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Inhibition of AR Restores the Sensitivity to Fulvestrant in ER-Positive Breast Cancer Cells

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Endocrine therapy has been widely used in treating estrogen receptor (ER)-positive breast cancer which accounts for nearly 75% of breast cancer. Though endocrine therapy has shown great potency, acquired resistance occurs. Fulvestrant, the first selective ER down-regulator (SERD), was confirmed to completely suppress ER α and notably efficient. However, it has been observed that some ER-positive breast cancer would eventually develop unresponsiveness and acquired resistance to it, resulting in poor outcome. Several mechanisms have been proposed to be involved in antiestrogen resistance, such as activated pathways and altered expression of microRNAs. Of note, it is postulated androgen receptor (AR) which is often observed in most primary and metastatic breast cancer, might be a crucial protein associated with the efficacy of Fulvestrant, due to its common co-expression and intricate crosstalk with ER. In this study, we demonstrate that treatment suppressing ER would shift tumors from ER dependence to AR dependence, resulting in resistance to Fulvestrant and tumor growth. A blockade of AR could increase the sensitivity to Fulvestrant, and dual inhibition of AR and ER would be more effective than either drug alone, which might provide an insight into choosing optimal therapy for patients with AR-expressing ER-positive breast cancer. Furthermore, activated AR could also upregulate its downstream factor SOX9 to promote cell migration and proliferation.

Keywords: Fulvestrant resistance; estrogen receptor-positive breast cancer; Androgen receptor; SOX9; targeted combination therapy

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Abbreviations: ER, estrogen receptor; SERM, selective ER modulator; AI, aromatase inhibitor; SERD, selective ER downregulator; AR, androgen receptor; DHT, Dihydrotestosterone; EMT, epithelial-mesenchymal transition; LTED, long-term estrogen-deprived

Currently, breast cancer is the one of the leading causes among all cancer-related deaths [1]. Despite of early diagnosis and comprehensive treatment, especially the development of endocrine therapy, the mortality of breast cancer has still remained relatively high [1]. Highly heterogeneous as breast cancer is, different subtypes have different biological

behaviors and clinical prognosis. Among all the subtypes, estrogen receptor (ER)-positive breast cancer is the most common one [2, 3]. ER forms estradiol/ER complex with estradiol, mediating gene transcription via receptor dimerization and nuclear translocation, further activating multiple pathways to promote cell growth [4, 5].

Endocrine therapy or antiestrogen therapy is the treatment that targets on ER. Current widely used antiestrogen agents include selective ER modulators (SERMs, e.g., Tamoxifen) which could interfere with estrogen signaling, aromatase inhibitors (AIs, e.g., Letrozole) that function as estrogen biosynthesis inhibitors, and selective ER downregulators (SERDs, e.g., Fulvestrant) which could degrade ER [6]. However, despite the potent anti-tumor activity, the resistance in ER-positive breast cancer to these agents is inevitable, eventually leading to tumor progression and metastasis. Whereas mechanisms regarding SERMs and AIs resistance have been widely studied including pathway activation and altered microRNA, what leads to Fulvestrant resistance is still waiting to be elucidated [6].

Intriguingly, androgen receptor (AR) which is often observed in most primary and metastatic breast cancer, is considered a crucial protein associated with the efficacy of endocrine therapy due to its common co-expression with ER [7]. Cumulative studies have shown in ER-positive breast cancer patients, AR expression is not only a favorable prognostic factor in terms of tumor size, tumor grade, Ki-67 levels and response to treatment, but also is associated with a better outcome [8-11]. However, on the contrary, there also exists evidence indicating higher level of AR might suggest a worse prognosis. A cohort study of 192 patients with ER-positive breast cancer showed that a high AR/ER ratio of at least 2.0 predicted a higher risk of failure of tamoxifen [12]. It has also been found that AR overexpression could contribute to the development of hormonal therapy resistance via various mechanisms [12-14]. For example, overexpressed AR, by enhancing tamoxifen's effect as an agonist, induced the ER-downstream signaling pathways which subsequently drove cell growth and proliferation [13, 15]. Besides, independent of ER, Dihydrotestosterone (DHT), an AR agonist, was found to induce epithelial-mesenchymal transition (EMT) in MCF-7 cells [16], which might precipitate resistance to hormonal therapy. Herein, we hypothesized that treatment suppressing ER turns tumors from ER dependence to AR dependence, resulting in resistance to antiestrogen agents and activation of AR pathway.

Materials and Methods

Cell Lines and Reagent

Breast cancer cell line MCF-7 was purchased from the Chinese Academy of Science Committee Type Culture Collection Cell Bank (Shanghai, China) in 2018. Fulvestrant-resistant (FulR) breast cancer cell line was purchased from AXOL Bioscience in 2018. MCF-7 cells were cultured in DMEM medium (Gibco, USA) with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin. FulR cells were cultured in phenol red-free DMEM/F12 medium (KeyGEN BioTECH, China) containing with 100 nM

Fulvestrant, supplemented with 1% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin, with additional 2.5 mM GlutaMAX™ (Gibco, USA) and 6 ng/mL insulin. Cells were then incubated in a humidified atmosphere with 5% CO₂ at 37°C. Fulvestrant, Bicalutamide and DHT were purchased from MedChem Express.

Microarray Analysis

Samples for microarray analysis were MCF-7 and FulR cells. Total RNA was extracted from MCF-7 and FulR cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. RNA was amplified to produce double-stranded cDNA which was subsequently purified and transcribed to cRNA using T7 Enzyme Mix. Then, CbcScript II reverse transcriptase and Klenow Fragment enzyme was used to produce cDNA and labeling.

The labeled and fluorescent cDNA was then hybridized in a Agilent hybridization oven overnight. Array was then scanned and acquired array images were analyzed by Agilent Feature Extraction software V11.0.1.1. Normalization and quality control were performed using the Agilent GeneSpring GX software version 12.1 (Agilent Technologies). mRNAs with fold-change ≥ 2.0 and a P-value ≤ 0.05 were considered significantly different. Benjamini-Hochberg was used to correct for multiple statistical tests.

Lentivirus Transfection and Transient Transfection

To investigate the role of SOX9, we transfected MCF-7 with recombinant lentivirus of SOX9 from Applied Biological Materials (ABM; Zhenjiang, China) as well as an empty vector control. Cells were harvested after 72 hours of transfection for further study. To select the stable transfected SOX9-overexpressed cells, Puromycin was used and the concentration for selection was 2 $\mu\text{g}/\text{mL}$.

The shRNA sequences were synthesized by GenePharma (Shanghai, China) as followed: shSOX9-1, CCACCTTCACCTACATGAA; shSOX9-2, CAGCGAACGCACATCAAGA; shNC, AGTGCACGTGCATGTCCTA. Cells were transfected with lentiviral vectors following standard procedures from manufacturer.

Cell Survival Assay

Cell viability was measured using Cell Counting Kit-8 (CCK-8) (MedChem Express, China). Briefly, cells were seeded into a 96-well plate at a density of 5×10^3 cells/well with 6 repeats for each condition. After 24 hours, the cells were treated with Fulvestrant, Bicalutamide, DHT or the combination of Fulvestrant and Bicalutamide for another 48 or 72 hours. Then, the supernatants were removed and 100 μl medium with 10 μl CCK-8 was added into each well of the

plate and incubated at 37°C. After 2 hours, the absorbance value (OD) of each well was measured at 450 nm using an ELX-800 spectrometer reader (Bio-Tek Instruments, Winooski, USA).

Cell Apoptosis Assay

Cells transfected with SOX9 and/or treated with medication were incubated for 48 or 72 hours, then harvested by trypsinization (no EDTA) and washed three times with phosphate-buffered saline (PBS). For apoptosis analysis, the cells were resuspended in 500 µl of 1 × binding buffer and stained with 5 µl of Annexin V and 5 µl of PI for 15 minutes at room temperature in the dark. Assays were tested in triplicate.

Wound Healing Assay

Cells were seeded in six-well plates and incubated to generate confluent cultures. Using 200 µl sterile pipette tips, wounds were scratched in the cell monolayer and rinsed with PBS. Subsequently, the cells were cultured in serum-free medium for 48 hours. The migration of the cells was photographed at time 0 and 24 hours.

Quantitative Real-Time PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. Then, cDNA was synthesized in a reaction mix containing 2 mg RNA and PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan). Quantitative RT-PCR was implemented with Step One (TM) sequence detection system (Applied Biosystems Inc, Milan, Italy) using validated primers and SYBR Premix Ex Taq II (Takara, Japan). The cycle number at threshold (Ct) was used to quantify the transcript levels of genes of interest. Primer sequences were listed below: β-Actin, forward 5'-CTGGAACGGTGAAGGTGACA-3' and reverse 5'-AAGGGACTTCCTGTAACAACGCA-3'; ESR, 5'-TGCCTGGCTAGAGATCCTGA-3' and reverse 5'-CTCCACCATGCCCTCTACAC-3'; AR, forward 5'-GCAACTCCTTCAGCAACA-GC-3' and reverse 5'-GACACCGACACTGCCTTACA-3'; SOX9, forward 5'-GACTTCTGAA-CGAGAGCGAGA-3' and reverse 5'-CCGTTCTTACCGACTTCCTC-3'; GREB1, forward 5'-ATCAGCTGCTCGGACTTGCTG-3' and reverse 5'-TGAGCTCCGGTCCCTGACAGATG-3'; PGR, forward 5'-GGCGAGAGGCAACTTCTTTC-3' and reverse 5'-CATCTGCCCACTG-ACGTGTT-3'; TFF1, forward 5'-GTGTACGCCCTCCAGT-3' and reverse 5'-GGACCCC-ACGAACGGTG-3'; FKBP5, forward 5'-CATGCAGCGGTGATTGAGTA-3' and reverse 5'-GTTGAGAAAGGCAGCAAGGAG-3'. Each sample was tested in triplicate and normalized for β-Actin expression.

Western Blotting

The whole protein was extracted by RIPA buffer supplemented with protease and phosphatase inhibitors. 20 µg cell lysates were loaded per lane and resolved by sodium dodecylsulfate-polyacrylamide (SDS-PAGE) electrophoresis and blotted onto polyvinylidene fluoride (PVDF) membranes. Following 2-hour blockade with 5% skim milk in tris-buffered saline/0.1% tween-20, the membranes were incubated with the primary antibodies overnight at 4°C and with a horseradish peroxidase-conjugated secondary antibody (1:10000) for 2 hours the next day. Antibodies for detecting SOX9 (#82630) and β-Actin (#4967) were from Cell Signaling Technology. Antibodies for detecting ER (ab32063) and AR (ab74272) were from Abcam. Results from at least two separate experiments were analyzed.

Immunofluorescence

MCF-7 and FulR cells were plated on eight-well chamber slides (Lab-Tek Products, Illinois, USA) and were stained according to immunofluorescence as described [17]. Immunofluorescence images were obtained using an Olympus.

Statistical Analysis

All the in vitro experiments were performed in triplicate. Statistical tests were performed using GraphPad Prism version 7 (GraphPad Prism, San Diego). Data were analyzed using either F-test or Student t-tests. $P < 0.05$ was considered statistically significant.

Results

Fulvestrant-resistant ER-positive breast cancer cell growth was associated with AR expression

To establish Fulvestrant-resistant (FulR) ER-positive cell line model, we cultured MCF-7 cells in long-term estrogen-deprived (LTED) condition with Fulvestrant. Dose-response experiments showed that a significant 4.7-fold increase in the Fulvestrant IC₅₀ in FulR cells compared with MCF-7 cells (MCF-7 Fulvestrant IC₅₀ = 5.73×10^{-5} M, FulR Fulvestrant IC₅₀ = 27.02×10^{-5} M, $P = 0.0486$) (Fig. 1a).

Since ER is the key driver of tumor development in ER-positive breast cancer [4], we first assessed its role in cell growth in FulR cell line model. The results showed that in FulR cells, the expression of ER was lower than those in MCF-7 cells. Correspondingly, the representative downstream proteins of ER pathway such as GREB1, PGR and TFF1, were substantially less activated (Fig. 1b), indicating FulR cells proliferation might not be dependent on ER.

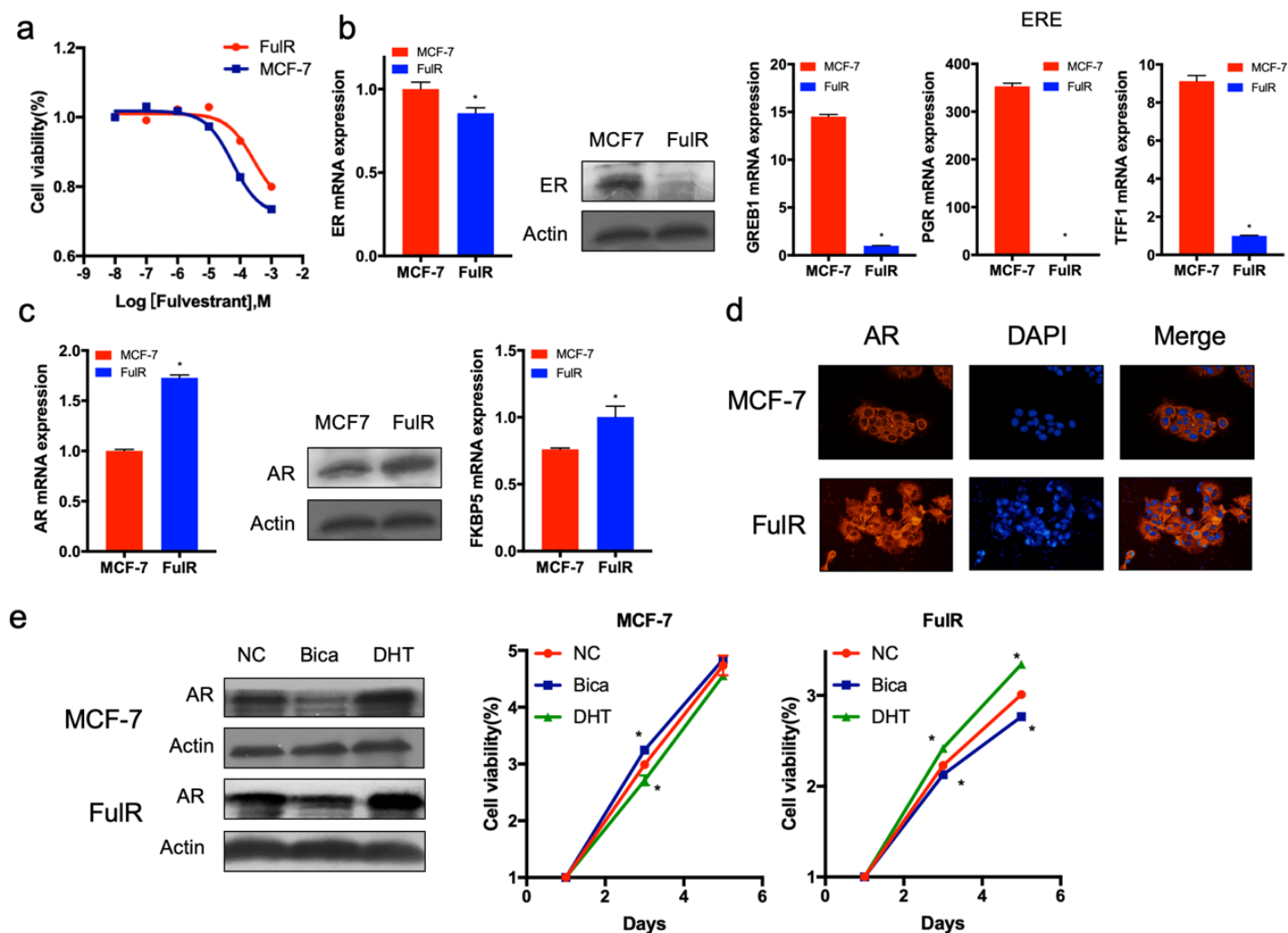


Figure 1. Fulvestrant-resistant ER-positive breast cancer cell growth depends on AR. (a) Dose-response curves for Fulvestrant treatment in MCF-7 and FulR cells. MCF-7 Fulvestrant $IC_{50} = 5.73 \times 10^{-5}$ M and FulR Fulvestrant $IC_{50} = 27.02 \times 10^{-5}$ M, $P = 0.0486$. (b) PCR and western blotting showed a decreased ER and ER downstream proteins GREB1, PGR and TFF1 in FulR cells compared with MCF-7 cells. (c) PCR and western blotting showed an increased AR and AR downstream protein FKBP5 in FulR cells compared with MCF-7 cells. (d) Immunofluorescence of AR in MCF-7 and FulR cells. (e) Western blotting confirmed the decreased or increased expression of AR by 20 μ M Bicalutamide or 100 nM DHT. Cell viability detected by CCK-8 on days 1, 3, and 5 in MCF-7 and FulR cells treated with either 20 μ M Bicalutamide or 100 nM DHT, compared to untreated cells.

Based on the evidence shown in previous studies that AR could confer antiestrogen therapy resistance [12, 13], as well as its intricate interaction with ER [18], we subsequently determined the impact of AR on cell growth and found AR was highly expressed in FulR cells, with an activated downstream pathway, shown by upregulated FKBP5 which is a typical AR-binding element (Fig. 1c). As is known, nuclear AR is canonical in terms of cell proliferation promotion [19]. Therefore, immunofluorescence was performed to detect the nuclear translocation of AR. Compared with MCF-7 cells, in which scarce nuclear AR was found, FulR cells had a higher expression of both cytoplasmic and nuclear AR (Fig. 1d), which was

consistent to aforementioned upregulated AR downstream pathway.

To better understand whether proliferation of FulR cells is related with AR, Bicalutamide (an AR antagonist) and DHT (an AR agonist) were used to treat both MCF-7 and FulR cells. Identical to what has been discussed in preclinical studies [11], different roles of AR were found in the two cell lines. In Fulvestrant-sensitive MCF-7 cell line, activation of AR contributed to growth inhibition, while in FulR cells, activated AR led to cell proliferation (Fig. 1e). Thus, we verify that cell proliferation in FulR cells might rely on the activation of AR instead of ER. As a consequence,

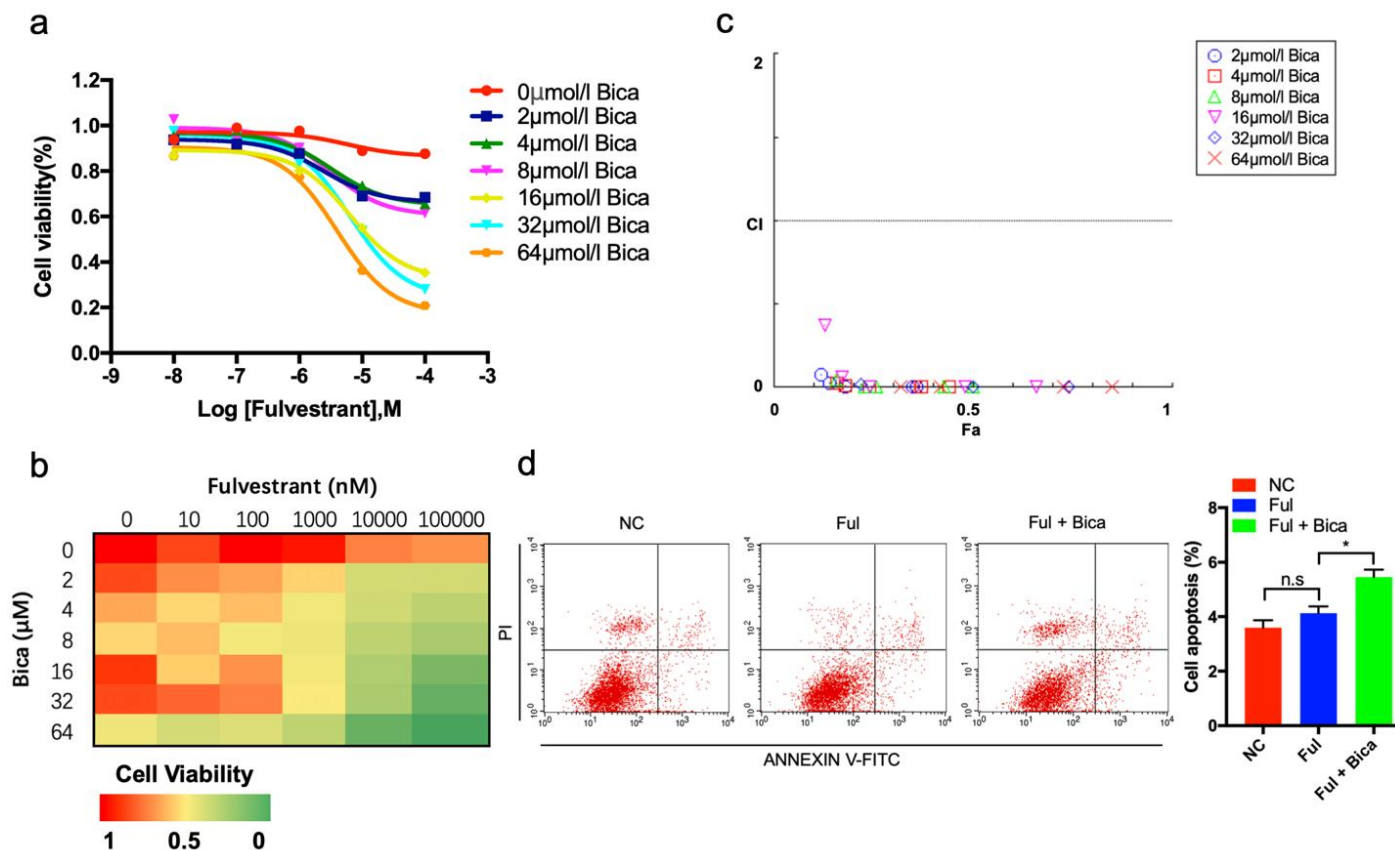


Figure 2. Inhibiting AR restores cell sensitivity to Fulvestrant. (a) Dose-response curves of FulR cells treated by 1×10^{-8} - 1×10^{-4} M Fulvestrant with or without a constant dose of Bicalutamide for 48 h. (b and c) Dose matrix and CI vs. Fa plot (Combination index vs. Fraction affected) of 1×10^{-7} - 1×10^{-3} M Fulvestrant in combination with 2 - 64 μ M Bicalutamide for 48 h. The CI values were calculated by CompuSyn Software, with a value below 1 considered synergistic. (d) Apoptosis analysis of untreated FulR cells and FulR cells after treatment of 100 nM Fulvestrant for 72 h or combined 20 μ M Bicalutamide with 100 nM Fulvestrant for 48 h.

resistance to Fulvestrant develops.

Inhibition of AR re-sensitized Fulvestrant-resistant ER-positive breast cancer cells

Since we have demonstrated a shift to AR dependence could reduce the sensitivity to Fulvestrant in ER-positive breast cancer, we next hypothesized inhibition of AR could reverse Fulvestrant resistance and restore the sensitivity in cells. Via the dose-response experiments, we found that blocking AR by Bicalutamide significantly increased responsiveness to Fulvestrant in a dose-dependent manner in ER-positive breast cancer cells (Fig. 2a). The dose matrix showed that combination of Bicalutamide with Fulvestrant was more effective than either agent alone, showing a synergistic interaction (Fig. 2b and 2c). To further characterize the effects of the combination, we performed apoptosis assay in FulR cells treated with Bicalutamide, Fulvestrant or the combination. Consistent with our previous results, addition of AR inhibitor to Fulvestrant triggered higher cell apoptosis compared with either

treatment alone in FulR cells (Fig. 2d). Taken together, inhibition of AR could help Fulvestrant-resistant cells regain sensitivity to Fulvestrant in a dose-dependent manner.

SOX9 might be involved in AR-related EMT.

Furthermore, we characterized FulR cells and observed that the morphology of FulR cells. Different from the morphology of Fulvestrant-sensitive MCF-7 cells, FulR cells exhibited a mesenchymal-like appearance (Fig. 3a). Western blotting showed decreased expressions of cell adhesion molecules and increased expressions of mesenchyme-related proteins (Fig. 3a), suggesting EMT was promoted in FulR cells. In line with the morphological and molecular changes, the enrichment of Gene Ontology (GO) terms derived from RNA microarray also revealed substantial changes of cell adhesion-related functions (Fig. 3b).

Based on prior literature that showed AR could promote

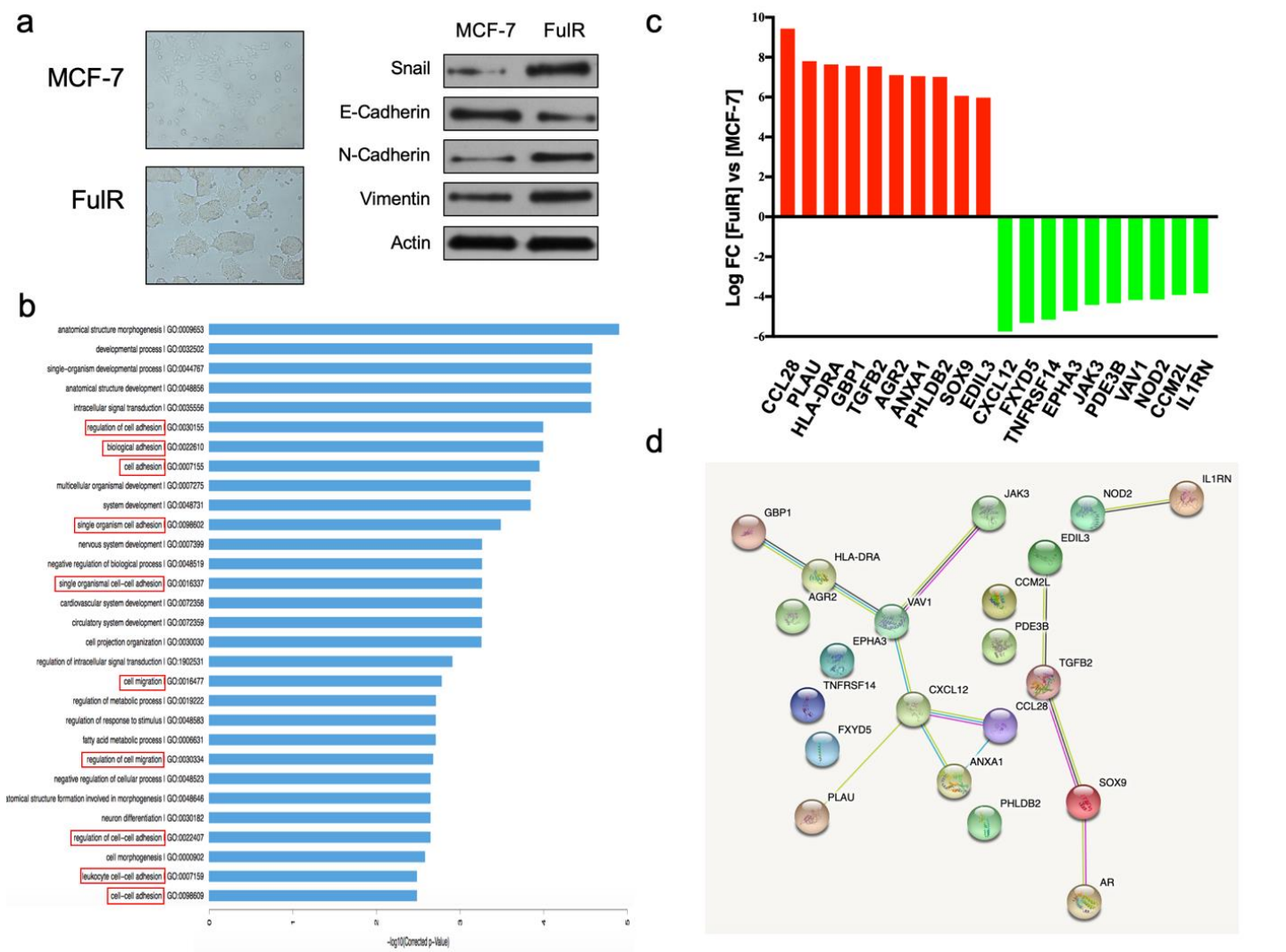


Figure 3. RNA microarray and String prediction results indicate SOX9 might be involved in AR-related EMT promotion. (a) Brightfield microscopy pictures of 2D culture of MCF-7 and FulR cells. Magnification, 10 \times . Western blotting of EMT-associated proteins. (b) Top 30 significantly enriched GO terms related with biological process in FulR cells, MCF-7 as control. (c) Top 10 up- and down-regulated proteins associated with cell adhesion regulation. (d) Prediction of the 20 proteins that might interact with AR using String prediction algorithm (<http://string-db.org>).

EMT [16], we next investigated the underlying mechanisms of AR in EMT and selected top ten up- and down-regulated proteins involved in regulation of cell adhesion (Fig. 3c). Using the String prediction algorithm (<http://string-db.org>), we found SOX9, which was upregulated by more than 6-fold change, was predicted to have an interaction with AR (Fig. 3d).

SOX9, upregulated by AR, promotes EMT and cell proliferation

Previous studies have proved that AR could regulate SOX9 in prostate cancer [20]. To further investigate the relation between AR and SOX9 in ER-positive breast cancer, first we validated the results from RNA microarray

and confirmed increased mRNA and protein expressions of SOX9 in FulR cells (Fig. 4a). Then, we overexpressed SOX9 in Fulvestrant-sensitive MCF-7 cells and found cell migration in SOX9-overexpressed MCF-7 was increased (Fig. 4b), consistent with the previous findings that AR could promote EMT [16].

To determine if SOX9 is also regulated by AR in breast cancer, we treated MCF-7 cells with Bicalutamide or DHT. Western blotting showed a decreased SOX9 expression in Bicalutamide-treated FulR cells and an increased SOX9 expression in DHT-treated FulR cells (Fig. 4c). Then, by knocking down SOX9 in FulR cells, we found that silencing SOX9 did not have an impact on AR expression, showing that AR expression might not depend on SOX9

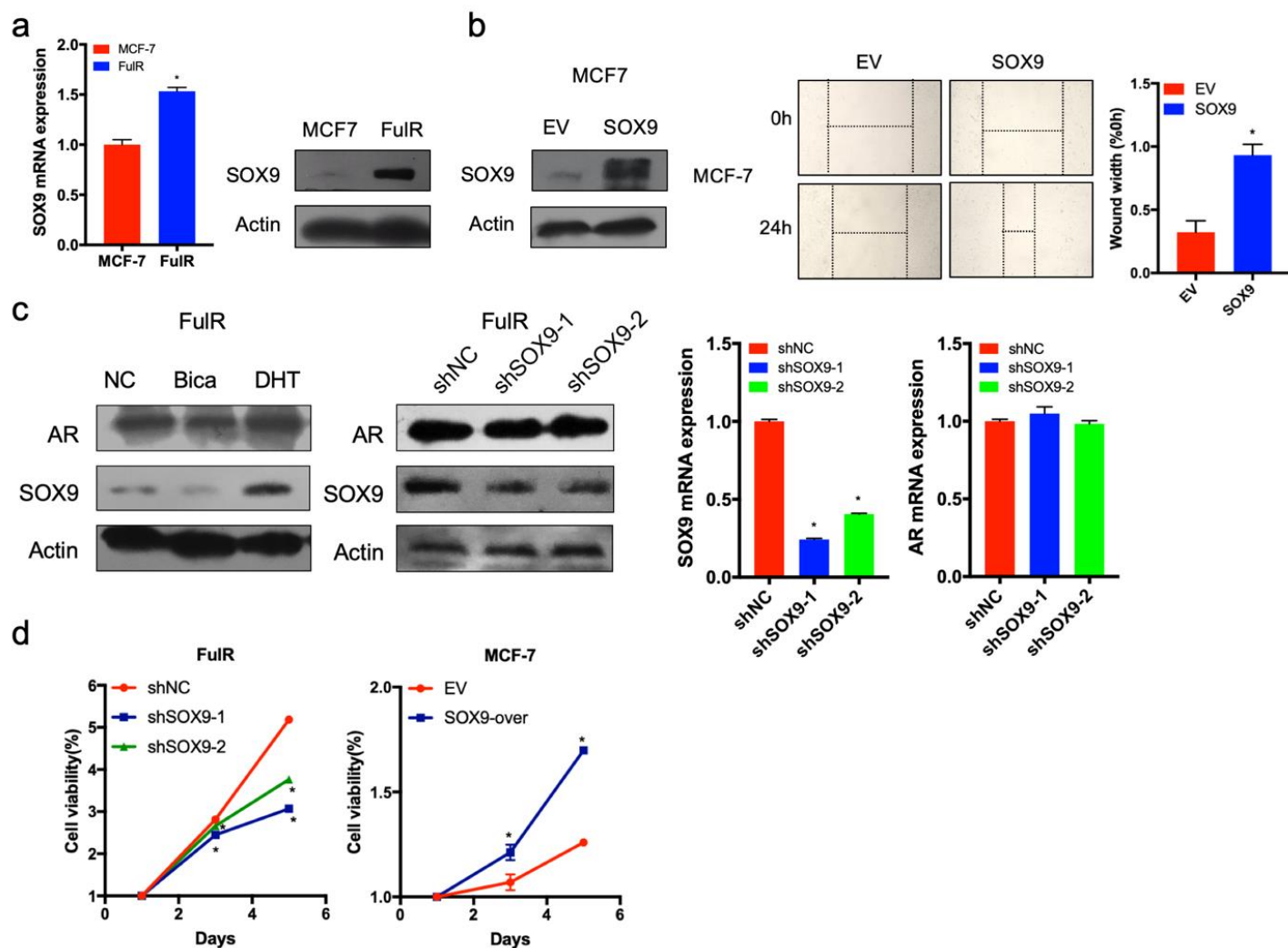


Figure 4. AR upregulates SOX9 to facilitate EMT and cell proliferation. (a) PCR and western blotting of SOX9 in MCF-7 and FulR cells. (b) Western blotting and cell migration in MCF-7 cells transfected with SOX9 or an EV control. (c) Western blotting of AR and SOX9 in FulR cells after treatment with 20 μ M Bicalutamide or 100 nM DHT for 48 h, compared to untreated FulR cells. PCR and western blotting of AR and SOX9 in FulR cells with SOX9 silencing using two different shRNAs or an shRNA control (shNC). (d) Cell viability detected by CCK-8 on days 1, 3, and 5 in FulR cells after silencing SOX9 with two different shRNAs or an shRNA control (shNC). Cell viability detected by CCK-8 on days 1, 3, and 5 in MCF-7 cells overexpressed with SOX9 or an EV control.

(Fig. 4c). Shortly, SOX9 might function as one of the important downstream elements of AR pathway in ER-positive breast cancer.

As a transcription factor, SOX9 has been demonstrated to regulate tumor growth [21]. In Figure 4d, we assessed whether proliferation of FulR cells is contingent on SOX9. As a result, knockdown of SOX9 in FulR cells, compared with control, markedly inhibited cell growth, while overexpression of SOX9 largely promoted cell growth in MCF-7 cells.

Discussion

Potent as endocrine therapy is in treating ER-positive breast cancer, however, the resistance to anti-estrogen therapeutics has gradually developed. To further improve the life span and quality for those patients, it is urgent to clarify mechanisms underlying such resistance so as to identify and develop new diagnostic methods and therapeutics.

Androgens are important for both normal breast cells and breast cancer cells, and its receptor AR is critical for breast

cancer development as well [22]. Besides, due to the complex interaction between AR and ER, the implication of AR in breast cancer has been increasingly recognized. Evidence has shown that in primary breast cancer, high expression of AR is associated with low tumor burden and a favorable tumor cell differentiation [9]. Studies evaluating AR in patients with early breast cancer also suggested that AR expression is correlated with better outcome in terms of both overall survival (OS) and disease-free survival (DFS) [8, 10]. However, controversy about the role of AR in ER-positive breast cancer exists and no consensus has been reached. Data based on ER-positive primary invasive breast cancer patients showed that more aggressive biological features and worse prognosis were observed in patients with a AR/ER ratio ≥ 2 [14]. Additionally, highly expressed AR may also have an impact on the sensitivity to tamoxifen in ER-positive breast cancer [12, 13, 15]. In our study, we identified that blocking ER in the long term using Fulvestrant would potentially induce breast cancer cell adaption from ER dependence to AR dependence. Such adaption renders it possible for ER-positive breast cancer cells to escape from inhibition by ER blockade and sustain growth.

Considering the critical role of AR in Fulvestrant resistance development, we used AR inhibitors Bicalutamide alone to treat FulR cells but found it could only moderately inhibit cell viability. However, the suppression effect by Bicalutamide was substantially improved when it was combined with Fulvestrant. Similarly, Bicalutamide also increased cell sensitivity to Fulvestrant reciprocally. We speculated that blocking AR might shift cells from ER independence to ER dependence, subsequently activating ER signaling and increasing sensitivity to Fulvestrant in FulR cells. Thus, simultaneous blockade of AR and ER is more effective than either drug alone.

We also found that the increased AR could induce EMT and enhance migration in AR-dependent and Fulvestrant-resistant ER-positive breast cancer cells, which is consistent with previous study [16]. Further experiments based on prior microarray data found that SOX9 could function as an AR downstream element to promote migration and proliferation in breast cancer cells. Intriguingly, another study demonstrated that in ER-dependent and Tamoxifen-resistant ER-positive breast cancer cells, SOX9 could be regulated by ER and then induce Tamoxifen resistance [21]. In consideration of the involvement of SOX9 in both AR and ER pathways, and the association between AR and ER, more in-depth research about the role of SOX9 in endocrine therapy resistance is anticipated.

In conclusion, our study demonstrated that AR might be

closely related with obtained resistance to Fulvestrant in ER-positive breast cancer cells. By inhibiting AR, sensitivity to Fulvestrant could be regained. Besides, AR could upregulate SOX9 and further promote cell proliferation and migration in AR-dependent ER-positive breast cancer cells.

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Conflicting interests

The authors have declared that no conflict of interests exist.

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