TGFα-EGFR pathway in breast carcinogenesis, association with WWOX expression and estrogen activation

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Abstract
WWOX is a tumor-suppressive steroid dehydrogenase, which relationship with hormone receptors was shown both in animal models and breast cancer patients. Herein, through nAnT-iCAGE high-throughput gene expression profiling, we studied the interplay of estrogen receptors and the WWOX in breast cancer cell lines (MCF7, T47D, MDA-MB-231, BT20) under estrogen stimulation and either introduction of the WWOX gene by retroviral transfection (MDA-MB-231, T47D) or silenced with shRNA (MCF7, BT20). Additionally, we evaluated the consequent biological characteristics by proliferation, apoptosis, invasion, and adhesion assays. TGFα-EGFR signaling was found to be significantly affected in all examined breast cancer cell lines in response to estrogen and strongly associated with the level of WWOX expression, especially in ER-positive MCF7 cells. Under the influence of 17β-estradiol presence, biological characteristics of the cell lines were also delineated. The study revealed modulation of adhesion, invasion, and apoptosis. The obtained results point at a complex role of the WWOX gene in the carcinogenesis of the breast tissue, which seems to be closely related to the presence of estrogen α and/or β receptors.

Keywords Estrogen receptor · Breast cancer · WW domain-containing oxidoreductase · nAnT-iCAGE · MCF7 cells · BT20 cells · MDA-MB-231 cells · T47D

Introduction

Worldwide, breast cancer (BC) is not only the most often diagnosed tumor type among women, but also is the main causative of death due to oncological disorders (World Cancer Report et al. 2014). Data published in March 2019 by the Global Cancer Observatory (GLOBOCAN) show that there were 2 088 849 cases of breast cancer diagnosed, and 626 679 deaths due to this type of tumor in the year 2018 (Sharma 2021).

BC is a clinically and morphologically highly heterogeneous disease that differs in prognosis and response to treatment. Advances in the molecular and genetic field enhanced understanding of molecular pathways and genetic changes underlying breast cancer carcinogenesis, leading to more advanced and targeted BC management. One of the first breakthroughs in its treatment was made over a hundred years ago when it was found to be the hormone-dependent type of cancer (Beatson 1896). Indeed, up to now, hormone therapy is the oldest systemic management of BC. This phenomenon was for the first time associated with the presence of estrogen receptors in the late 1960s, upon their discovery (Toft and Gorski 1966; JENSEN 1962). Further studies of ER receptor confirmed not only its association with physiologic developmental and differentiation changes undergoing in the mammary gland but also with hormone sensitivity of breast cancer (Heldring et al. 2007).

ER receptors belong to a large family of nuclear receptors; however, for many years, it was believed that only one ER exists. In 1996, new estrogen-specific receptor was discovered, and was termed ERβ, and already known receptor ERα (Kuiper et al. 1996; Mosselman et al. 1996). Both have a similar structure, though are encoded by different genes—ESRI and ESR2—found at different chromosomal locations (chromosome 6 and 14, respectively). They also differ in
tissue distributions, post-translational modifications, and cellular localization in healthy and malignant tissues (Burns and Korach 2012).

Currently, ERα is considered a key player in BC outgrowth and in developing anti-hormonal resistance. On the other hand, the exact role of ERβ in breast cancer is not yet fully understood; however, its discovery possibly explains ambiguous estrogen action in ERα-negative tissues.

Signal transduction through ER begins with binding with its ligand (estrogens), which results in a conformational change in receptor structure, enabling direct binding with specific DNA sequences, named estrogen response elements (ERE), promoting transcription of target genes. Apart from that classical pathway, ERs can interact with transcription factors Fox/Jun activating transcription at AP-1 sites (Kushner et al. 2000) or SP-1 at GC-rich (Sp1) promoter elements (Saville et al. 2000). What is interesting, unlike for ER, signal transduction via AP-1 is opposite for both estrogen receptors—in case estradiol, ERα binding transcription activation is observed, whereas for ERβ, it is inhibited (Paech et al. 1997). Transcription of genes can be also influenced without ligand binding, through ER receptor phosphorylation (Heldring et al. 2007).

Estrogens are crucial for the development and maintenance of the reproductive system and proper sexual activity. But their biological range of action is much broader—encompasses both sexes and pertains to cardiovascular, musculoskeletal, immune, and central nervous systems (Heldring et al. 2007). Structurally, ligand-binding domains of both receptors display 53% homology but they differ only by two amino acid residues. This is sufficient for distinctive affinity towards various ligands (Abderrahman and Jordan 2018). Almost equal and very high affinity for ERα and ERβ was observed in the case of 17β-estradiol (E2). Other endogenous estrogens exhibit much lower affinity than estradiol, yet estrone (E1) binds preferentially to ERα, whereas estriol (E3) to ERβ (Kuiper et al. 1997).

ERα status is the most important predictor of breast cancer prognosis. ERα as a prognostic marker in BC is associated with increased survival in ERα-positive tumors, due to sensitivity to anti-estrogen therapy (Burns and Korach 2012).

The WWOX tumor suppressor gene coding region is located on chromosome 16q23.3–24, which is recognized as the one of the most common fragile sites of genetic change in breast cancer (Chen et al. 1996). Moreover, a decline in WWOX expression corresponds to shorter disease-free survival (DFS) and overall survival (OS) of BC patients (Guler et al. 2005), and gene was proposed as a potential prognostic marker in this tumor type (Pluciennik et al. 2010; Aldaz et al. 2014).

WWOX protein possesses enzymatic SDR (short-chain dehydrogenase/reductase) domain, with the coenzyme NAD(H)/NADP(H) binding site and two WW domains at the NH2 terminus (Bednarek et al. 2000, 2001). In Eukaryotes, proteins with WW domains are involved in numerous cellular processes, associated with cellular signaling, protein transport, transcription, and RNA processing. Indeed, for WWOX, there were over 40 partners identified, among them several transcription factors: such as P73 (Salah et al. 2010), AP2-gamma (Aqeilan et al. 2004a), c-Jun (Gaudio et al. 2006), DVL-2 (Bouteille et al. 2009), and RUNX2 (Aqeilan et al. 2008).

Analysis of the WWOX expression pattern in normal human tissues showed the highest expression in the testis, prostate, and ovary, and significantly lower in other tissues (Bednarek et al. 2000). In another wide range analysis, the highest expression was observed in the fallopian tubes, ovaries, mammary gland epithelial cells, endometrial, prostate, testes, liver, stomach, salivary glands, adrenal gland, thyroid, parathyroid, pituitary, cerebellum, and brain cells (Nunez et al. 2006). The highest expression observed in hormone-regulated tissues, additionally with the presence of an enzymatic dehydrogenase/reductase domain, suggested that its protein product may act as steroid dehydrogenase involved in the metabolism of steroid hormones (Bednarek et al. 2000; Duax and Ghosh 1997). Later on, the WWOX gene product was classified more precisely as 17β-hydroxysteroid dehydrogenase (Kallberg et al. 2002; Marijanovic et al. 2003).

It was shown that WWOX fusion protein was active in the presence of both NAD+ and NADP+ towards steroid substrates such as 17β-estradiol, estrone, progesterone, and testosterone; however, its purification without losing activity has proved unsuccessful, suggesting that WWOX protein is in vivo present only in liaison with other cellular proteins (Saluda-Gorgul et al. 2011).

That WWOX protein participates in sex steroid metabolism, which was reinforced in animal studies (Chang et al. 2005). WWOX knocking-out in mice leads to deficiency in Leydig cell formation, untraceable levels of serum testosterone, decreased theca cell proliferation, and undersized ovarian follicles, which all point to the significant role of WWOX in steroidogenesis and proper gonadal function. In the same study, researchers revealed differential expression of 15 steroidogenesis-associated genes associated with WWOX absence (Aqeilan et al. 2009).

WWOX was correlated with estrogen receptor status in several studies. Immunohistochemical staining of archived BC specimens (n = 97) revealed reduced WWOX staining was more common in tumors with less favorable ER-negative (ER−) status (p = 0.033). Whereas 30.4% of cancer cases with normal WWOX staining showed negative or almost negative for estrogen receptors, 56.3% ER− tumors and less than 25% ER-positive (ER+) tumors exhibited low WWOX expression (Guler et al. 2009). Another study confirmed those results in a group of 16 human normal breast...
epithelium samples, 15 DCIS tumors, and 203 invasive breast cancer cases. A significant correlation ($p = 0.0054$) between WWOX level and ER status was observed, with the proportion of 46% ER$^-$ cases to 27% ER$^+$ breast carcinomas being negative for WWOX. When WWOX-deficient cases were analyzed together with nearly negative, the difference became more convincing ($p = 0.003$), with 73% of ER$^-$ cases to 51% of ER$^+$ cases (Nunez et al. 2005).

Level of WWOX expression correlated both with estrogen and progesterone receptor status in a study, where 132 breast cancer cases were evaluated utilizing quantitative real-time RT-PCR. The ER$^+$ tumors had WWOX expression several times higher than the ER$^-$, which was also true for PR$^+$ cancers vs. PR$^-$ and ER$^+$ PR$^+$ tumors in relation to ER-PR$^-$ cases (Pluciennik et al. 2006). Positive and high relation of WWOX expression and with ER ($p < 0.001$) and PR ($p = 0.001$) was further confirmed by Guler G et al. through immunohistochemistry staining of tissue microarrays constructed from 837 breast cancer blocks (Guler et al. 2009).

Association of WWOX and hormone receptors was shown on an animal model as well. WWOX heterozygous C3H mammary tumor-susceptible mice (WWOX(C3H)/+−) exhibited loss of estrogen and progesterone receptors (Abdeen et al. 2011).

As we known, one allele of WWOX is deleted in more than 70% breast cancers and increasing WWOX expression in breast cancer cell lines reduce aggressiveness in animal xenografts. Therefore, we decided to find how WWOX expression differentiation affects global gene expression changes in association with estrogen.

Herein, we have tried to assess the relationship between the presence of estrogen receptors and the WWOX gene in breast cancer cell lines. The study was based on MCF7 and T47D estrogen-responsive cell lines, as well as MDA-MB-231 and BT20 estrogen-unresponsive cell lines, for which depending on native expression, the WWOX gene was either induced by retroviral transfection (MDA-MB-231, T47D) or silenced with shRNA (MCF7, BT20), and changes in the phenotype were estrogen-stimulated. We have employed nAnT-iCAGE methodology for high-throughput gene expression profiling.

MCF-7 breast cancer cell line is ER$^+$-positive, which exhibits high proliferative potential under estrogen influence. Moreover, it shows one of the highest WWOX gene expressions among all studied breast cancer cell lines. On the other hand, BT-20 is recognized as a ER$^-$-negative cell line, although estrogen receptor mRNA is expressed, but with exon 5 deletion (Castles et al. 1993). Additionally, WWOX expression for BT-20 is relatively high. T47D cell line possesses functional estrogen alpha receptor and exhibits a low expression of the investigated gene. Conversely, MDA-MB-231 cell line is well acknowledged as being ER$^+$, but ER$^-$, and is an excellent model for WWOX gene studies for one of the lowest native expressions of this gene (Vladusic et al. 2000).

Under the influence of 17β-estradiol presence, we have also assessed biological characteristics of the cells, such as adhesion to extracellular matrix (ECM) proteins, invasion, proliferation, and apoptosis. These findings may implicate in the prediction of BC outcomes arising from biological consequences of the crosstalk between WWOX and ER during breast carcinogenesis. It also reveals new therapeutical opportunities due to identified aberrations of signaling pathways (e.g., EGFR) in response to ER signaling.

### Materials and methods

#### Cell lines and culture conditions

T47D and MCF7 breast cancer cell lines were obtained from the American Type Culture Collection (ATCC), and grown according to the manufacturer’s protocol in RPMI-1640 Medium (Gibco) with 2 mM L-glutamine (Gibco), 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate supplemented with 10% heat-inactivated fetal bovine serum (Gibco), antibiotics (Gibco, 0.05 mg/mL penicillin; 0.05 mg/mL streptomycin; 0.1 mg/mL neomycin), and human insulin (ITS insulin/transferrin/selenium acid Premix, BD Biosciences), in a humidified atmosphere containing 5% CO2 at 37 °C.

BT20 breast carcinoma cell line was obtained from CLS (Cell Lines Service GmbH, Eppelheim, Germany) and grown according to the manufacturer’s protocol in DMEM: Ham’s F12 medium (1:1 mixture) supplemented with 2 mM L-glutamine (Gibco), 10% heat-inactivated fetal bovine serum (Gibco), and antibiotics (Gibco, 0.05 mg/mL penicillin; 0.05 mg/mL streptomycin; 0.1 mg/mL neomycin) in a humidified atmosphere containing 5% CO2 at 37 °C.

Mammary gland adenocarcinoma cell line MDA-MB-231 was obtained from the American Type Culture Collection (ATCC) and was cultured in Advanced DMEM (Dulbecco’s modified Eagle’s medium) with 10% heat-inactivated fetal bovine serum, 4500 mg/L glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, and antibiotics (penicillin, 50 U/mL; streptomycin, 50 µg/mL; and neomycin, 100 µg/mL), in a humidified atmosphere of 5% CO2 at 37 °C.

For estrogen influence assays, cells were incubated for 48 h in above-listed culture media deprived of phenol red, supplemented with 10% charcoal stripped serum (Gibco). Cells were then treated either with 10$^{-8}$ M 17β-estradiol (Sigma) or vehicle control (i.e., equivalent amounts of ethanol). Apart from untreated (time 0), samples were collected at 24 h after treatment.
Stable retroviral transfection

The WWOX gene cDNA was introduced into T47D and MDA-MB-231 breast cancer cells via retroviral transfection, in which PT67 packaging line was used to produce the pLNCX2 retroviral vector with the cloned WWOX gene. T47D and MDA-MB-231 cells were grown up to 40% confluence and subsequently infected with 106 CFU/mL viruses suspended in culture medium supplemented with polybrene as a vehicle (8 μg/mL, Sigma-Aldrich). After 24-h incubation, stable transfectants were selected with 400 μg/mL G418 (Sigma-Aldrich) for 3 weeks, and transfection efficiency was confirmed by western blot analysis. A pool of stable transfectants was used for further biological experiments.

Stable lentiviral transduction

MCF7 and BT20 breast cancer cells were plated in 24-wells plate at 4 × 104 cells in 1 mL growth medium 24 h prior to transduction. Subsequently cells were incubated in serum- and antibiotic-free media supplemented with polybrene (6 μg/mL and 4 μg/mL, respectively) with GIPZ lentiviral WWOX shRNA particles (Thermo Fisher Scientific, catalog no. VGH5523, clones ID: V2LHS_115633, V2LHS_255229 and V2LHS_411864), and GIPZ Non-silencing Lentiviral shRNA Control (catalog no. RHS4348) at MOI 5 (multiplicity of infection) for 24 h according to the manufacturer’s protocol.

Transduction efficiency was determined by the green fluorescence protein (GFP) positive cells, by means of fluorescence microscopy using a FLoid Cell Imaging Station (Life Technologies Corp.). Twenty-four hours after infection, 2 μg/mL puromycin (Life Technologies) was added for cell selection. Stable cell lines were obtained after 2 weeks. A pool of stable transfectants was used for further biological experiments.

Western blot analysis

For cell lysis and total protein extraction, RIPA protein extraction buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) and protease and phosphatase inhibitor cocktails (Sigma-Aldrich) was used. Protein concentration was determined by the Bradford method (Bio-Rad Laboratories). Sixty micrograms of extracted proteins was resolved on 10% SDS-PAGE (Bio-Rad Laboratories) and transferred on PVDF membranes (Sigma-Aldrich) by semi-dry blotting (Whatman; Biometra GmbH, Göttingen, Germany). The membranes were blocked for 1 h in 5% non-fat milk (Sigma-Aldrich) and incubated at 4 °C, overnight with a primary antibody (1:200 goat polyclonal anti-WWOX, Santa Cruz Biotechnology, cat. no. sc-20529). Subsequently, the membranes were washed with Tris-buffered saline-Tween 20 (TBST) buffer and incubated with the secondary antibody (1:15,000 anti-goat IgG, Sigma-Aldrich, cat. no. A4187) conjugated with alkaline phosphatase for 1 h at room temperature, followed by washes with TBST and developed with Novex® AP Chromogenic Substrate (Life Technologies). As a reference, mouse anti-GAPDG monoclonal antibody was used (1:1000; Santa Cruz Biotechnology, cat. no. sc-59540). The relative protein amount was assessed with ImageJ (Schneider et al. 2012) based on the integrated density of the bands.

nAnT-iCAGE library preparation and sequencing

Libraries for next-generation sequencing (NGS) were prepared according to nAnT-iCAGE protocol (Murata et al. 2014). Briefly, 5 μg of total RNA was reverse transcribed using random N6 primer with 3-bp anchor and SuperScript III reverse transcriptase (Invitrogen), followed by oxidation of cap structure with sodium periodate (Sigma) and biotinylation with biotin (Long arm) hydrazide (Vector Laboratories). Subsequently, single-stranded RNA was digested with RNase I (Promega), whereas biotinylated RNA/cDNA duplexes were captured using magnetic streptavidin beads (Dynabeads® M-270 Streptavidin; Invitrogen). Unbound RNA/cDNA hybrid molecules were washed away, and single-stranded cDNA released by treatment with RNase H (Invitrogen), RNase I (Promega), and heat, followed by both end linker ligation—barcoded 5’ linker and 3’ linker. Eight different barcodes were used in the procedure. Second strand was synthesized by nAnT-iCAGE 2nd primer, which anneals to 5’ linker. Final products were assessed for their quality by means of Agilent Bioanalyzer High Sensitivity DNA kit (Agilent Technologies) and their concentration by Quant-iT™ PicoGreen® dsDNA Kit (Invitrogen). Five ng libraries were sequenced on NextSeq 550 Illumina sequencer and NextSeq 500/550 High Output Kit, 75 reads. Samples for cluster generation were prepared according to (CAGE™ Preparation Kit n.d.).

nAnT-iCAGE data processing and expression analysis

Processing and analysis of sequenced nAnT-iCAGE data was done with the workflow proposed by the Data Analysis Center of the Cell Innovation Program (https://cell-innovation.nig.ac.jp/maser/Applications/CAGE-seq_en.html), employing Galaxy—an open source, web-based platform (Afgan et al. 2018). Briefly, FASTQ sequenced data files were first assessed for proper quality by FastQC tool (Andrews n.d.) and split by a barcode sequence by means of Barcode Splitter tool (Gordon n.d.). Subsequently, AfterQC tool was used for filtering, barcode trimming, error removal, and quality check of fastq files (Chen et al.
Burrows-Wheeler Alignment tool (BWA) (Li and Durbin 2009) was used for reads alignment and mapping to the human reference genome (GRCh38). The data were recorded in SAM (Sequence Alignment/Map) format, transformed to BAM (Binary Sequence Alignment Map) files and alignments sorted by means of SAMtools package (Li et al. 2009a). CAGEr R package (Haberle et al. 2015) was employed for identification of transcription start sites (TSS) and their usage frequency, raw tag count normalization, and construction of promoterome. Full genome sequences for human “BSgenome.Hsapiens.UCSC.hg38” were used (Team 2020). For each experimental point, at least $1.5 \times 10^{-6}$ transcripts mapped to the human genome were obtained. Finally, gene expression is shown as TPM (transcript counts per million transcripts mapped). Statistical analysis of differentially expressed genes was performed using NOIseq algorithm (Tarazona et al. 2015).

**ERα and ERβ target gene list** was downloaded from Harmonizome database (Rouillard et al. 2016). For ERα, 1791 ENCODE-verified target genes were analyzed and for ERβ, it was 419 ChEA-verified target genes. Differential expression between experimental points was assayed using UpSetR software (Conway et al. 2017). Gene ontology analysis was performed with ShinyGO web tool at (Ge et al. 2020).

### Proliferation and apoptosis assay

A triplex test simultaneously evaluating cellular redox potential (data not shown), proliferation (data not shown), and apoptosis of the cells was used in order to reduce population and systematic discrepancies between cell cultures. Apoptosis was assessed with the DELFIA® DNA fragmentation assay (PerkinElmer), which exploits terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL reaction) and samarium-labeled streptavidin.

Ten thousand cells/well were incubated for 48 h in culture media deprived of phenol red, supplemented with 10% charcoal stripped serum (Gibco) on a white, clear bottom 96-well plates. Cells were then treated either with $10^{-8}$ M 17β-estradiol (Sigma) or vehicle control (i.e., equivalent amounts of ethanol). Samples were collected at time 0 (untreated) and 24 h after treatment.

### Adhesion assay

Adhesion assay evaluating capability of cells to adhere to four extracellular matrix (ECM) proteins—fibronectin, collagen I, collagen IV, and laminin—was carried out in order to assess the ability of the cells to integrate into the ECM. After 48-h incubation of cells in culture media deprived of estrogens (without phenol red, supplemented with 10% charcoal stripped serum) and subsequent treatment with $10^{-8}$ M 17β-estradiol (Sigma) or vehicle control, the cells were seeded in serum free media at the density of 300 000 cells/well on the 24-well plates coated with selected ECM proteins and uncoated control (Corning® Biocoat™ 24 well clear Flat Bottom TC-Treated Multiwell Plates; cat. no. 354411, 354,408, 354,430, 354,412) and allowed to adhere for 90 min at 37 °C. Adherent cells were stained with 0.09% crystal violet, the stain extracted with 10% acetic acid and quantified colorimetrically.

### Invasion assay

The invasive potential of the investigated cells was evaluated using the colorimetric CytoSelect™ 24-well invasion assay (Cell Biolabs, Inc.). The assay contains a membrane coated with a layer of basement membrane matrix solution and allows for discrimination of invasive cells. The cells were seeded on inserts placed in a 24-well plate at a density of 300 000 cells/well and left to invade for 48 h (according to the manufacturer’s protocol). Next, the cells that crossed the membrane were dyed and their number was analyzed colorimetrically.

### Statistical analysis

Data was presented as the means ± standard error. Statistical significance between the samples was assessed using the Aspin-Welch $t$ test and recognized as being statistically significant at a confidence level $>95%$ ($p < 0.05$). All the statistical analyses were performed using Statistica 12 software (StatSoft Inc.).

### Results

**WWOX expression evaluation**

The successfulness of WWOX protein level down- and upregulation was confirmed by western blot analysis before any assays (Fig. 1). The increase in relative WWOX protein expression for T47D and MDA-MB-231 cell lines was 2.68 and 5.58, respectively, whereas a decrease of WWOX protein expression for MCF7 and BT20 cell lines was 3.89 and 6.00, respectively.

**WWOX-ER interplay in adhesion, invasion, and apoptosis**

All the cell variants, for which stable modulation of WWOX expression was observed, were treated with 17β-estradiol (or equivalent amounts of ethanol, further in text denoted as VC, standing for vehicle control) in time points ($t = 0$ h, $t = 24$ h) and sequenced. Due to low sequencing quality for T47D, this
Adhesion

As mentioned above, to confirm NGS data, adhesion to ECM proteins was assessed biologically, by means of four different ECM components—laminin, collagen I, collagen IV, and fibronectin (Fig. 2).

For the T47D cell line, after WWOX upregulation, most significant changes were observed in adhesive properties to collagen I (1.56-fold increase, \( p = 0.001 \)) and fibronectin (1.44-fold increase, \( p = 0.043 \)). A slight increase was also observed for collagen IV (1.16-fold, \( p = 0.026 \)). Similarly, overexpression of WWOX gene in MDA-MB-231 cell line resulted in increased adhesion to all investigated ECM proteins (laminin, 1.89-fold, \( p = 0.001 \); collagen I, 1.85-fold, \( p = 0.017 \); collagen IV, 1.81-fold, \( p = 0.002 \), and fibronectin, 1.54-fold, \( p = 0.005 \)). In MCF7 cell line, WWOX gene silencing reverted the relation—a decrease was observed for collagen I (2.09-fold, \( p = 0.010 \)), collagen IV (1.26-fold, \( p = NS \)), and fibronectin (1.51-fold, \( p = 0.014 \)). Interestingly, for BT20 cell line, WWOX downregulation significantly increased adhesive properties of the cells to laminin (2.27-fold, \( p = 0.002 \)) and collagen I (1.28-fold, \( p = 0.002 \)).

All above given results are summarized in Supplementary Table 1 available at GitHub repository (https://github.com/orzechmag/wwox-er), along with the other supplementary materials.

The greatest changes in adhesive properties upon estrogen treatment after WWOX gene transduction were observed for estrogen-responsive cell lines, for which a significant decrease in all of ECM proteins was detected. For T47D cell line, we have observed decrease in adhesion to laminin (1.83-fold, \( p = 0.003 \)), collagen I (1.41-fold, \( p = 3.0 \times 10^{-4} \)), collagen IV (2.19-fold, \( p = 4.6 \times 10^{-4} \)), and fibronectin (3.85-fold, \( p = 0.019 \)), whereas for MCF7 cell line, it was for laminin (1.34-fold, \( p = 0.015 \)), collagen I (3.29-fold, \( p = 1.4 \times 10^{-5} \)), collagen IV (1.59-fold, \( p = 0.03 \)), and fibronectin (2.48-fold, \( p = 0.04 \)) (Supplementary Table 2, Fig. 2).

Contrastingly for estrogen-unresponsive MDA-MB-231 cell line, WWOX gene transduction increased adhesion after 17β-estradiol treatment for all investigated proteins, but statistically significantly were only for laminin (1.46-fold, \( p = 0.017 \)).

Invasion

The epithelial-to-mesenchymal transition (EMT) is considered a crucial event in gaining by the cells’ invasive potential. Therefore, the invasive potential of the investigated cells...
was evaluated biologically by measuring the number of cells invading across the basement membrane matrix solution (Fig. 3).

In estrogen-positive cell lines—T47D and MCF7—WWOX expression modulation did not affect the invasiveness of the cells. Instead, the MDA-MB-231 cell line after WWOX gene restoration exhibited a 1.3-fold increase in invasive potential ($p = 0.0026$).

In the estrogen-responsive T47D cell line, estrogen exposure and WWOX upregulation resulted in a 1.4-fold decrease of invasion ($p = 0.0058$), whereas in the estrogen-negative MDA-MB-231 cell line, 1.5-fold increase ($p = 8 \times 10^{-6}$) in invasion was observed.

**Apoptosis**

As for adhesion and invasion, also apoptosis was evaluated in biological experiments, by measuring the degree of DNA fragmentation (DELFIA® DNA fragmentation assay, PerkinElmer). It was part of a triplex test, which additionally evaluated redox potential and proliferation (data not shown), but did not return any significant findings.

On the other hand, we have observed substantial changes in apoptotic potential of cell lines under investigation, both as a result of differential WWOX gene expression and as simultaneous with the transfection estrogen treatment (Fig. 4).

The results of apoptosis after 24-h treatment with estradiol for investigated transfection variants are equally interesting (Fig. 4). As a result of estrogen treatment, there was a statistically significant decrease in apoptosis in both estrogen-dependent cell lines, T47D and MCF7 (1.44-fold, $p = 0.0201$, and 3.11-fold, $p = 0.0133$, respectively), and an increase in apoptosis for estrogen-independent breast cancer cells, MDA-MB-231 and BT20, though only for the latter, the change turned out to be statistically significant (1.49-fold, $p = 0.0173$).
**Fig. 3** The invasiveness of breast cancer cell lines, with respect to WWOX gene expression and estrogen influence. The assay was conducted using basement membrane proteins coated inserts which create a barrier that can be passed by invasive cells (see the “Material and methods” section). Key: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.005, ****p ≤ 0.001

**Fig. 4** Apoptosis in breast cancer cell lines with respect to WWOX gene influence and estrogen treatment. The graph shows increase in apoptosis relative to the control transductions with (E2) and without (vehicle control) estradiol treatment. Key: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.005, ****p ≤ 0.001
Differential expression of ERα and ERβ target genes associated with WWOX silencing

Differential expression CAGE analysis revealed 13,076 genes expressed in three cell lines MCF7, BT20, and MDA-MB-231. For differential gene expression (DGE) analysis in BT20 and MDA-MB-231 cell lines, we excluded genes showing very low expression, and analysis was limited only to genes that showed TPM above five in any cell line analyzed in any point measured. To find expression differences between WWOX protein level and E2 treatment, we compared fold change (FC) of gene expression between a cell with low versus high WWOX protein level according to estradiol (E2) treatment versus vehicle only. Table 1 shows the number of ESR1 and ESR2 genes which expression was identified in assayed cell lines.

Supplementary Table 3 contains a list of all 13,076 transcripts identified in examined cell lines with their TPM values as well as ENCODE and ChEA ERα and ERβ target genes and their TPM expression numbers and TPM > 5 filtered and fold change values used in this analysis. Figure 5 shows analysis of ERα and ERβ target genes showing lowered or elevated (FC > 2) expression in cell line constructs with low versus high expression of WWOX after 24-h estradiol treatment. As expected, the highest expression differentiation was found in an estrogen-dependent MCF7 cell line with 147 genes expressed higher in MCF7 WWOXlow versus MCF7 WWOXhigh and 107 genes showing lowered expression respectively. A relatively low number of target genes showed differential expression in two or three different constructs (Fig. 5A). A similar effect was found for ERβ and both estrogen receptors where the largest differential expression was also found in MCF7 cells (Fig. 5B and C). Lists of estrogen target genes showing mutually exclusive expression or co-expression in MCF7, BT20, and MDA-MB-231 cell lines according to WWOX protein level (FC change WWOXlow vs WWOXhigh) after 24-h E2 treatment is enclosed as Supplementary Tables 4 (ESR1), 5 (ESR2), and 6 (ESR1a2).

Gene ontology analysis returned that most ERα target genes which estradiol differentiated expression was affected by WWOX protein can be divided into four groups according to Biological Processes ShinyGO classification: cell death, cell adhesion, signal transduction, and metabolism regulation.

The transcription of many genes is regulated by more than one transcription factor. Using ShinyGO analysis, we identified 29 TFs that co-regulate ERα target genes (FDR < 0.05). Supplementary Table 7 lists transcription factors and ERα co-targeted genes. The function of three of them can be regulated by WWOX: CREB1 and MYC directly and FOXA1 through TFAP2C-WWOX integration (Woodfield et al. 2010; Li et al. 2009b; Khawaled et al. 2019). According to our findings, CREB1 and ERα co-regulate expression of 329 genes identified in MCF7, FOXA1 309 genes, and MYC 381 genes. Interestingly, we identified 40 CREB1-ERβ co-targeted genes differentially expressed according to WWOX protein level in MDA-MB-231 cell line which is known to express ESR2 receptor (Vladusic et al. 2000).

In MCF7 cells, we found differential expression of 98 genes classified by GO to regulation of signaling (Supplementary Table 8). One of them is survivin (BIRC5) upregulated in MCF7 WWOXlow under E2 treatment. Some other genes crucial for cancer metabolism identified in regulation of signaling set (GO:0,023,051) are ERBB2, TGFA, GLI3, MUC1, BAD, BCL6, CASP4, JDP2, ROBO1, and HES1.

Our results show that WWOX differential expression together with the effects of estrogen significantly affects gene expression profiles of model breast cancer cell lines. We observed association of WWOX with expression changes of genes directly regulated by estrogen receptors. However, these changes also involve a number of signaling pathways and transcription factors which consequently indirectly regulates more genes. As a result, we observe biological changes important for the development and progression of the neoplastic disease, such as proliferation, apoptosis, adhesion, and invasion.

### Table 1

| Database    | MCF7 | BT20 | MDA-MB-231 |
|-------------|------|------|-------------|
| ERα targets total | 1791 |      |             |
| ERα targets TPM > 5 | 902  | 896  | 817         |
| ERβ targets total | 419  |      |             |
| ERβ targets TPM > 5 | 278  | 253  | 224         |
| ERα and ERβ targets total | 103  |      |             |
| ERα and ERβ targets TPM > 5 | 68   | 65   | 53          |

**Discussion**

In the study, we performed biological and global gene expression analysis of breast cancer cell lines in the context of differential expression of WWOX tumor suppressor gene in association with estrogen receptor response. Numerous reports are showing WWOX as a critical player in breast carcinogenesis and progression. There are also several studies showing the association of WWOX expression with ER status of breast cancer and cancer invasiveness. We show here changes in cell viability, apoptosis, invasion, and ECM adhesion of ER-positive and ER-negative breast cancer cell lines.
Fig. 5 Graphs show UpSetR analysis of mutually exclusive expression or co-expression genes regulated by estrogen receptors according to databases ENCODE for ERα, ChEA for ERβ, or both. Analysis was performed only for genes which calculated expression was not lower than TPM = 5 (at least at one measurement point) and expression fold change was bigger than 2 after 24-h estradiol treatment in cells showing low WWOX expression relative to high WWOX expression. Intersection size bar numbers represent a number of genes co-expressed or showing mutually exclusive expression between examined cells. In cell lines, names lowExp and highExp mean lowered or elevated expression respectively.
supporting this data with analysis of global gene expression profiles.

In the study, we have used NGS CAGE to reveal the relation between estrogen receptor and WWOX, which might shed some light on its function and significance in hormone-related tissues. We have assayed ERα (ESR1) and ERβ (ESR2) target genes according to WWOX low and high expression and estradiol treatment in breast cancer cell lines estrogen receptor alpha (ERα)-positive MCF7 which has native high WWOX gene expression in comparison with BT20 ERα-negative and high WWOX and MDA-MB-231 which is ERα-negative with low WWOX expression.

The functional analysis encompassed ShinyGO Analysis of ERα targets in MCF7 cell line revealed significant modulation of adhesion-, invasion-, and apoptosis-related genes. To confirm their significant variation, further efforts were focused on gene sets associated with identified processes. Analysis of adhesion-related gene set (GO:0,007,155) comprised of 52 genes, whereas for programmed cell death (GO:0,012,501) pathway 62 genes (list of all identified genes in Supplementary Table 8). Figures 6 and 7 show gene ontology biological processes networks for 30 most significant (FDR < 0.5) pathways of differentially expressed ERα target genes in WWOX-silenced MCF7 cells and ERβ target genes in MDA-MB-231 wild-type cells after 24-h estradiol treatment. As shown in both cell lines, major networks are associated with cell death/apoptosis, signal transduction, or EMT and invasion.

**WWOX-ER interplay in adhesion**

For adhesion, well diversifying turned out genes encoding laminins (LAMB3, LAMC1), myosin-x (MYO10), and cadherin 18 (CDH18) which are associated with breast cancer invasion and metastasis (Arjonen et al. 2014; Polyak and Weinberg 2009), collagens (COL9A, COL12A) claudin-4...
(CLDN4) was previously found to control proliferation, migration, and apoptosis of MCF7 cells (Ma et al. 2015). Increased expression of mucin MUC1 has been linked to tumor aggressiveness and autophagy-mediated chemotherapy resistance as reported using the MCF7 cell line model (Garbar et al. 2017). Interestingly, AGR2 gene expression was elevated after estradiol treatment in MCF7 and BT20 cell lines with lowered WWOX. This anterior gradient 2 protein was found to be a doxorubicin resistance regulator through hypoxia induction by the binding and stabilization of HIF1A protein (Li et al. 2015). AGR2 expression is induced by estradiol in MCF7 cells with lowered WWOX protein. Moreover, WWOX binds HIF1A in the cytoplasm, therefore regulates its transcriptional activity (Abu-Remaileh and Aqeilan 2014).

Cell adhesion is an important and complex process involved in multiple processes, such as migration, tissue remodeling, and embryogenesis, as well as invasion and metastasis during malignant transformation. All those processes can be modulated by estrogens, particularly in the mammary gland. For instance, in MCF7 cells, 17β-estradiol was found to trigger morphological changes, like a rearrangement of actin filaments and formation of cell–cell and cell-ECM adhesion plaques (DePasquale et al. 1994). There are also numerous associations between adhesion molecules, whose expression was proved significantly altered in tumor cells and estrogen receptors. To give only one example, high expression of E-cadherin is observed in ERα+, low-grade tumors (Parker et al. 2001), whereas P-cadherin is present in ERα−, high-grade tumors (Paredes et al. 2002). There are also associations between adhesion and ERβ, as its loss expression FC > 2 after 24-h estradiol treatment in cells showing low WWOX expression relative to high WWOX expression.
was found to be related to the reduction of ECM and by decreased levels of the adhesion molecules, such as E-cadherin, or integrin α2 (Forster et al. 2002).

WWOX relationship with cell adhesion was already suggested in several studies (Pluciennik et al. 2015; Gourley et al. 2009). Both our NGS results as well as biological experiments confirmed that association, additionally interrelating it with estrogen receptor signaling. Biologically, the most significant changes were observed in adhesion to ECM proteins after estrogen treatment for estrogen-responsive cell lines; however, also WWOX up- and downregulation alone resulted in significant alterations in adhesive characteristics (Fig. 1).

**WWOX and TGFα and TGFβ**

Epithelial-to-mesenchymal transition in the course of tumor progression is related to extensive morphological changes, enhanced migration, a gain of invasive potential, and loss of adherens junctions. The key event in this process is related to the loss of E-cadherin (Kang and Massague 2004; Cowin et al. 2005). The principal repressor of E-cadherin transcription is SNAI1 which expression was elevated under estradiol treatment in MCF7 WWOX-silenced cells, and also SNAI2, SIP1, and TWIST1. Estrogen interplay can begin, when one of those EMT regulators—SNAI1—is repressed by MTA3 (metastatic tumor antigen 3), which is E2 regulated (Fujita et al. 2003). An opposite mechanism was also proposed showing that in non-invasive MCF7 cancer cells, SNAI1 directly represses expression of ERα expression, thus promoting EMT (Dhasarathy et al. 2007). An analogous mechanism of ERα repression was reported for SNAI2 in human breast cancer (Bai et al. 2017). ERα can also downregulate EMT through suppression of TGFβ and NF-kB signaling pathways. In the first case, it is achieved through promoting proteasome degradation of Smad2 and Smad3 and in the latter by regulation of NF-kB subunit RelB (Guttilla et al. 2012). Additionally, it was observed that E2 downregulates E-cadherin both in normal and BC epithelial cells (Oesterreich et al. 2003). Also, hypoxia and estrogen signaling may contribute to Notch-mediated EMT (Francesco et al. 2018).

Among numerous cellular processes that WOX plays important role in, there are also reports associating it with EMT in the breast (Li et al. 2018) and endometrial adenocarcinoma (Pluciennik et al. 2015). Our NGS analysis revealed many EMT-related genes well diversifying according to WWOX expression, among others, collagens (COL12A1, COL9A2—elevated expression in MCF7 WWOX-depleted cells under E2); laminins (LAMC1, LAMB3) showing lowered expression in MCF7 WWOX-depleted with E2; and keratins: KRT18 which was upregulated in MDA-MB-231 wild-type (low WWOX) under E2 treatment. It is quite interesting because KRT18 expression is regulated by ERα, therefore, its upregulation in MDA-MB-231 cells is modulated by WWOX independently of ERα signaling. Another two keratin genes KRT8—which expression is ERα-dependent and KRT15 ERβ-dependent—were overexpressed in WWOX-depleted MCF7 cells but KRT15 showed to be downregulated in BT20, respectively. We found that tumor necrosis factor receptor superfamily member 12A (TNFRSF12A) was overexpressed under E2 treatment in WWOX-depleted MCF7 cells. This protein is involved in tissue remodeling during EMT and was found to be associated with poor survival prognosis in breast cancer patients (Yang et al. 2018). DUSP6 (overexpressed in MCF7 and BT20 WWOXlow) is associated with brain metastasis in triple-negative breast cancer (Wu et al. 2019).

G0/S2 switch protein (BT20 high expression, MCF7 low expression associated with WWOX silencing and E2) mediates the Hippo pathway and induces EMT. Silencing decreased cell proliferation, migration, and invasion of MDA-MB-231 cells (Cho et al. 2019). Another protein ANXA1 (downregulated in MC7 and BT20 E2 treated and WWOX-depleted cells) which was previously reported to regulate EMT and is associated with highly invasive basallike breast cancer phenotype (Graauw et al. 2010) was found also to contribute to trastuzumab resistance through AKT activation (Berns et al. 2016; Sonnenblick et al. 2015).

As far as biological experiments are concerned (Fig. 3), no changes in invasive potential were observed for the cell lines, where WWOX expression was downregulated (MCF7 and BT20), neither as a result of WWOX modulation nor E2 treatment. Estrogen influence on invasiveness was thought observed for T4D cells after estrogen exposure and WWOX upregulation. Most significant changes were observed for MDA-MB-231 cell line, where solely WWOX gene restoration caused 1.3-fold increase in invasive potential and 1.5-fold increase with simultaneous E2 application.

It was previously reported in several papers that TGFβ signaling is strongly associated with WWOX gene expression (Hsu et al. 2016, 2009; Khawaled et al. 2020). In our NGS experiment, we found that TGFβ2 is overexpressed under estradiol treatment in MCF7 WWOX-silenced cells through ERβ (ESR2) signaling. As was reported by us and others, another tyrosine receptor kinases pathway which depends on the WWOX expression gene is ERBB4 signaling in breast cancer cells (Bednarek et al. 2000, 2001; Nunez et al. 2005; Aqeilan et al. 2005; Iliopoulos et al. 2007).

TGFα-EGFR signaling is associated with carcinogenesis and cancer progression. Interestingly, in our experiment, we identified TGFα elevated expression associated with E2 treatment both in MCF7 and BT20 cell WWOX-depleted cells. TGFα signaling is triggered by its binding to the EGFR receptor and then TGFα is cleaved by ADAM17 metalloproteinase which showed stable expression in MCF7 and BT20 cells despite WWOX protein level. There was
Previously reported that TGFα elevated expression together with ADAM17 expression in breast cancer is associated with lymph node metastasis and worse survival prognosis (Auvinen et al. 1996; Kenny and Bissell 2007).

Among TGFα-EGFR signaling pathway proteins, some are regulated by estrogen receptors ERα and ERβ. Moreover, we found that several of those proteins expression are also modulated by WWOX. One of them is STAT5B which in our experiment shows elevated expression in MCF7 WWOX-silenced cells. It was previously reported that STAT5B ectopically expressed in MCF7 and T47D activates cell proliferation and anchorage-independent growth (Tang et al. 2010). Another protein ERα-regulated protein from TGFα-EGFR is PLCB1 which was recently identified as a metastatic breast cancer driver gene (Mirsadeghi et al. 2021). PLCB1 was also found to be overexpressed in MCF7 WWOX-silenced cells under estradiol treatment. Other genes from this pathway that we found differentially expressed in our experiments are CBLC that was reported as associated with lung cancer progression (Hong et al. 2018) and CRKL which promotes tumorigenesis, cancer cell survival, and gefitinib therapy resistance in non-small cell lung cancer (Cheung et al. 2011). We found that expression of CBLC is elevated but CRKL lowered in WWOX-depleted MCF7 cells after E2 treatment. On another hand, CRKL was overexpressed in MDA-MB-231 wild-type cells cultured with estradiol. Figure 8 shows TGFα-EGFR core members of three branches of this signaling pathway, and Table 2 shows the expression differentiation of these genes. Diagrams in Fig. 8 were prepared based on KEGG networks and signaling pathways (KEGG networks TGFA-EGFR-PLCG-PKC N00227, TGFA-EGFR-RAS-ERK N00229, and TGFA-EGFR-PI3K N00231 contributing to KEGG calcium signaling pathway hsa04020, MAP kinase signaling pathway hsa04010, and PI3K-Akt signaling pathway hsa04151, respectively). As shown, all examined breast cancer cell lines show differential expression genes of TGFα-EGFR signaling which is associated with WWOX protein level. Interestingly, TGFA expression is regulated by ERα. However, despite estradiol treatment, we found that TGFA transcription was highly elevated in MCF7 cells with a low level of WWOX protein. This together with increased expression of EGFR in those cells explains the strong tumor-suppressing activity of WWOX in breast cancer. As we see in Fig. 8, MCF7 WWOX-silenced cells also show elevated expression of TGFα-EGFR signaling core members in all three branches of this pathway when compared to MCF7 WT cells with estradiol treatment. A mentioned above, MDA-MB-231 WT cells having low WWOX expression showed lowered TGFβ mRNA level when WWOX was ectopically overexpressed. Moreover, a low level of WWOX in MDA-MB-231 cells is also associated with the TGFα-EGFR pathway several genes elevated expression which is also associated with estradiol treatment. Similar to MDA-MB-231, BT20 breast cancer cells also express variable levels of ERβ receptor (Hansen et al. 1999). Our analysis depicted in Fig. 8 shows that TGFα-EGFR pathway core gene expression is also affected both by estradiol and WWOX silencing. In all three branches of this pathway, we observed differential expression and some overexpression of TGFA under estradiol treatment in WWOX-silenced BT20 cells. To summarize, TGFα-EGFR is regulated in breast cancer mostly by estradiol through ERα regulation of TGFα expression. Our findings showed that such TGFα-EGFR carcinogenic action can be enhanced in breast cancer with low expression of WWOX tumor suppressor gene which is supposed to be a very common event in this tumor. As our experiments show, ER-positive MCF7 breast cancer cells showed increased aggressiveness when estradiol action was combined with lowered WWOX protein levels.

Going downstream of TGFα-EGFR, it was reported that JUNB may enhance EGFR transcription in MCF7 cells (Johnson et al. 2000) and we found this gene to be downregulated in MCF7 WWOX-silenced cells after estradiol stimulation but EGFR expression was elevated anyway in those cells compared to MCF7 with WWOX native level. SHC1 protein (downregulated in MCF7 WWOXlow cells) sequesters ERK inhibiting MAPK/ERK pathway involved in cancer pathogenesis (Lin et al. 2019). Finally, we found the MYC gene to be overexpressed in MCF7 and BT20 WWOX-silenced cells under estradiol which connects TGFα-EGFR and TGFβ signaling pathways.

**WWOX and estrogen-mediated apoptosis**

Both in normal and malignant mammary epithelial tissue, 17β-estradiol promotes cell proliferation by expression regulation of hormone-responsive genes involved in the cell cycle and/or apoptosis. E2 proved itself to be a potent inhibitor of apoptosis, which regulates the expression of such apoptotic proteins, as Bcl-2 and bclx(L) (Gompel 2000). On the other hand, estrogen was also found to induce apoptosis in BC cells that have been for a long-time deprived of estrogen (LTED cells) or were treated exhaustively with anti-estrogen drugs (Song et al. 2001; Jordan et al. 2005). The pro-apoptotic mechanisms of estradiol action in LTED cells are associated with the death receptors (activation of the complex of Fas death receptor and Fas ligand (FasL) (Gompel 2000)), mitochondrial pathway (release of cytochrome c from the mitochondria and downregulation of Bcl-2 (Lewis 2000)), and TGFβ signaling pathway.
et al. 2005; Song et al. 2005), as well as with repression of the anti-apoptotic factor NF-kB (Lewis-Wambi and Jordan 2009). E2 affects apoptosis also by modulating the c-Jun N-terminal kinase (JNK) signaling pathway, which might be additionally ERα-receptor status dependent. Under certain conditions, E2 induced apoptosis and increased phospho-rylation of c-jun for ER$^+$ MCF7 cells but not in MDA-MB 231 ER$^-$ cells (Altiok et al. 2007). Cellular survival might be also mediated by activation of the MAPK and PI3K/Akt signaling pathways via non-genomic action of estrogen-ER complex, which as reported interacts with c-Src protein (Liang and Shang 2013).

Our NGS analysis revealed 60 unique cell death/apoptosis-related genes (programmed cell death GO:0,012,501, apoptotic process GO:0,006,915) well diversifying WWOX expression variants, which were estrogen associated (Supplementary Table 8). One of the major apoptosis regulators is BAD pathway which several genes transcription controlled by ERα we found to be overexpressed under E2 treatment in MCF7 WWOX-depleted cells. There are BCL6, BCL9L, and BMF, the pro-apoptotic BCL2-modifying factor (Oudenaarden et al. 2018). Downstream in the caspase cascade, the estrogen controlled expression of caspase-4 was induced in WWOX-silenced MCF7 cells. CASP4 gene was previously identified as a major regulator of estrogen induced apoptosis in long-term estrogen-deprived MCF7 cells (Ariazi et al. 2011). Interestingly, despite of CASP4 expression induction by E2 in MCF7 WWOX-silenced cells, we observed significant reduction of apoptosis. This may be associated with increased expression of anti-apoptotic survivin gene (BIRC5) and JDP2 gene which is another cellular survival protein which represses AP1 TF and inhibits apoptosis (Lerdrup et al. 2005). Lastly, WWOX silencing-depended survival of cancer cells can be also associated with PRKG2 gene E2-induced expression. PRKG2 encodes cGMP-dependent protein kinase and regulates broad spectrum of cellular processes such as cell proliferation, differentiation, and cell cycle (Wang et al. 2014; Jiang et al. 2014). Another road of cell death inhibition in MCF7 WWOX-silenced cells can lead through Fn14 receptor gene (TNFRSF12A) over-expression. Fn4 is the receptor for TWEAK. The TNF-like weak inducer of apoptosis and expression of TNFRSF12A is controlled by estrogen. It was reported that TNFRSF12A

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**Table 2** Differences in expression (FC) of genes participating in three major branches of TGFα-EGFR signaling in cell line constructs with low versus high expression of WWOX after 24-h estradiol treatment

| Gene   | FC   | BT20  | MCF7 | MDA-MB-231 |
|--------|------|-------|------|------------|
| TGFA   | 2.64 | 100   | 1.16 |            |
| EGFR   | 1.04 | 1.38  | 0.48 |            |
| PLG1   | 1.38 | 1.24  | 0.53 |            |
| PLG2   | 0.96 | 0.55  | 8    |            |
| PRKCA  | 0.85 | 1.41  | 0.8  |            |
| PLCG1  |      | 0.68  | 1.34 |            |
| S1OS1  | 3.16 | 1.52  | 0.8  |            |
| S1OS2  | 1.09 | 0.79  | 1.24 |            |
| KARS   | 0.76 | 0.65  | 0.98 |            |
| NRAS   | 0.7  | 0.7   | 1.2  |            |
| ARAF   | 0.86 | 0.54  | 1.46 |            |
| BRAF   | 1.47 | 0.75  | 1.01 |            |
| RAF1   | 1.29 | 0.5   | 0.54 |            |
| MAP2K1 | 2.02 | 1.68  | 0.84 |            |
| MAP2K2 | 1    | 0.5   | 0.67 |            |
| MAPK1  | 0.98 | 0.84  | 0.9  |            |
| MAPK3  | 0.62 | 0.32  | 2.31 |            |
| ELK1   | 0.65 | 0.63  | 2    |            |
| PIK3CA | 1.06 | 1.17  | 1.68 |            |
| PIK3CB | 0.74 | 1.1   | 0.82 |            |
| PIK3CD | 100  | 1     | 0.16 |            |
| AKT1   | 2.33 | 1.73  | 0.5  |            |
| AKT2   | 0.85 | 0.47  | 1.31 |            |
| AKT3   | 100  | 1     | 0.57 |            |
| MTO1   | 0.83 | 0.62  | 1.79 |            |

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ectopically induced expression in MCF7 and T47D cells resulted in a significant induction of invasion and activation of NF-kB signaling (Willis et al. 2008).

There are also some reports suggesting association between TRADD and WWOX. All thanks to short peptide Zfra (zinc finger-like protein that regulates apoptosis), which regulates TNF-mediated cell death via interacting with TRADD and FADD, as well as JNK1, WWOX, and NF-kB during stress response (Hong et al. 2007; Dudekula et al. 2010). In a proposed model of Zfra/WWOX/TRADD apoptosis, after TNF induction, Zfra physically interacts with TRADD and WWOX and WWOX binds TRADD; therefore, both are recruited to death-inducing signaling complex (DISC) (Dudekula et al. 2010).

Apoptosis is one of numerous cellular processes WWOX has been suggested to play roles in Chang et al. (2001); Aqeilan et al. (2004b). Indeed, our NGS results stay in tune with literature reports and all identified apoptosis-related genes were proved to be in tight relationship with WWOX and co-regulated by estrogen receptors. Also, our biological experiments (Fig. 4) point at WWOX-ER association with apoptosis. In this case, we can however observe rather the modulation of the apoptosis pathway, which is WWOX gene level dependent, than straightforward relationship. Additionally, for both ERα-positive cell lines, estrogen treatment decreased apoptosis, while for estrogen-independent breast cancer cells, increase of apoptosis was noticed. One could suggest that pro-oncogenic activity of E2 is prevalent in that case; however, it is known that oncogenic effect of estrogen is mediated predominantly by ERα activation of cell proliferation-promoting or apoptosis-downregulating genes (Liang and Shang 2013). On the other hand, it was reported that ERα and ERβ can differentially modulate apoptosis-related pathways. Apoptosis-associated JNK kinase was found estrogen activated by ERβ, contrary to ERα, via which this kinase was inhibited (Razandi et al. 1999). This might be the case of our divergent results.

The modulation of adhesion, invasion, and apoptotic properties in all examined breast cancer cell lines, depending on the level of WWOX tumor suppressor gene expression, as well as the treatment of estrogen, was confirmed. The obtained results point at a complex role of WWOX gene in breast carcinogenesis, which seems to be in tight relationship with the presence of estrogen α and/or β receptors; however, further studies are required to reveal the sophisticated function of the WWOX gene.

Conclusions

As showed in our data and discussed above, WWOX in breast cancer modulates significant number of estrogen-regulated genes both ERα- and ERβ-dependent. As our data shows and was reported by others, WWOX gene silencing is associated with cancer aggressiveness, epithelial-to-mesenchymal transition, tumor metastasis, and chemoresistance. WWOX depletion changes cancer cell proliferation, survival, and apoptosis. We revealed that TGFα-EGFR carcinogenic action is being enhanced in BC cases of low WWOX expression which is supposed to be a very common event in this tumor. Additionally, ER-positive MCF7 breast cancer cells showed increased aggressiveness when estradiol action was combined with lowered WWOX protein levels.

Supplementary Information

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Author contribution

All authors contributed to the study conception, design, material preparation, data collection, and analysis. The first draft of the manuscript was written by Karolina Pospiech and Magdalena Orzechowska and Andrzej K. Bednarek commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability

The datasets generated for this study can be found in the Gene Expression Omnibus (accession number: GSE140406; https://www.ncbi.nlm.nih.gov/geo/). Supplementary materials supporting the presented findings are available at GitHub repository (https://github.com/orzechmag/wwox-er).

Declarations

Conflict of interest

The authors declare no competing interests.

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References

Abdeen SK, Salah Z, Maly B, Smith Y, Tufail R, Abu-Odeh M, Zanesi N, Croce CM, Nawaz Z, Aqeilan RI (2011) Wwox inactivation enhances mammary tumorigenesis. Oncogene 30:3900–3906. https://doi.org/10.1038/onc.2011.115

Abderrahman B, Jordan VC (2018) Steroid receptors in breast cancer. The Breast. Elsevier, Amsterdam, pp 272-281.e2

Abu-Rmeileh M, Aqeilan RI (2014) Tumor suppressor WWOX regulates glucose metabolism via HIF1α modulation. Cell Death Differ 21:1805–1814. https://doi.org/10.1038/cdd.2014.95
Afgan E, Baker D, Batut B, van den Beek M, Bouvier D, Cech M, Chilton J, Clements D, Coraor N, Gruning BA et al (2018) The galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. Nucleic Acids Res 46:W537–W544. https://doi.org/10.1093/nar/gky379

Aldaz CM, Ferguson BW, Abba MC (2014) WWOX at the crossroads of cancer, metabolic syndrome related traits and CNS pathologies. Biochim Biophys Acta 1846:188–200. https://doi.org/10.1016/j.bbcan.2014.06.001

Aliotk N, Koyuturk M, Aliotk S (2007) JNK Pathway regulates estradiol-induced apoptosis in hormone-dependent human breast cancer cells. Breast Cancer Res Treat 105:247–254. https://doi.org/10.1007/s10549-006-9451-1

Andrews S (n.d.) FastQC: a quality control tool for high throughput sequence data (version 0.11.9; 2019). Available online: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Aqeilan RI, Palamaruch A, Weigel RJ, Herrero JJ, Pekarsky Y, Croce CM (2004a) Physical and functional interactions between the Wwox tumor suppressor protein and the AP-2gamma transcription factor. Cancer Res 64:8256–8261. https://doi.org/10.1158/0008-5472.CAN-04-2055

Aqeilan RI, Pekarsky Y, Herrero JJ, Palamaruch A, Letoyski J, Druck T, Trapasso F, Han S-Y, Melino G, Huebner K et al (2004b) Functional association between Wwox tumor suppressor protein and P73, a P53 homolog. Proc Natl Acad Sci U S A 101:4401–4406. https://doi.org/10.1073/pnas.0400805101

Aqeilan RI, Donati V, Palamaruch A, Trapasso F, Kaou M, Pekarsky Y, Sudol M, Croce CM (2005) WW Domain–containing proteins, WWOX and YAP, compete for interaction with ErbB-4 and modulate its transcriptional function. Cancer Res 65:6764–6772. https://doi.org/10.1158/0008-5472.CAN-05-1150

Aqeilan RI, Hassan MQ, de Bruin A, Hagan JP, Volinia S, Palumbo T, Hussain S, Lee S-H, Gaur T, Stein GS et al (2008) The WWOX tumor suppressor is essential for postnatal survival and normal bone metabolism. J Biol Chem 283:21629–21639. https://doi.org/10.1074/jbc.M800855200

Aqeilan RI, Hagan JP, de Bruin A, Rawahneh M, Salah Z, Gaudio E, Siddiqui H, Volinia S, Alder H, Lian JB et al (2009) Targeted ablation of the WW domain-containing oxidoreductase tumor suppressor leads to impaired steroidogenesis. Endocrinology 150:1530–1535. https://doi.org/10.1210/en.2008-1087

Ariazi EA, Cunliffe HE, Lewis-Wambi SA, Willis MJ, Willis AL, Rouse P, Tapia C, Kim HR, Yerrum S, Sharma CGN et al (2011) Estrogen induces apoptosis in estrogen deprivation-resistant breast cancer through stress responses as identified by global gene expression across time. Proc Natl Acad Sci 108:18879–18886. https://doi.org/10.1073/pnas.1115188108

Arjonen A, Kaukonen R, Mattila G, Rouhi P, Högnäs G, Sihto H, Miller T, Trapasso F, Han S-Y, Melino G, Huebner K et al (2004b) JNK Pathway regulates estradiol-induced apoptosis in hormone-dependent human breast cancer cells. Breast Cancer Res Treat 105:247–254. https://doi.org/10.1007/s10549-006-9451-1

Beattson GT (1896) On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases. Trans Med Chir Soc Edinb 15:153–179

Bednarek AK, Laflin KJ, Daniel RL, Liao Q, Hawkins KA, Aldaz CM (2000) WWOX, a Novel WW domain-containing protein mapping to human chromosome 16q23.3–24.1, a region frequently affected in breast cancer. Cancer Res 60:2140–2145

Bednarek AK, Keck-Waggoner CL, Daniel RL, Laflin KJ, Bergsagel PL, Kiguchi K, Brenner AJ, Aldaz CM (2001) WWOX, the FRA16D gene, behaves as a suppressor of tumor growth. Cancer Res 61:8068–8073

Benn K, Sonnenblick A, Gennissen A, Brohée S, Hijnmans EM, Evers B, Fumagalli D, Desmedt C, Loibl S, Denkert C et al (2016) Loss of ARID1A activates ANXA1, which serves as a predictive biomarker for trastuzumab resistance. Clin Cancer Res 22:5238–5248. https://doi.org/10.1158/1078-0432.CCR-15-2996

Bouteille N, Driouch K, Hage PE, Sin S, Formstecher E, Camonis J, Liderau R, Lallemand F (2009) Inhibition of the Wnt/beta-catenin pathway by the WWOX tumor suppressor protein. Oncogene 28:2569–2580. https://doi.org/10.1038/onc.2009.120

Burns KA, Korach KS (2012) Estrogen receptors and human disease: an update. Arch Toxicol 86:1491–1504. https://doi.org/10.1007/s00204-012-0868-5

CAGE™ Preparation Kit | CAGE - a highly sensitive and precise means of gene expression analysis (2019). Available online: https://cage-seq.com/cage_kit/

Castles CG, Fuqua SA, Klotz DM, Hill SM (1993) Expression of a constitutively active estrogen receptor variant in the estrogen receptor-negative BT-20 human breast cancer cell line. Cancer Res 53:5934–5939

Chang NS, Pratt N, Heath J, Schultz L, D3e J, Carey GB, Zevotek N (2001) Hyaluronidase induction of a WW domain-containing oxidoreductase that enhances tumor necrosis factor cytotoxicity. J Biol Chem 276:3361–3370. https://doi.org/10.1074/jbc.M007140200

Chang N-S, Schultz L, Hsu L-J, Lewis J, Su M, Sze C-I (2005) 17beta-estradiol upregulates and activates WOX1/WWOXv1 and WOX2/WWOXv2 in vitro: potential role in cancerous progression of breast and prostate to a premetastatic state in vivo. Oncogene 24:714–723. https://doi.org/10.1038/ojc.2008.124

Chen T, Sahin A, Aldaz CM (1996) Deletion map of chromosome 16q in ductal carcinoma in situ of the breast: refining a putative tumor suppressor gene region. Cancer Res 56:5605–5609

Chen S, Huang T, Zhou Y, Han Y, Xu M, Gu J (2017) AfterQC: automatic filtering, trimming, error removing and quality control for Fastq data. BMC Bioinformatics 18:80. https://doi.org/10.1186/s12859-017-1469-3

Cheung HW, Du J, Boehm JS, He F, Weir BA, Wang X, Butaney M, Sequist LV, Luo B, Engelman JA et al (2011) Amplification of CRKL induces transformation and epidermal growth factor receptor inhibitor resistance in human non–small cell lung cancers. Cancer Discov 1:608–625. https://doi.org/10.1158/2159-8290.CD-11-0046

Cho E, Kwon Y-J, Ye D-J, Baek H-S, Kwon T-U, Choi H-K, Chun Y-J (2019) G0/G1 Switch 2 induces cell survival and metastasis through integrin-mediated signal transduction in human invasive breast cancer cells. Biochem Ther 27:591–602. https://doi.org/10.4062/biomolther.2019.063

Conway JR, Lex A, Gehlenborg N (2017) UpSetR: an R package for the visualization of intersecting sets and their properties. Bioinformatics 33:2938–2940. https://doi.org/10.1093/bioinformatics/btx364

Cowan P, Rowlands TM, Hatsell SJ (2005) Cadherins and catenins in breast cancer. Curr Opin Cell Biol 17:499–508. https://doi.org/10.1016/j.jocbi.2005.08.014

De Francesco EM, Maggioni M, Musti AM (2018) Crossstalk between Notch, HIF-1alpha and GPER in breast cancer EMT. Int J Mol Sci 19:2011. https://doi.org/10.3390/ijms19072011

de Graauw M, van Miltenburg MH, Schmidt MJ, Pont C, Lalai R, Karptopawiro J, Pardali E, Le Devedec SE, Smit VT, van der
Wal A et al (2010) Annexin A1 regulates TGF- signaling and promotes metastasis formation of basal-like breast cancer cells. Proc Natl Acad Sci 107:6340–6345. https://doi.org/10.1073/pnas.0913360107

DePasquale JA, Samsonoff WA, Gierthy JF (1994) 17-beta-estradiol induced alterations of cell-matrix and intercellular adhesions in a human mammary carcinoma cell line. J Cell Sci 107(Pt 5):1241–1254

Dhasarathy A, Kajita M, Wade PA (2007) The transcription factor snail mediates epithelial to mesenchymal transitions by repression of estrogen receptor-alpha. Mol Endocrinol 21:2907–2918. https://doi.org/10.1210/me.2007-0293

Duax WL, Ghosh D (1997) Structure and function of steroid dehydrogenases involved in hypertension, fertility, and cancer. Steroids 62:95–100. https://doi.org/10.1016/s0039-128x(96)00166-3

Dudekula S, Lee M-H, Hsu L-J, Chen S-J, Chang N-S (2010) Zfira is a small wizard in the mitochondrial apoptosis. Aging 2:1023–1029. https://doi.org/10.18632/aging.100263

Forster C, Makeda S, Warri A, Kietz S, Becker D, Hultenby K, Warner M, Gustafsson J-A (2002) Involvement of estrogen receptor beta in terminal differentiation of mammary gland epithelium. Proc Natl Acad Sci U S A 99:15578–15583. https://doi.org/10.1073/pnas.192561299

Fujita N, Jaye DL, Kajita M, Geigerman C, Moreno CS, Wade PA (2003) MTA3, a Mi-2/NuRD complex subunit, regulates an invasive growth pathway in breast cancer. Cell 113:207–219. https://doi.org/10.1016/S0092-8674(03)00234-4

Garbar C, Musca C, Giustianini J, Merrouche Y, Bensussan A (2017) Chemotherapy treatment induces an increase of autophagy in the luminal breast cancer cell MCF7, but not in the triple-negative MDA-MB231. Sci Rep 7:7201. https://doi.org/10.1038/s41598-017-07489-x

Gaudio E, Palamarchuk A, Palumbo T, Trappaso F, Pekarsky Y, Croce CM, Aqeilan RI (2006) Physical association with WWOX suppresses C-Jun transcriptional activity. Cancer Res 66:11585–11589. https://doi.org/10.1158/0008-5472.CAN-06-3376

Ge SX, Jung D, Yao R (2020) ShinyGO; a graphical gene-set enrichment tool for animals and plants. Bioinformatics 36:2628–2629. https://doi.org/10.1093/bioinformatics/btz931

Gompel A (2000) Hormonal regulation of apoptosis in breast cells and tissues. Steroids 65:593–598. https://doi.org/10.1016/s0039-128x(00)00172-0

Gordon A (n.d.) FASTX: A short-reads pre-processing tools (version 0.0.14; 2014). Available online: http://hannonlab.cshl.edu/fastx_toolkit/

Gourley C, Paige AJW, Taylor KJ, Ward C, Kuske B, Zhang J, Sun M, Janczar S, Harrison DJ, Muir M et al (2009) WWOX gene expression abolishes ovarian cancer tumorigenicity in vivo and decreases attachment to fibronectin via integrin alpha3. Cancer Res 69:4835–4842. https://doi.org/10.1158/0008-5472.CAN-08-2974

Guler G, Uner A, Guler N, Han S-Y, Ilipooulos D, McCue P, Huebner K (2005) Concentrated loss of fragile gene expression early in breast cancer development. Pathol Int 55:471–478. https://doi.org/10.1111/j.1440-1827.2005.01855.x

Guler G, Huebner K, Himmetoglu C, Jimenez RE, Costinean S, Volinia S, Pilarski RT, Hayran M, Shapiro CL (2009) Fragile histidine triad protein, WW domain-containing oxido-reductase protein Wwox, and activator protein 2gamma expression levels correlate with basal phenotype in breast cancer. Cancer 115:899–908. https://doi.org/10.1002/cncr.24103

Guttilla IK, Adams BD, White BA (2012) EURalpha, MicroRNAs, and the epithelial-mesenchymal transition in breast cancer. Trends Endocrinol Metab 23:73–82. https://doi.org/10.1016/j.tem.2011.12.001

Haberle V, Forrest ARR, Hayashizaki Y, Carninci P, Lenhard B (2015) CAGEpipe: precise TSS data retrieval and high-resolution promoterome mining for integrative analyses. Nucleic Acids Res 43:e51. https://doi.org/10.1093/nar/gkv054

Hanstein B, Liu H, Yancisin MC, Brown M (1999) Functional analysis of a novel estrogen receptor-beta isoform. Mol Endocrinol 13:129–137. https://doi.org/10.1210/mend.13.1.0234

Heldring N, Pike A, Andersson S, Matthews J, Cheng D, Oster S, Suh S-S, Smith Y, Khawaled S, Nigita G, Distefano R, Hayashizaki Y, Carninci P, Lenhard B, Kenny PA, Bissell MJ (2007) Targeting TACE-dependent EGFR ligand receptor signaling in lung adenocarcinoma. Cancer Res 69:4835–4842. https://doi.org/10.1158/0008-5472.CAN-06-3376

Hsu L-J, Schultz L, Pratt N, Mattison J, Chang N-S (2007) Zfira affects TNF-mediated cell death by interacting with death domain protein TRADD and negatively regulates the activation of NF-KappaB, JNK1, P53 and WOX1 during stress response. BMC Mol Biol 8:50. https://doi.org/10.1186/1471-2199-8-50

Hong S-Y, Kao Y-R, Lee T-C, Wu C-W (2018) Upregulation of E3 ubiquitin ligase CBLC enhances EGFR dysregulation and signaling in lung adenocarcinoma. Cancer Res 78:4984–4996. https://doi.org/10.1158/0008-5472.CAN-17-3858

Hsu L-J, Schultz L, Hong Q, Van Moer K, Heath J, Li M-Y, Lai F-J, Lin S-R, Lee M-H, Lo C-P et al (2009) Transforming growth factor beta1 signaling via interaction with cell surface Hyal-2 and recruitment of WWOX/WOX1. J Biol Chem 284:16049–16059. https://doi.org/10.1074/jbc.M806688200

Hsu L-J, Chang M-F, Sze C-I, Su W-P, Yap YV, Lee J-T, Kao H-L, Chang N-S (2016) HYAL-2-WWOX-SMAD4 signaling in cell death and anticancer response. Front Cell Dev Biol 4:141. https://doi.org/10.3389/fcell.2016.00141

Ilipooulos D, Fabbri M, Druck T, Qin HR, Han S-Y, Huebner K (2007) Inhibition of breast cancer cell growth in vitro and in vivo: effect of restoration of Wwox expression. Clin Cancer Res 13:268–274. https://doi.org/10.1158/1078-0432.CCR-06-2038

JENSEN EV (1962) On the mechanism of estrogen action. Perspect Biol Med 6:47–59. https://doi.org/10.1353/pbm.1963.0005

Jiang L, Chen Y, Li Y, Lan T, Wu M, Wang Y, Qian H (2014) Type II CGMP-dependent protein kinase inhibits ligand-induced activation of EGFR in gastric cancer cells. Mol Med Rep 9:1405–1409. https://doi.org/10.3892/mmr.2014.1942

Johnson AC, Murphy BA, Matelis CM, Rubinstein Y, Piebenga EC, Akers LM, Neta G, Vinson C, Birrer M (2000) Activator protein-1 mediates induced but not basal epidermal growth factor receptor gene expression. Mol Med 6:17–27

Jordan VC, Lewis JS, Osipo C, Cheng D (2005) The apoptotic action of estrogen following exhaustive antihormonal therapy: a new clinical treatment strategy. Breast 14:624–630. https://doi.org/10.1016/j.breast.2005.08.022

Kallberg Y, Oppermann U, Jornvall H, Persson B (2002) Short-chain dehydrogenase/reductase (SDR) relationships: a large family with eight clusters common to human, animal, and plant genomes. Protein Sci 11:636–641. https://doi.org/10.1110/ps.26902

Kang Y, Massague J (2004) Epithelial-mesenchymal transitions: twist in development and metastasis. Cell 118:277–279. https://doi.org/10.1016/j.cell.2004.07.011

Kenny PA, Bissell MJ (2007) Targeting TACE-dependent EGFR ligand shedding in breast cancer. J Clin Investig 117:337–345. https://doi.org/10.1172/JCI29518

Khawaled S, Suh SS, Abdeen SK, Monin J, Distefano R, Nigita G, Croce CM, Aqielan RI (2019) WWOX inhibits metastasis of triple-negative breast cancer cells via modulation of MiRNAs. Cancer Res 79:1784–1798. https://doi.org/10.1158/0008-5472.CAN-18-0614

Khawaled S, Nigita G, Distefano R, Oster S, Suh S-S, Smith Y, Khalaileh A, Peng Y, Croce CM, Geiger T et al (2020)
Pleiotropic tumor suppressor functions of WWOX antagonize metastasis. Sig Transduct Target Ther 5:43. https://doi.org/10.1038/s41392-020-0136-8

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA (1996) Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 93:5925–5930. https://doi.org/10.1073/pnas.93.12.5925

Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA (1997) comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 138:863–870. https://doi.org/10.1210/endo.138.3.4979

Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, Webb P (2000) Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol 74:311–317. https://doi.org/10.1016/s0960-7670(00)01008-4

Lerdrup M, Holmberg C, Dietrich N, Shaulian E, Herdegen T, Jäättelä M, Kallunki T (2005) Depletion of the AP-1 repressor JDP2 induces cell death similar to apoptosis. Biochim et Biophys Acta (BBA) Molec Cell Res 1745:29–37. https://doi.org/10.1016/j.bbamcr.2005.06.008

Lewis JS, Meeker K, Osipo C, Ross EA, Kidawi N, Li T, Bell E, Chandel NS, Jordan VC (2005) Intrinsic mechanism of estradiol-induced apoptosis in breast cancer cells resistant to estrogen deprivation. J Natl Cancer Inst 97:1746–1759. https://doi.org/10.1093/jnci/dji400

Lewis-Wambi JS, Jordan VC (2009) Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? Breast Cancer Res 11:206. https://doi.org/10.1186/bcr2255

Li H, Durbin R (2009) Fast and accurate short read alignment with burrows-wheeler transform. Bioinformatics 25:1754–1760. https://doi.org/10.1093/bioinformatics/btp324

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R (2009a) The sequence alignment/map format and SAMtools. Bioinformatics 25:2078–2079. https://doi.org/10.1093/bioinformatics/btp352

Li M-Y, Lai F-J, Hsu L-J, Lo C-P, Cheng C-L, Lin S-R, Lee M-H, Chang J-Y, Subhan D, Tsai M-S et al (2009b) Dramatic co-activation of WWOX/WOX1 with CREB and NF-KB in delayed loss of small dorsal root ganglion neurons upon sciatric nerve transaction in rats. PLOs ONE 4:e7820. https://doi.org/10.1371/journal.pone.0007820

Li Z, Zhu Q, Hu L, Chen H, Wu Z, Li D (2015) Anterior Gradient 2 is a binding stabilizer of hypoxia inducible factor-1z that enhances CoCl2-induced doxorubicin resistance in breast cancer cells. Cancer Sci 106:1041–1049. https://doi.org/10.1111/cas.12714

Li J, Liu J, Li P, Zhou C, Liu P (2018) The downregulation of WWOX induces epithelial-mesenchymal transition and enhances stemness and chemoresistance in breast cancer. Exp Biol Med (maywood) 243:1066–1073. https://doi.org/10.1177/1535370218806455

Liang J, Shang Y (2013) Estrogen and cancer. Annu Rev Physiol 75:225–240. https://doi.org/10.1146/annurev-physiol-030212-183708

Lin C-C, Suen KM, Stainthorp A, Wieteska L, Biggs GS, Leitão A, Montanari CA, Ladbury JE (2019) Targeting the Shc-EGFR interaction with indomethacin inhibits MAP kinase pathway signalling. Cancer Lett 457:86–97. https://doi.org/10.1016/j.canlet.2019.05.008

Ma X, Miao H, Jing B, Pan Q, Zhang H, Chen Y, Zhang D, Liang Z, Wen Z, Li M (2015) Claudin-4 controls the proliferation, apoptosis, migration and in vivo growth of MCF-7 breast cancer cells. Oncol Rep 34:681–690. https://doi.org/10.3892/or.2015.4037

Marianijanovic Z, Laubner D, Moller G, Gege C, Husen B, Adamski J, Breitling R (2003) Closing the gap: identification of human 3-ketosteroid reductase, the last unknown enzyme of mammalian cholesterol biosynthesis. Mol Endocrinol 17:1715–1725. https://doi.org/10.1210/me.2002-0436

Mirsadeghi L, Haji Hosseini R, Banaei-Moghaddam AM, Kavousi K (2021) EARN: an ensemble machine learning algorithm to predict driver genes in metastatic breast cancer. BMC Med Genomics 14:122. https://doi.org/10.1186/s12920-021-00974-3

Mosselman S, Polman J, Dijkema R (1996) ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett 392:49–53. https://doi.org/10.1016/0014-5793(96)00782-x

Murata M, Nishiyori-Sueki H, Kojima-Ishiyama M, Carninci P, Hayashizaki Y, Itoh M (2014) Detecting expressed genes using CAGE. Methods Mol Biol 1164:67–85. https://doi.org/10.1007/978-1-4939-0805-9_7

Nunez MI, Ludes-Meyers J, Abba MC, Kil H, Abbey NW, Page RE, Sahin A, Klein-Szanto AJP, Aldaz CM (2005) Frequent loss of WWOX expression in breast cancer: correlation with estrogen receptor status. Breast Cancer Res Treat 98:99–105. https://doi.org/10.1007/s10549-004-1474-x

Nunez MI, Ludes-Meyers J, Aldaz CM (2006) WWOX protein expression in normal human tissues. J Mol Histol 37:115–125. https://doi.org/10.1007/s10735-006-9046-5

Oesterreich S, Deng W, Jiang S, Cui X, Ivanova M, Schiff R, Kang K, Hadsell DL, Behrens J, Lee AV (2003) Estrogen-mediated down-regulation of e-cadherin in breast cancer cells. Cancer Res 63:5203–5208

Oudenaaarden CRL, van de Ven RAH, Derksen PWB (2018) Re-inforcing the cell death army in the fight against breast cancer. J Cell Sci 131:212563. https://doi.org/10.1242/jcs.212563

Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS (1997) Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science 277:1508–1510. https://doi.org/10.1126/science.277.5331.1508

Paredes J, Milanezi F, Viegas L, Amendoeira I, Schmitt F (2002) P-cadherin expression is associated with high-grade ductal carcinoma in situ of the breast. Virchows Arch 440:16–21. https://doi.org/10.1007/s004280100487

Parker C, Rampaul RS, Pinder SE, Bell JA, Wencyk PM, Blamey RW, Nicholson RI, Robertson JF (2001) E-cadherin as a prognostic indicator in primary breast cancer. Br J Cancer 85:1958–1963. https://doi.org/10.1054/bjoc.2001.2178

Pluciennik E, Kusinska R, Potemski P, Kubiak R, Kordek R, Bednarek AK (2006) WWOX-the FRA16D cancer gene: expression correlation with breast cancer progression and prognosis. Eur J Surg Oncol 32:153–157. https://doi.org/10.1016/j.ejso.2005.11.002

Pluciennik E, Krol M, Nowakowska M, Kusinska R, Potemski P, Kordek R, Bednarek AK (2010) Breast cancer relapse prediction based on multi-gene RT-PCR algorithm. Med Sci Monit 16:CR132–CR136

Pluciennik E, Nowakowska M, Pospiech K, Stepien A, Wolkwicz M, Galdyszynska M, Popeda M, Wojcik-Krowiranda K, Bienkiewicz A, Bednarek AK (2015) The role of WWOX tumor suppressor gene in the regulation of EMT process via regulation of CDH1-ZEB1-VIM expression in endometrial cancer. Int J Oncol 46:2639–2648. https://doi.org/10.3822/ijo.2015.2964

Polyak K, Weinberg RA (2009) Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer 9:265–273. https://doi.org/10.1038/nrc2620

Razandi M, Pedram A, Greene GL, Levin ER (1999) Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. Mol Endocrinol 13:307–319. https://doi.org/10.1210/mend.13.2.0239

Rouillard AD, Gundersen GW, Fernandez NF, Wang Z, Monteiro CD, McDermott MG, Ma’ayan A (2016) The harmonizome:
a collection of processed datasets gathered to serve and mine knowledge about genes and proteins. Database 2016:baw100. https://doi.org/10.1093/database/baw100

Salah Z, Aqeilan R, Huebner K (2010) WWOX gene and gene product: tumor suppression through specific protein interactions. Future Oncol 6:249–259. https://doi.org/10.2217/fon.09.152

Saluda-Gorgul A, Seta K, Nowakowska M, Bednarek AK (2011) WWOX oxidoreductase–substrate and enzymatic characterization. Z Naturforsch C J Biosci 66:73–82

Saville B, Wormke M, Wang F, Nguyen T, Enmark E, Kuiper G, Gustafsson JA, Safe S (2000) Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements. J Biol Chem 275:5379–5387. https://doi.org/10.1074/jbc.275.8.5379

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH image to ImageJ: 25 years of image analysis. Nat Methods 9:671–675. https://doi.org/10.1038/nmeth.2089

Sharma R (2021) Global, regional, national burden of breast cancer in 185 countries: evidence from GLOBOCAN 2018. Breast Cancer Res Treat 187:557–567. https://doi.org/10.1007/s10549-020-06083-6

Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, Yue W, Wang J, Santen RJ (2001) Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol. J Natl Cancer Inst 93:1714–1723. https://doi.org/10.1093/jnci/93.22.1714

Song RX-D, Zhang Z, Mor G, Santen RJ (2005) Down-regulation of Bcl-2 enhances estrogen apoptotic action in long-term estradiol-depleted ER(+) breast cancer cells. Apoptosis 10:667–678. https://doi.org/10.1007/s10495-005-1903-2

Sonnenblick A, Brohé S, Fumagalli D, Rothé F, Vincent D, Ignatiadis M, Desmedt C, Salgado R, Sirtainé N, Loi S et al. (2015) Integrative proteomic and gene expression analysis identify potential biomarkers for adjuvant trastuzumab resistance: analysis from the Fin-Her Phase III randomized trial. Oncotarget 6:30306–30316. https://doi.org/10.18632/oncotarget.5080

Tang J-Z, Zuo Z-H, Kong X-J, Steiner M, Yin Z, Perry JK, Zhu T, Liu D-X, Lobie PE (2010) Signal transducer and activator of transcription (STAT)-5A and STAT5B differentially regulate human mammary carcinoma cell behavior. Endocrinology 151:43–55. https://doi.org/10.1210/en.2009-0651

Tarazona S, Furió-Tarí P, Turrà D, Pietro AD, Nueda MJ, Ferrer A, Conesa A (2015) Data quality aware analysis of differential expression in RNA-Seq with NOISEq R/Bioconductor. Nucleic Acids Res 2015:gkv711. https://doi.org/10.1093/nar/gkv711

Team TBD (2020) BSgenome.Hsapiens.UCSC.Hg38: Full Genome Sequences for Homo Sapiens (UCSC V-eraion Hg38, Based on GRCh38.P12)

Toft D, Gorski J (1966) A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. Proc Natl Acad Sci U S A 55:1574–1581. https://doi.org/10.1073/pnas.55.6.1574

Vladusic EA, Hornby AE, Guerra-Vladusic FK, Lakins J, Lupu R (2000) Expression and regulation of estrogen receptor alpha in breast tumors and cell lines. Oncol Rep. https://doi.org/10.3892/or.7.1.157

Wang R, Kwon I-K, Singh N, Islam B, Liu K, Sridhar S, Hofmann F, Browning DD (2014) Type 2 CGMP-dependent protein kinase regulates homeostasis by blocking c-Jun N-terminal kinase in the colon epithelium. Cell Death Differ 21:427–437. https://doi.org/10.1038/cdd.2013.163

Willis AL, Tran NL, Chatigny JM, Charlton N, Vu H, Brown SAN, Black MA, McDonough WS, Fortin SP, Niska JR et al. (2008) The fibroblast growth factor–inducible 14 receptor is highly expressed in HER2-positive breast tumors and regulates breast cancer cell invasive capacity. Mol Cancer Res 6:725–734. https://doi.org/10.1158/1541-7786.MCR-08-0005

Woodfield GW, Chen Y, Bair TB, Domann FE, Weigel RJ (2010) Identification of primary gene targets of TFAP2C in hormone responsive breast carcinoma cells. Genes Chromosom Cancer 49:948–962. https://doi.org/10.1002/gcc.20807

World Cancer Report, Stewart BW, Wild C (2014) International Agency for Research on Cancer. World Health Organization (eds.) International Agency for Research on Cancer, Lyon. ISBN 978-92-832-0429-9

Yang J, Min K-W, Kim D-H, Son BK, Moon KM, Wi YC, Bang SS, Oh YH, Do S-I, Chae SW et al (2018) High TNFRSF12A level associated with MMP-9 overexpression is linked to poor prognosis in breast cancer cell invasive capacity. Mol Cancer Res 6:725–734. https://doi.org/10.1158/1541-7786.MCR-18-0013

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