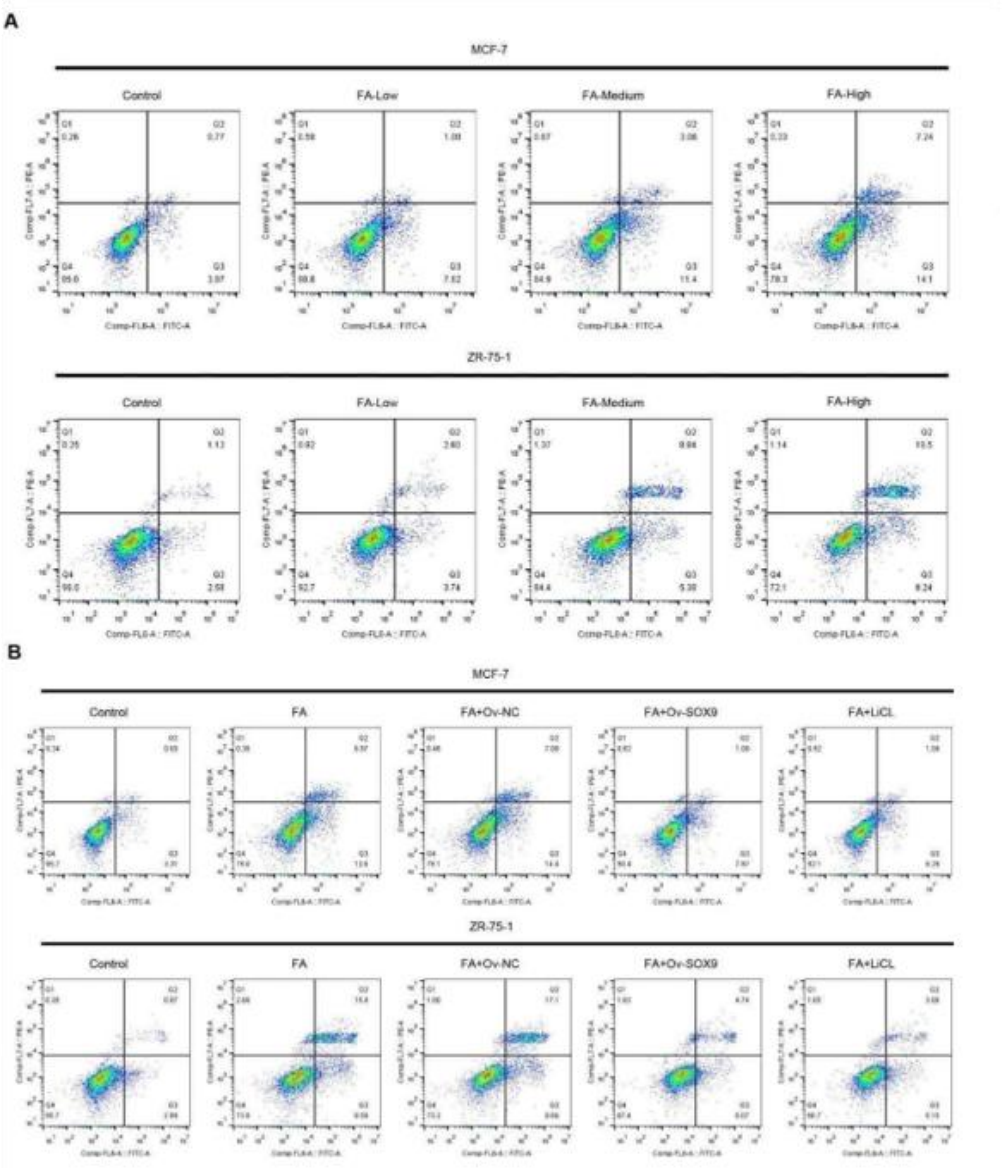

Figure S2.A: After treating MCF-7 and ZR-75-1 cells with varying doses of FA, cell apoptosis was detected using flow cytometry with PI staining. **B.** After treating MCF-7 and ZR-75-1 cells with folic acid (FA), flow cytometry was used to detect the apoptosis of MCF-7 and ZR-75-1 cells that over-expressed SOX9 or were treated with LiCl**.**

4.3. FA inhibits LABC Cell Survival Via SOX9/Wnt/β-Catenin/ c-Myc Axis

To elucidate the molecular mechanism underlying FA-mediated suppression of LABC progression, we focused on the SOX9/ Wnt/β-catenin/c-Myc signaling axis. Western blot analysis revealed that FA treatment significantly downregulated the protein expression of SOX9, β-catenin, and c-Myc in both MCF-7 and ZR-75-1 cells in a dose-dependent manner (Figure 3A), suggesting that FA disrupts this oncogenic pathway at the molecular level. The aforementioned results suggest that FA inhibits the viability of LABC cell lines. In order to further explore whether SOX9 is a key regulator in this context, we successfully overexpressed SOX9 by transfection of Ov-SOX9 plasmid (Figure S3), and the expression of SOX9, β-catenin, and c-Myc proteins were all elevated (Figure 3B). Overexpression of SOX9, or the usage of a Wnt/β-catenin pathway agonist (LiCl) in conjunction with high-dose FA treatment, partially restored the proliferative capacity of MCF-7 and ZR-75-1 cells (Figure 4C, D, S1E). Furthermore, the overexpression of SOX9 or LiCl treatment resulted in a decrease in the number of apoptotic deaths in both cell lines treated by FA (Figure 4A, S2), while concurrently increasing the BCL2/Bax ratio (Figure 4B), suggesting that SOX9 plays a pivotal role in regulating the viability of LABC cells by FA.In the treatment of FA, we performed colony formation assays and spheres formation assays on MCF-7 and ZR-75-1 cells overexpressing SOX9 or treated with LiCl. The results demonstrated that the overexpression of SOX9 and the activation of the β-catenin/c-Myc pathway were capable of partially inhibiting the FA-induced damage to the

proliferation of the LABC cell lines (Figure 5C) and partially restoring the stemness of FA-damaged cells (Figures 5D, E). In order to further confirm whether the upregulation of SOX9 and the activation of the β-catenin/c-Myc pathway promote LABC progress, we demonstrated by scratch assay and Matrigel invasion assay that the upregulation of SOX9 or the treatment of LiCl further acceleration of migration and invasion rate (Figure 5A, B). The partial restoration of LABC cell line stemness by SOX9 overexpression and activation of the β-catenin/c-Myc pathway further suggested that SOX9 and the β-catenin/c-Myc pathway are key mediators of FA-impaired function in LABC cell lines. Since high expression of SOX9 activates the β-catenin/c-Myc pathway, it is inferred that FA impairs the survival, proliferation, metastasis, and invasion of LABC cell lines by down-regulating SOX9 expression and thereby inhibiting the activation of the β-catenin/c-Myc pathway. This finding lends further support to the notion that FA has the potential to serve as a novel targeting agent for LABC.

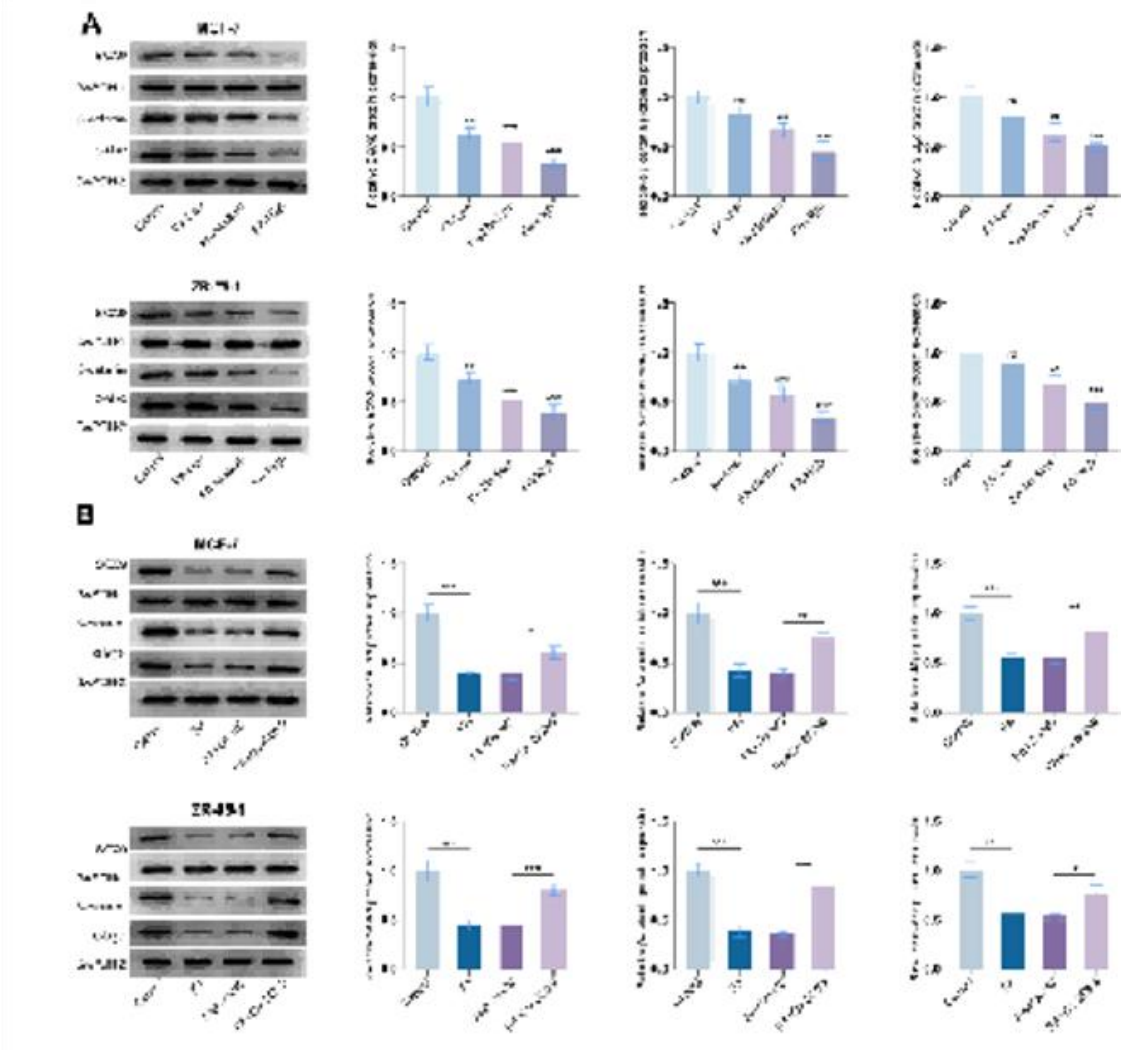

Figure 3: FA inhibits activation of Wnt/β-catenin/c-Mycsignaling by suppressing SOX9 expression.

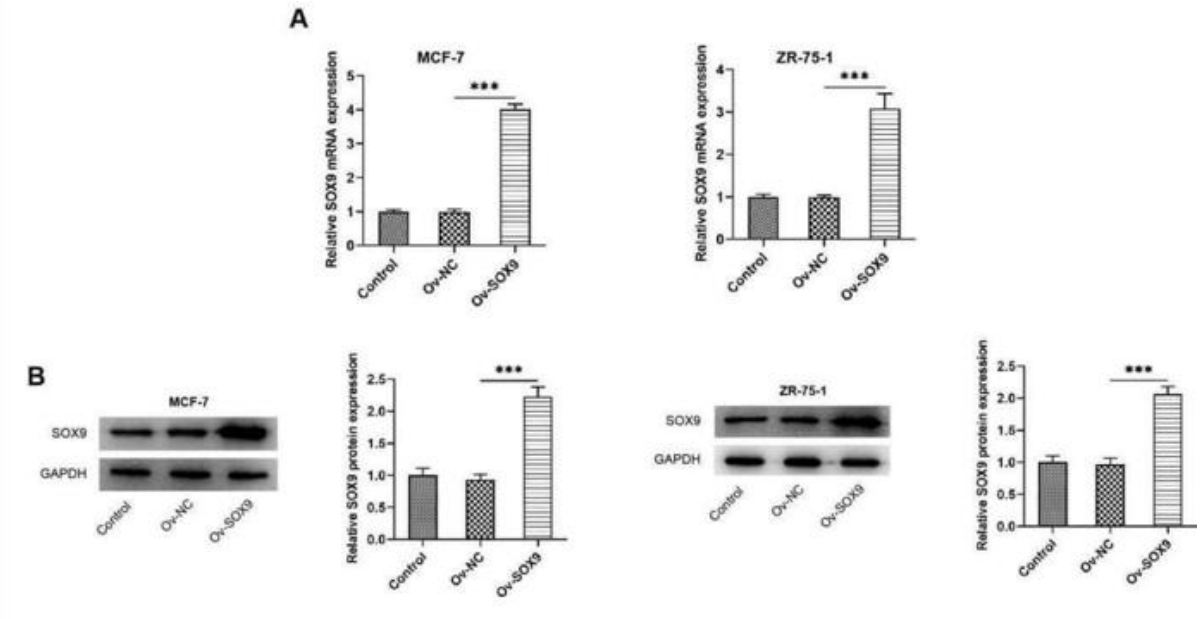

Figure S3. A: The expression of *Sox9* in MCF-7 and ZR-75-1 cells after infection was confirmed using real-time quantitative PCR.**B.**The expression of Sox9 in MCF-7 and ZR-75-1 cells after infection was detected by western blot assay.***is *P*<0.001.

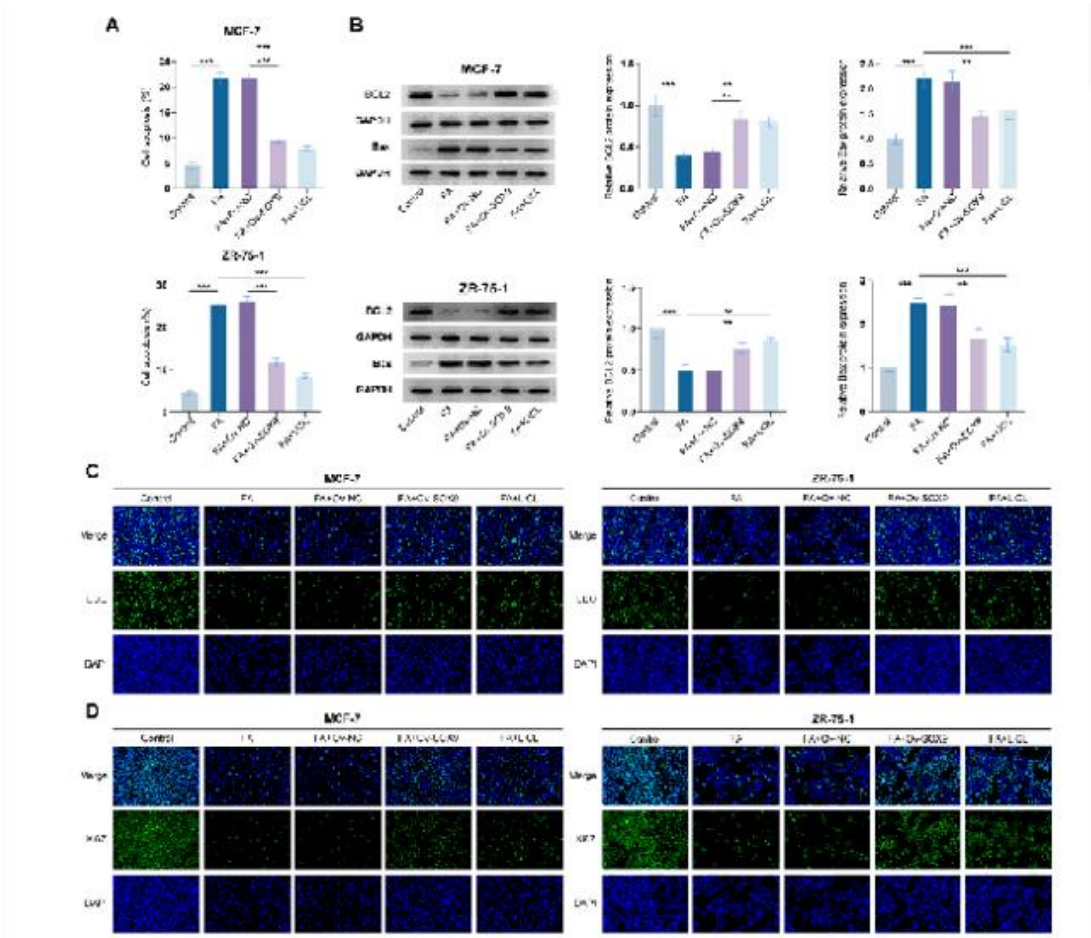

Figure 4: FA impairs the viability of LABC cell lines by inhibiting SOX9 expression in LABC cell lines.

4.4. FA Inhibits LABC Formation and the Activation of the Wnt/β-Catenin/c-Myc Signaling Pathway Mediated by SOX9 in Vivo

To further explore the inhibitory effect of FA on LABC in vivo, a xenograft model of LABC was established by subcutaneous inoculation of MCF-7 and ZR-75-1 cells in nude mice. The administration of 10 mg/kg FA was executed once the tumor volume reached 100 mm3, and the tumor size was meticulously recorded at three-day intervals. As time progressed, the subcutaneous tumor volume of mice treated with FA underwent a substantial reduction (Figures 6B). On day 18, the size of isolated tumor tissue was documented, and it was observed that the tumors of mice treated with FA were smaller than those of the control mice (Figure 6A). This finding suggests that FA inhibited the growth of LABC in vivo. The expression of SOX9, NANOG, CD44, β-catenin, and c-Myc was confirmed to be suppressed in the tumor tissues of mice treated with FA compared with the control group by western blot (Figure 9C), which further demonstrated that FA inhibited the activation of SOX9-mediated Wnt/β-catenin/c-Myc pathway. Immunofluorescence staining revealed that the fluorescence intensity of Ki67 was attenuated and that of caspase3 was enhanced in mouse tumor tissues after administration of FA (Figure 9D), demonstrating that FA promotes apoptosis of LABC cells with good prognosis. The present findings demonstrate that FA effectively suppresses LABC tumor growth in vivo by inhibiting the SOX9-mediated Wnt/β-catenin/c-Myc signaling axis and enhancing apoptosis, thereby highlighting its therapeutic potential for targeting aggressive breast cancer phenotypes.

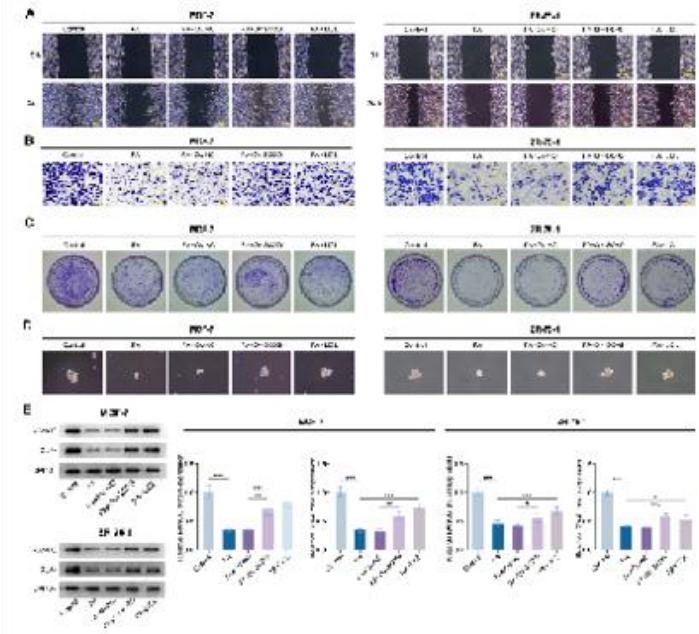

Figure 5: FA inhibits metastatic potential and stemness of LABC cells by suppressing SOX9 expression.

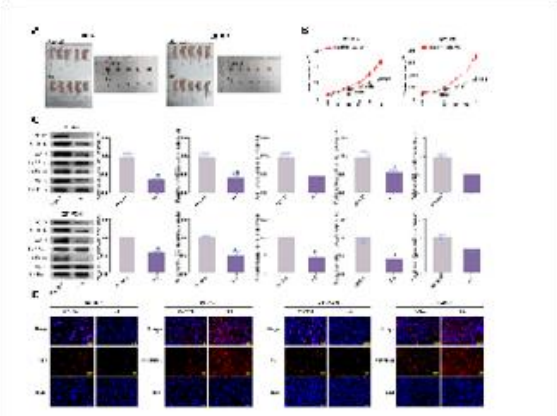

Figure 6: FA inhibits LABC growth and suppress activation of Wnt/β-catenin/c-Mycsignaling*in vivo.*

5. Discussion and Conclusion

Our study focuses on the precise function of FA in LABC (a prevalent subtype of breast cancer), and investigates the potential mechanisms of action. Through a combination of in vitro and in vivo experiments, we substantiate that FA significantly curtails the proliferation, migration, invasion, and stem cell characteristics of LABC cells, while concomitantly inducing apoptosis. Mechanism studies demonstrate that FA reduces SOX9 expression, which in turn inhibits the Wnt/β-catenin signaling pathway and its downstream effector c-Myc, thereby blocking key drivers of tumor progression. In vivo, the efficacy of FA shows a reduction in tumor size, damaged cell stemness, an enhancement of apoptosis, and a downregulation of SOX9 and Wnt/β-catenin/c-Myc signaling. Nevertheless, safety of FA in vivo requires further investigation. COX-2 expression has been reported to as a factor associated with poor prognosis in breast cancer[26], and the pharmacological inhibition of COX-2 has been widely demonstrated to exert a protective effect on breast cancer development[27,28]. As a prodrug inhibiting COX-2 enzymes, FA has been revealed to suppress breast cancer cell migration and invasion[23], repress colorectal cancer inflammation, proliferation, invasion, migration while aggravating cell apoptosis[17]. In addition, FA has been proved to obstruct thyroid cancer cell proliferation[22]. In addition to the inhibitory role of FA in cervical cancer tumor growth[21], recent literature has exposed that FA can retard basal-like breast cancer lung metastasis in mice[23]. These comprehensive review of the extant literature reveals a consistent pattern of findings, which supports the hypothesis that FA is a potential anti-tumor drug in human malignancies. Our study proves that FA attenuates the capabilities for proliferate, migrate and invade and induces tumor cells apoptosis in LABC cells. In addition, FA was found to result in a decrease in the expression of Ki67, a proliferation marker. Concurrently, we observed that FA can increase the expression of pro-apoptotic Bax, while decreasing the expression of anti-apoptotic BCL2. In vivo, we observed that FA can diminish the proliferation of tumors, concomitant with the repression of Ki67 expression, and the upregulation of cleaved caspase3 expression in murine tumor tissues. Combined with these results, FA is noticed to play the anti-oncogenic role in LABC.Cancer stem cells (CSCs) also known as tumor-initiating or tumor-propagating cells, are a relatively rare stem-like cell subpopulation within the tumor. They are capable of self-renewal and multilineage differentiation. Furthermore, previous studies have indicated that CSCs play a pivotal role in the tumor initiation, progression, spreading and resistance to therapy of tumors[29]. A substantial number of evidences have been demonstrated that breast cancer CSCs can undergo evolution over time and space, resulting in significant genotypic, phenotypic, and functional heterogeneity[30,31]. NANOG, was first discovered in 2003 as a critical factor which underlies pluripotency in both the inner cell mass and embryonic stem cells (ESCs). NANOG has been observed to be capable of maintaining ESC self-renewal independent of leukemia inhibitory factor (LIF)/STAT3 (or STAT4/8). It is expressed in pluripotent embryonic cells, derivative ESCs and the developing germ line of mammals and birds[32]. CD44, a cell-surface glycoprotein molecule, has been found that is widely expressed in the cell membrane where it contributes to cell adhesion, motility and trafficking[33]. As a stemness-related protein, CD44 is associated with CSC maintenance in BC[34]. We observed that FA weakened the colony-forming ability, decreased the number and size of the spheres and depleted NANOG and CD44 expressions in LABC cells. The impacts of FA on NANOG and CD44 expressions were similarly observed in the tumor tissues of mice, which further broaden the suppressive role of FA in LABC cell stemness.SOX9 is a well-recognized master regulator of cell fate determination and tissue morphogenesis, which has been demonstrated to be negatively modulated by FA in cerebral ischemia reperfusion injury[24]. As a pivotal transcription factor, SOX9 has been identified to regulate multiple signaling pathways during cancer progression[6]. Importantly, SOX9 can induce the aberrant activation of the classical oncogenic Wnt/β-catenin signaling pathway in breast cancer[11]. In the presence of Wnt ligands, β-catenin is isolated from the destruction complex and accumulates in the cytoplasm, which further translocates into the nucleus. In this particular instance, β-catenin engages in an interaction with the T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) transcription factors, thereby promoting the expression of downstream targets, including c-Myc and cyclin D1. In the current study, it was discovered that FA could reduce SOX9, β-catenin and c-Myc expressions in LABC cells and in the tumor tissues. Furthermore, after SOX9 was overexpressed, β-catenin and c-Myc expressions were all raised in FA-treated LABC cells, suggesting that FA can inactivate Wnt/β-catenin signaling pathway mediated by SOX9. SOX9 has been regarded as a master regulatory in cell fate in breast cancer. This regard is due to its ability to facilitate a number of processes, including the initiation, proliferation, migration, invasion, angiogenesis, stemness, drug resistance, and apoptosis, as well as its ability to modulate the immunomodulation and microenvironment[7]. The aberrant activity of the Wnt/β-catenin signaling pathway has been demonstrated to play a pivotal role in various hallmarks of breast cancer, including uncontrolled proliferation, metastatic spread, immune microenvironment regulation, stemness maintenance, therapeutic resistance, and the shaping of patient phenotype[35-37]. In our study, we found that LiCl or SOX9 overexpression partially compensated the impacts of FA on the phenotypes of LABC cells, further proving SOX9 is a key factor of Wnt/β-catenin pathway regulated by FA. These findings suggest that the blockade of SOX9/Wnt/β-catenin/c-Myc signaling might be accountable for the anti-oncogenic potential of FA in LABC.While this study provides novel insights into the

anti-tumor effects of FA in LABC, several limitations should be acknowledged. The absence of clinical data limits direct clinical applicability. In addition, while no acute toxicity was observed in short-term experiments, the chronic effects of FA on normal tissues and its safety in combination therapies require systematic evaluation.Our research uncovers that FA suppresses the occurrence and development of LABC both in vitro and in vivo via downregulating SOX9 to block Wnt/β-catenin/c-Myc signaling pathway. The establishment of the critical tumor-suppressing role of FA, has made as FA a potential drug targeting LABC. Also, our findings pave the way for a better understanding of the mechanism of the role of FA in LABC.

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