

A novel *FOXA1/ESR1* interacting pathway: A study of Oncomine™ breast cancer microarrays

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Abstract. Forkhead box protein A1 (FOXA1) is essential for the growth and differentiation of breast epithelium, and has a favorable outcome in breast cancer (BC). Elevated *FOXA1* expression in BC also facilitates hormone responsiveness in estrogen receptor (*ESR*)-positive BC. However, the interaction between these two pathways is not fully understood. *FOXA1* and GATA binding protein 3 (*GATA3*) along with *ESR1* expression are responsible for maintaining a luminal phenotype, thus suggesting the existence of a strong association between them. The present study utilized the Oncomine™ microarray database to identify *FOXA1:ESR1* and *FOXA1:ESR1:GATA3* co-expression co-regulated genes. Oncomine™ analysis revealed 115 and 79 overlapping genes clusters in *FOXA1:ESR1* and *FOXA1:ESR1:GATA3* microarrays, respectively. Five *ESR1* direct target genes [trefoil factor 1 (*TFF1/PS2*), B-cell lymphoma 2 (*BCL2*), seven in absentia homolog 2 (*SIAH2*), cellular myeloblastosis viral oncogene homolog (*CMYB*) and progesterone receptor (*PGR*)] were detected in the co-expression clusters. To further investigate the role of FOXA1 in *ESR1*-positive cells, MCF7 cells were transfected with a *FOXA1* expression plasmid, and it was observed that the direct target genes of *ESR1* (*PS2*, *BCL2*, *SIAH2* and *PGR*) were significantly regulated upon transfection. Analysis of one of these target genes, *PS2*, revealed the presence of two FOXA1 binding sites in the vicinity of the estrogen response element (ERE), which was confirmed by binding assays. Under estrogen stimulation, FOXA1 protein was recruited to the FOXA1 site and could also bind to the ERE site (although in minimal amounts) in the *PS2* promoter.

Co-transfection of *FOXA1/ESR1* expression plasmids demonstrated a significant regulation of the target genes identified in the *FOXA1/ESR1* multi-arrays compared with only *FOXA1* transfection, which was suggestive of a synergistic effect of *ESR1* and *FOXA1* on the target genes. In summary, the present study identified novel *FOXA1*, *ESR1* and *GATA3* co-expressed genes that may be involved in breast tumorigenesis.

Introduction

The majority of breast cancers (BCs) are generally hormone-related cancers, with estradiol (E2) essentially being the primary inducing factor (1,2). In women, E2 promotes cell proliferation, growth and development of the mammary epithelium (3,4). The mammary epithelium is composed of basal and myoepithelial/basal cell lineages (5). Approximately 15-25% of mammary epithelial cells express estrogen receptor 1 (*ESR1*) in the normal resting breast, and are considered to proliferate slowly and in a well-differentiated cell-type (6). However, the number of *ESR1*-positive mammary cells changes throughout the menstrual cycle (7-9). Notably, E2 induces the proliferation of *ESR1*-negative breast cells that surround the *ESR1*-positive cells, probably through the secretion of paracrine factors (6,7). E2 is also known to promote proliferation in a large number of BCs, with positive correlation between *ESR1* positivity and endocrine therapy (10). In addition, the number of mammary epithelial cells and the expression of *ESR1* increase to >50% during initial diagnosis, which suggests a transformation role that provides a target for therapy (8,9). Apart from cellular transformation, *ESR1* also plays a pivotal role in cell proliferation and growth (11,12). Approximately 70% of BCs are *ESR*⁺ or E2-responsive (13). The presence of *ESR1* is a good predictive and prognostic factor for BC patients, who are likely to respond to anti-hormone therapy with tamoxifen or aromatase inhibitors (8). The use of adjuvant therapy such as tamoxifen results in ~40-50% reduction in recurrence and prolonged disease-free and overall patient survival (14), and also provides a clinical benefit for >50% of all metastatic *ESR1*⁺ tumors (15). Although tamoxifen is initially effective, ~50% of breast tumors acquire tamoxifen resistance during the course of treatment (16-18). Such a situation has resulted in the quest for developing novel selective *ESR* modulators.

Forkhead box A1 (FOXA1) is a forkhead family member protein encoded by the *FOXA1* gene, which is located on

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chromosome 14q21.1 (19,20). FOXA1 was initially identified as a vital factor for liver development by transcriptionally activating the liver-specific transcripts albumin and transthyretin (21); however, its role in the development of the breast and other organs has also been reported (22-25). FOXA proteins bind to DNA elements [A(A/T)TRTT(G/T)RYTY] as monomers to mediate their physiological response (6). These proteins are similar to histone linker proteins, but unlike histones, they lack basic amino acids that are essential for chromatin compaction (26). FOXA1 protein also has the potential to compact chromatin and reposition the nucleosome by recruiting itself to enhancer regions of the target genes (20). The repositioning of nucleosomes is considered to facilitate the temporal and spatial differential binding of transcription factors in a lineage-specific manner (27). As observed in rescue experiments in *FOXA1*-null mice, *FOXA1* is responsible for post-natal development of mammary and prostate glands (25). Apart from development, FOXA1 was observed to be highly elevated in prostate cancer and BC (28,29). In *ESR*⁺ BC cells, FOXA1 facilitates hormone responsiveness by modulating *ESR1* binding sites in the target genes (30,31). Thorat *et al* demonstrated that ~50% of *ESR1*-regulated target genes and E2-induced cell proliferation requires prior FOXA1 protein recruitment (32). Furthermore, FOXA1 expression is also associated with low breast tumor grade, exhibiting a positive correlation with the luminal A BC subtype (33). Such observation suggests a strong correlation between *FOXA1* expression and luminal A breast tumor subtype; however, the co-regulatory partners of both molecules are still undefined.

GATA binding protein 3 (*GATA3*) is one of the six members of the zinc finger DNA binding protein family (22). It binds to the DNA sequence (A/T)GATA(A/G) in the target gene, and promotes cell proliferation, development and differentiation of different tissues and cell types (34,35), including the luminal glandular epithelial cells of the mammary gland (36-38). The genes *GATA3*, *FOXA1* and *ESR1* are highly expressed in BC, with positive correlation between them (39). *ESR1* messenger RNA (mRNA) is transcribed from ~6 promoter regions with different tissue specificity (40). The regulatory factors involved in *GATA3* and *FOXA1* expression may interact with the *ESR1* promoter region, although this remains to be determined (28). However, a previous whole genome expression analysis demonstrated that FOXA1 and *GATA3* protein express in close association with *ESR1* (41).

Previous studies have utilized the OncomineTM software (Thermo Fisher Scientific, Inc., Waltham, MA, USA) to correlate published microarray data (42,43) in order to confirm the authenticity of the correlation data. The OncomineTM software enables to understand and analyze a number of microarray data (multi-array), which contain multiple clinical tumor samples and normal biopsies (44). The software function search tool allows the queried gene to be correlated in terms of its expression with other genes in the multi-arrays (www.oncomine.org). Such analyses will yield a significant overlap of co-expressed genes that can link proteins in the same molecular pathway.

The objective of the present study was to compare the co-expressed target genes of *FOXA1* and to correlate them with *ESR1* and *GATA3* in order to determine the extent of overlap using OncomineTM microarray data. For that purpose, an intensive individual meta-analysis of *FOXA1*, *ESR1* and

GATA3 (putative pathway partners that may be associated in BC tumorigenesis) was performed, followed by a comparison of the overlapping genes. Such comparisons would provide a highly significant number of genes that may be involved in the same pathway. Analyses of the OncomineTM microarray data identified 115 co-regulated genes between *FOXA1* and *ESR1*. Comparison of these genes with another co-related and co-regulated gene, *GATA3*, identified 79 genes that are co-expressed along with *FOXA1* and *ESR1* co-regulated genes, which are consistent with the previously reported estrogen- and *ESR1*-regulated pathway. Semiquantitative and quantitative polymerase chain reaction (qPCR) analysis also confirmed a number of the overlapping genes [*PS2*, B-cell lymphoma 2 (*BCL2*), progesterone receptor (*PGR*), seven in absentia homolog 2 (*SIAH2*), cellular myeloblastosis viral oncogene homolog (*CMYB*) and *GATA3*], which suggested a significant correlation. *In silico* analysis of one of the significantly associated genes, *PS2*, demonstrated the presence of two FOXA1 binding sites and an estrogen response element (ERE), which was observed to recruit FOXA1 upon E2 stimulation.

The present findings reveal novel co-expression partners and the existence of a molecular network involving interacting partners in the *FOXA1*, *ESR1* and *GATA3* signaling pathways.

Materials and methods

OncomineTM analysis. OncomineTM is an integrated cancer microarray database and web-based data-mining platform (44). OncomineTM analysis was performed as previously described (42,43). The co-expressed genes correlated with *FOXA1* and *ESR1* were searched for in the OncomineTM platform. A total of 24 microarrays were selected, 20 of which were *ESR*⁺ BC microarrays, while the remaining 4 were normal *ESR*⁺ breast microarrays (Table I) (45-68). All the *ESR*⁺ microarrays were selected for co-expression analysis. The first 500 genes co-regulated with *FOXA1* and *ESR1* within each microarray were retrieved and compared separately. These 500 genes were selected based on a >2 fold-change expression level and in an adjusted threshold by gene rank for the top 10%. Such a threshold will return mRNA datasets having breast cancer clinical samples, with *FOXA1* and *ESR1* coexpression results ranked or grouped in the top 10% of the datasets. Therefore by examining these coexpression results we can determine genes that are coordinately expressed with *FOXA1* and *ESR1*, which may help to identify potential targets in the same pathway. The repetitive genes within each study (*FOXA1* and *ESR1*) were removed, keeping only a single representative of the gene in each microarray analysis. The gene names were derived from GeneCards[®] (<http://www.genecards.org/>). To understand the significant correlations, genes represented on >4 microarrays were considered significant (16% frequency), and those represented on >5 microarrays were considered highly significant (20% frequency). Genes from the *FOXA1* and *ESR1* microarrays were sorted and overlapped to identify overlapping co-expressed genes. Such microarray coexpression analysis may help to identify potential targets that function in the same regulatory pathway.

Table I. Forkhead box protein A1:estrogen receptor 1 micro-array used for the analysis.

Author	Type ^a	Sample numbers	Ref.
Higgins <i>et al</i>	Normal	34	(45)
Roth <i>et al</i>	Normal	353	(46)
Shyamsundar <i>et al</i>	Normal	123	(47)
Tabchy <i>et al</i>	Breast	178	(48)
Perou <i>et al</i>	Breast	65	(49)
Su <i>et al</i>	Normal	101	(50)
Zhao <i>et al</i>	Breast	64	(51)
Yu <i>et al</i>	Breast 3	96	(52)
Wang <i>et al</i>	Breast	286	(53)
Waddell <i>et al</i>	Breast	85	(54)
Van't Veer <i>et al</i>	Breast	117	(55)
Schmidt <i>et al</i>	Breast	200	(56)
Pollack <i>et al</i>	Breast 2	41	(57)
Minn <i>et al</i>	Breast 2	121	(58)
Lu <i>et al</i>	Breast	129	(59)
Korde <i>et al</i>	Breast	61	(60)
Kao <i>et al</i>	Breast	327	(61)
Julka <i>et al</i>	Breast	44	(62)
Hatzis <i>et al</i>	Breast	508	(63)
Gluck <i>et al</i>	Breast	158	(64)
Farmer <i>et al</i>	Breast	49	(65)
Desmedt <i>et al</i>	Breast	198	(66)
Bos <i>et al</i>	Breast	204	(67)
Bonnefoi <i>et al</i>	Breast	160	(68)

^aAccording to the Oncomine database acronym.

Cell culture and transient transfection. The cell lines MCF7 and T47D were purchased from the National Center for Cell Sciences (Pune, India). The MCF7 and T47D cell lines were cultured in Dulbecco's modified Eagle medium (DMEM; PAN Biotech GmbH, Aidenbach, Germany) and RPMI 1640 medium (PAN Biotech GmbH) respectively, supplemented with 10% (v/v) fetal bovine serum (PAN Biotech GmbH) and 1% (v/v) penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The cells were maintained under a humidified atmosphere in 5% CO₂ at 37°C. The plasmids pRB-HNF3α (expressing *FOXA1*) and pAcGFPC1-ESR1 (expressing *ESR1*) were provided by Professor Kenneth S. Zaret (Department of Cell and Developmental Biology, Smilow Center for Translational Research, Philadelphia, PA, USA) and Professor Ratna K. Vadlamudi (The Department of Obstetrics and Gynecology, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA), respectively.

To investigate the role of *FOXA1* in the transcriptional regulation of target genes, *FOXA1* expression plasmid (1 μg) and empty vector (1 μg) were transfected in MCF7 and T47D cells cultured in 35-mm plates (BD Biosciences, Franklin Lakes, NJ, USA) using the TransPass D2 transfection reagent (New England BioLabs, Inc., Ipswich, MA, USA).

Transfected and untransfected cell lines were harvested at 24 h post-transfection. Similarly, co-transfection was performed by transfecting *FOXA1* (500 ng) and *ESR1* (500 ng) expression plasmids. After 24 h of transfection, total RNA was isolated and processed.

RNA isolation, reverse transcription-PCR and qPCR. Total RNA was isolated from *FOXA1*-transfected and *ESR1/FOXA1*-co-transfected samples at 24 h post-transfection using TRI reagent (Sigma-Aldrich). RNA was digested with DNase I (Sigma-Aldrich) digested converted into complementary DNA (cDNA) using a first-strand cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The qPCR conditions were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 30 sec and 56-58°C for 30 sec. GAPDH was used as a internal control. The relative quantification of gene expression was calculated by the 2^{-ΔΔC_q} method (69). The primers used for PCR are listed in Table II. qPCR was performed using SYBR® Green (Sigma-Aldrich) with an MJ Research thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Nuclear extract. Nuclear lysate was extracted from MCF7 cells. The cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with cell lysis buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.9), 50% (v/v) glycerol, 0.1% (v/v) Triton X-100, 10 mM NaCl, 1.5 mM MgCl₂, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM ethylenediaminetetraacetic acid (EDTA) and 1X protease inhibitor cocktail] (Sigma-Aldrich) for 15 min in 4°C. Nuclear pellets were collected upon centrifugation at 500 x g for 15 min, and resuspended in chilled extraction buffer [20 mM HEPES (pH=7.9), 50% (v/v) glycerol, 420 mM NaCl, 10 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol (DTT) and 1X protease inhibitor cocktail] (Sigma-Aldrich). After 30 min of incubation on ice, the nuclear proteins were collected by centrifugation at 16,000 x g at 4°C for 30 min. The lysate prepared was stored at -80°C prior to use.

Electrophoretic mobility shift assay (EMSA). *In vitro* DNA-protein interaction was performed using EMSA. Oligonucleotides consisting of FOXA1 binding sites present in the PS2 promoter were designed from -517 to -547 (EMSA1) and from -363 to -393 (EMSA2) residues upstream of the transcription start site. The oligonucleotide sequences are provided in Table II. The forward primers of EMSA1 and EMSA2 were kinase-labeled with γ³²P adenosine triphosphate (BRIT, Hyderabad, India), and then annealed with reverse complementary oligonucleotide residues in annealing buffer [200 mM Tris-Cl (pH 7.5), 1,000 mM NaCl and 100 mM MgCl₂]. The nuclear lysate was incubated in 10 μl binding buffer [1 M Tris-Cl (pH 7.5), 50% (v/v) glycerol, 0.5 M EDTA, 1 mM DTT and 50 mg/ml bovine serum albumin; Sigma-Aldrich) containing 0.2 pmol radiolabeled probe. Poly(deoxyinosinic-deoxycytidylic) acid was used as a nonspecific competitor. For specific competition, the radiolabeled probes were mixed to compete with various excess molar concentrations of unlabeled double-stranded FOXA1 consensus probe. After 25 min of incubation at room temperature, the samples were subjected

Table II. Lists of primers used.

Primers	Primer sequence (5'-3')	Amplicon size (bp)
RT- <i>FOXA1</i>	F: GGGTGGCTCCAGGATGTTAGG R: GGGTCATGTTGCCGCTCGTAG	194
RT- <i>GATA3</i>	F: CAGACCACCACAACCACACTCT R: GGATGCCTCCTTCTTCATAGTCA	124
RT- <i>PGR</i>	F: CGCGCTCTACCCTGCACTC R: TGAATCCGGCCTCAGGTAGTT	121
RT- <i>CMYB</i>	F: GAAGGTCTGAACAGGAAGGTTATCT R: GTAACGCTACAGGGTATGGAACA	224
RT- <i>SIAH2</i>	F: CCTCGGCAGTCCTGTTTCCCTGT R: CCAGGACATGGGCAGGAGTAGGG	124
RT- <i>BCL2</i>	F: TGTGGATGACTGAGTACCTG R: GGAGAAATCAAACAGAGGCC	116
RT- <i>PS2</i>	F: GAACAAGGTGATCTGCGCCC R: TTCTGGAGGGACGTCGATGG	223
RT- <i>GAPDH</i>	F: AAGATCATCAGCAATGCCTC R: CTCTTCCTCTTGTGCTCTTG	619
<i>FOXA1</i> chip (<i>FOXA1</i> site1) <i>PS2</i>	F: CATGTTGGCCAGGCTAGTCT R: CATCCGTCTAGGCCTAAGC	165
<i>FOXA1</i> chip (<i>FOXA1</i> site2) <i>PS2</i>	F: GCTTAGGCCTAGACGGAATG R: CTCATATCTGAGAGGCCCTC	180
<i>PS2</i> chip F (ERE)	F: TTAAGTGATCCGCCTGCTTT R: CTCCCGCCAGGGTAAATACT	271
<i>FOXA1</i> consensus site	F: CTTATGCAATGTGTTGGTCTCACG R: CGTGAGACCAACACATTGCATAAG	
<i>FOXA1</i> EMSA (<i>FOXA1</i> site1) <i>PS2</i>	GGCCTCCCAAAGTGTTGGGATTACAGGCGT ACGCCTGTAATCCCAACACTTTGGGAGGCC	
<i>FOXA1</i> EMSA (<i>FOXA1</i> site2) <i>PS2</i>	CCCCGTGAGCCACTGTTGTCACGGCCAAG CTTGGCCGTGACAACAGTGGCTCACGGGG	

RT, reverse transcription; *FOXA1*, forkhead box protein A1; EMSA; electrophoretic mobility shift assay; *GATA3*, GATA binding protein 3; *PGR*, progesterone receptor; *BCL2*, B-cell lymphoma 2; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; ERE, estrogen response element; F, forward; R, reverse; *PS2*, trefoil factor 1; *SIAH2*, seven in absentia homolog 2; *CMYB*, cellular myeloblastosis viral oncogene homolog

to electrophoresis in a 6% polyacrylamide gel at 180 V in 0.5X Tris/borate/EDTA running buffer [40 mM Tris-Cl (pH 8.3), 45 mM boric acid and 1 mM EDTA] for 1 h. Subsequently, the gel was dried and autoradiographed.

Chromatin immunoprecipitation (ChIP) assay. For *in vivo* binding assays, ChIP was performed. Prior to E2 treatment, MCF7 cells were maintained in phenol-free DMEM (PAN Biotech GmbH) for 48 h. The cells were stimulated with 100 nM E2 (Sigma-Aldrich) for additional 24 h, fixed with 1% (v/v) formaldehyde for 10 min, washed twice with 1X PBS (10 mM PO_4^{3-} , 137 mM NaCl and 2.7 mM KCl), lysed with cell lysis buffer [1% (v/v) sodium dodecyl sulfate (SDS), 10 mM EDTA, 50 mM Tris-Cl (pH 8.1) and 1X protease inhibitor cocktail] (Sigma-Aldrich) and sonicated at M2 amplitude strength (~250W intensity level) using a Bioruptor® ultrasonicator device (Diagenode S.A., Seraing, Belgium). The sonicated samples were pre-cleared using protein A-sepharose beads (GE Healthcare Life Sciences, Chalfont, UK) and incubated with

1 μg anti-FOX A1 (catalog no., sc101058; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-ESR1 (catalog no., 8644s; Cell Signaling Technology, Inc., Danvers, MA, USA), normal mouse immunoglobulin G (IgG) (catalog no., kch-819-015; Diagenode S.A.) and normal rabbit IgG (catalog no., sc-2027; Santa Cruz Biotechnology, Inc.) antibodies (diluted, 1:100) at 4°C for 1 h. The antibody-protein complexes were separated using protein A-sepharose beads for an additional 1 h, and washed with different washing buffers, including a low salt wash buffer [0.1% (v/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1) and 150 mM NaCl], a high salt wash buffer [0.1% (v/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1) and 500 mM NaCl], a LiCl wash buffer [0.25 M LiCl, 1% (v/v) NP-40, 1% (w/v) deoxycholic acid (sodium salt), 1 mM EDTA and 10 mM Tris-HCl (pH 8.1)] and 1X Tris/EDTA [10 mM Tris-HCl (pH 8.1) and 1 mM EDTA]. The samples were then eluted with elution buffer [1% (v/v) SDS and 0.1 M NaHCO_3], reverse crosslinked with 5 mM NaCl for 6 h at 65°C and subjected to proteinase K

Table III. *FOXA1* Oncomine™ meta-analysis.

Gene	Percentage of co-expression (%)
<i>FOXA1</i>	100
<i>ESR1</i>	67
<i>GATA3</i>	67
<i>MLPH</i>	67
<i>AGR2</i>	63
<i>CA12</i>	63
<i>TFF3</i>	63
<i>XBP1</i>	63
<i>NAT1</i>	58
<i>SLC39A6</i>	58
<i>TBC1D9</i>	58
<i>DNALI1</i>	54
<i>SCNN1A</i>	54
<i>SLC44A4</i>	54
<i>SPDEF</i>	54
<i>TSPAN1</i>	54
<i>ANXA9</i>	50
<i>DNAJC12</i>	50
<i>FBP1</i>	50
<i>GREB1</i>	50
<i>MAGED2</i>	50
<i>MAPT</i>	50
<i>MYB</i>	50
<i>TFF1</i>	50
<i>AR</i>	46
<i>FAM174B</i>	46
<i>INPP4B</i>	46
<i>KDM4B</i>	46
<i>SCUBE2</i>	46
<i>SIDT1</i>	46
<i>VAV3</i>	46
<i>ABAT</i>	42
<i>BCL2</i>	42
<i>GPD1L</i>	42
<i>IL6ST</i>	42
<i>RHOB</i>	42
<i>TTC39A</i>	42
<i>ACADSB</i>	38
<i>ERBB4</i>	38
<i>EVL</i>	38
<i>NME5</i>	38
<i>SYBU</i>	38
<i>TOX3</i>	38
<i>ZNF552</i>	38
<i>CACNA1D</i>	33
<i>DACH1</i>	33
<i>GALNT6</i>	33
<i>GAMT</i>	33
<i>GFRA1</i>	33
<i>RAB17</i>	33
<i>RBM47</i>	33
<i>SLC16A6</i>	33

Table III. Continued.

Gene	Percentage of co-expression (%)
<i>SLC7A8</i>	33
<i>STC2</i>	33
<i>TSPAN13</i>	33
<i>ZMYND10</i>	33
<i>AFF3</i>	29
<i>AKR7A3</i>	29
<i>C10orf116</i>	29
<i>C9orf116</i>	29
<i>CRIP1</i>	29
<i>CYB5A</i>	29
<i>ELOVL5</i>	29
<i>GALNT7</i>	29
<i>KCNK15</i>	29
<i>KIAA1324</i>	29
<i>LASS6</i>	29
<i>MCCC2</i>	29
<i>MTL5</i>	29
<i>PGR</i>	29
<i>RAB26</i>	29
<i>SERPINA5</i>	29
<i>SIAH2</i>	29
<i>SLC2A10</i>	29
<i>AGR3</i>	25
<i>CAMK2N1</i>	25
<i>CYP2B7P1</i>	25
<i>FAM134B</i>	25
<i>GPR160</i>	25
<i>GSTM3</i>	25
<i>INPP5J</i>	25
<i>KIF5C</i>	25
<i>MAST4</i>	25
<i>MED13L</i>	25
<i>NPDC1</i>	25
<i>PNPLA4</i>	25
<i>PP1457I</i>	25
<i>RABEP1</i>	25
<i>SCCPDH</i>	25
<i>SEMA3B</i>	25
<i>SEMA3F</i>	25
<i>STARD10</i>	25
<i>SYT17</i>	25
<i>THSD4</i>	25
<i>UGCG</i>	25
<i>ABCC8</i>	21
<i>ABLIM3</i>	21
<i>BCAS1</i>	21
<i>C5orf30</i>	21
<i>C6orf97</i>	21
<i>C9orf152</i>	21
<i>CLSTN2</i>	21
<i>CYP2B6</i>	21
<i>DHCR24</i>	21

Table III. Continued.

Gene	Percentage of co-expression (%)
<i>DUSP4</i>	21
<i>DYNLRB2</i>	21
<i>EFHC1</i>	21
<i>ERBB3</i>	21
<i>FAAH</i>	21
<i>FSIP1</i>	21
<i>GDF15</i>	21
<i>IRS1</i>	21
<i>KCTD3</i>	21
<i>KIAA0040</i>	21
<i>KIF16B</i>	21
<i>KRT18</i>	21
<i>LRBA</i>	21
<i>METRN</i>	21
<i>MREG</i>	21
<i>MYO5C</i>	21
<i>PECI</i>	21
<i>PRR15</i>	21
<i>PTPRT</i>	21
<i>PVRL2</i>	21
<i>REEP1</i>	21
<i>REEP6</i>	21
<i>REERG</i>	21
<i>RNF103</i>	21
<i>SLC19A2</i>	21
<i>SLC22A5</i>	21
<i>SLC4A8</i>	21
<i>SYTL2</i>	21
<i>TBX3</i>	21
<i>TMC5</i>	21
<i>TMEM30B</i>	21
<i>TP53TG1</i>	21
<i>TTC6</i>	21
<i>WFS1</i>	21
<i>ADCY9</i>	17
<i>ANKRD30A</i>	17
<i>APBB2</i>	17
<i>AZGP1</i>	17
<i>BBS4</i>	17
<i>C17orf28</i>	17
<i>C1orf21</i>	17
<i>C1orf64</i>	17
<i>C4A</i>	17
<i>CACNA2D2</i>	17
<i>CASC1</i>	17
<i>CCNG2</i>	17
<i>CELSR2</i>	17
<i>CLGN</i>	17
<i>COX6C</i>	17
<i>CPB1</i>	17
<i>CREB3L4</i>	17
<i>CXXC5</i>	17

Table III. Continued.

Gene	Percentage of co-expression (%)
<i>CYP4B1</i>	17
<i>DEGS2</i>	17
<i>EEF1A2</i>	17
<i>FAM110C</i>	17
<i>FUT8</i>	17
<i>HHAT</i>	17
<i>HPN</i>	17
<i>IGF1R</i>	17
<i>KIAA0232</i>	17
<i>KIAA1244</i>	17
<i>KRT8</i>	17
<i>LRIG1</i>	17
<i>MEIS3P1</i>	17
<i>MKL2</i>	17
<i>MYST4</i>	17
<i>NBEA</i>	17
<i>NPNT</i>	17
<i>NRIP1</i>	17
<i>PBX1</i>	17
<i>PCSK6</i>	17
<i>RAB27B</i>	17
<i>RALGPS2</i>	17
<i>RND1</i>	17
<i>SLC9A3R1</i>	17
<i>SPRED2</i>	17
<i>STK32B</i>	17
<i>WWP1</i>	17
<i>ZNF703</i>	17

FOXA1, forkhead box protein A1.

digestion at 45°C for 1 h. The ChIP eluates were purified by phenol-chloroform, and the purified DNA fractions were used to perform PCR analysis to confirm the presence of ESR1 and FOXA1 binding in the PS2 promoter (Table II).

Statistical analysis. Data are shown as representative experiments performed in triplicates, and represented as the mean \pm standard error. Differences were compared with the paired Student's *t*-test. All statistical tests were performed with GraphPad Prism version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results and Discussion

Co-expression meta-analysis was performed using Oncomine™ (www.oncomine.org), which is a web-based interface cancer-profiling database containing published microarray data that have been collected, analyzed, annotated and maintained by Compendia Bioscience™ (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The co-expression genes

Table IV. *ESR1* Oncomine™ meta-analysis.

Gene	Percentage of co-expression (%)
<i>ESR1</i>	100
<i>CA12</i>	79
<i>GATA3</i>	79
<i>NAT1</i>	71
<i>SLC39A6</i>	71
<i>TBC1D9</i>	71
<i>DNALI1</i>	67
<i>FOXA1</i>	67
<i>ANXA9</i>	63
<i>DNAJC12</i>	63
<i>GREB1</i>	63
<i>MAPT</i>	63
<i>ABAT</i>	58
<i>SCUBE2</i>	58
<i>TFF3</i>	58
<i>ERBB4</i>	54
<i>KDM4B</i>	54
<i>MLPH</i>	54
<i>MYB</i>	54
<i>XPB1</i>	54
<i>AGR2</i>	50
<i>DACH1</i>	50
<i>FBP1</i>	50
<i>IL6ST</i>	50
<i>MAGED2</i>	50
<i>TFF1</i>	50
<i>VAV3</i>	50
<i>ACADSB</i>	46
<i>GFRA1</i>	46
<i>INPP4B</i>	46
<i>KIAA1324</i>	46
<i>PGR</i>	46
<i>SCNN1A</i>	46
<i>SLC44A4</i>	46
<i>SLC7A8</i>	46
<i>SPDEF</i>	46
<i>BCL2</i>	42
<i>C9orf116</i>	42
<i>CACNA1D</i>	42
<i>EVL</i>	42
<i>GAMT</i>	42
<i>GPDI1</i>	42
<i>NME5</i>	42
<i>SERPINA5</i>	42
<i>STC2</i>	42
<i>SYBU</i>	42
<i>TTC39A</i>	42
<i>ZMYND10</i>	42
<i>AFF3</i>	38
<i>AGR3</i>	38
<i>AR</i>	38
<i>FAM174B</i>	38

Table IV. Continued.

Gene	Percentage of co-expression (%)
<i>SIDT1</i>	38
<i>THSD4</i>	38
<i>TSPAN1</i>	38
<i>CLSTN2</i>	33
<i>CYP2B6</i>	33
<i>CYP2B7P1</i>	33
<i>ELOVL5</i>	33
<i>FAM134B</i>	33
<i>KCNK15</i>	33
<i>RERG</i>	33
<i>RHOB</i>	33
<i>SLC16A6</i>	33
<i>SLC22A5</i>	33
<i>UGCG</i>	33
<i>ZNF552</i>	33
<i>ABCC8</i>	29
<i>C5orf30</i>	29
<i>C6orf97</i>	29
<i>CYB5A</i>	29
<i>DYNLRB2</i>	29
<i>GSTM3</i>	29
<i>IRS1</i>	29
<i>MAST4</i>	29
<i>MCCC2</i>	29
<i>MTL5</i>	29
<i>PNPLA4</i>	29
<i>PTPRT</i>	29
<i>RABEP1</i>	29
<i>SEMA3B</i>	29
<i>SIAH2</i>	29
<i>SUSD3</i>	29
<i>SYT17</i>	29
<i>TSPAN13</i>	29
<i>ABLIM3</i>	25
<i>ADCY9</i>	25
<i>AKR7A3</i>	25
<i>C10orf116</i>	25
<i>CACNA2D2</i>	25
<i>CASC1</i>	25
<i>CRIP1</i>	25
<i>CXXC5</i>	25
<i>ERBB3</i>	25
<i>FSIP1</i>	25
<i>GALNT6</i>	25
<i>HHAT</i>	25
<i>INPP5J</i>	25
<i>KCTD3</i>	25
<i>KIF5C</i>	25
<i>MED13L</i>	25
<i>NRIP1</i>	25
<i>RAB17</i>	25
<i>RBM47</i>	25

Table IV. Continued.

Gene	Percentage of co-expression (%)
<i>SCCPDH</i>	25
<i>SEMA3F</i>	25
<i>SLC2A10</i>	25
<i>TBX3</i>	25
<i>TOX3</i>	25
<i>WFS1</i>	25
<i>WWP1</i>	25
<i>ACOX2</i>	21
<i>ANKRD30A</i>	21
<i>APBB2</i>	21
<i>C4A</i>	21
<i>CAMK2N1</i>	21
<i>CCDC74B</i>	21
<i>CCNG2</i>	21
<i>COX6C</i>	21
<i>DEGS2</i>	21
<i>EEF1A2</i>	21
<i>EFHC1</i>	21
<i>FAAH</i>	21
<i>FUT8</i>	21
<i>GALNT7</i>	21
<i>IGF1R</i>	21
<i>KIAA0040</i>	21
<i>LASS6</i>	21
<i>LRBA</i>	21
<i>LRIG1</i>	21
<i>MEIS3P1</i>	21
<i>METRN</i>	21
<i>MREG</i>	21
<i>NPDC1</i>	21
<i>NPNT</i>	21
<i>PDZK1</i>	21
<i>PRSS23</i>	21
<i>RAB26</i>	21
<i>REPS2</i>	21
<i>RNF103</i>	21
<i>SALL2</i>	21
<i>STK32B</i>	21
<i>ZNF703</i>	21
<i>ASTN2</i>	17
<i>AZGP1</i>	17
<i>BBS1</i>	17
<i>BBS4</i>	17
<i>BCAS1</i>	17
<i>C14orf45</i>	17
<i>C16orf45</i>	17
<i>C1orf64</i>	17
<i>C6orf211</i>	17
<i>CAPN8</i>	17
<i>CELSR2</i>	17
<i>CPB1</i>	17
<i>CYP4B1</i>	17

Table IV. Continued.

Gene	Percentage of co-expression (%)
<i>DHCR24</i>	17
<i>GDF15</i>	17
<i>HPN</i>	17
<i>KIAA0232</i>	17
<i>LONRF2</i>	17
<i>MKL2</i>	17
<i>MYO5C</i>	17
<i>MYST4</i>	17
<i>NKAIN1</i>	17
<i>PARD6B</i>	17
<i>PBX1</i>	17
<i>PCP2</i>	17
<i>PCSK6</i>	17
<i>PECI</i>	17
<i>PLAT</i>	17
<i>PLCD4</i>	17
<i>PP14571</i>	17
<i>PP1R3C</i>	17
<i>PREX1</i>	17
<i>PRLR</i>	17
<i>RALGPS2</i>	17
<i>RARA</i>	17
<i>REEP1</i>	17
<i>REEP6</i>	17
<i>SEC14L2</i>	17
<i>SEMA3C</i>	17
<i>SERPINA3</i>	17
<i>SLC19A2</i>	17
<i>SLC22A18</i>	17
<i>SLC27A2</i>	17
<i>SSH3</i>	17
<i>STARD10</i>	17
<i>SYTL2</i>	17
<i>TCEAL1</i>	17
<i>TMEM25</i>	17
<i>TMEM30B</i>	17
<i>TP53TG1</i>	17
<i>TPRG1</i>	17
<i>WNK4</i>	17

ESR1, estrogen receptor 1.

for *ESR1* and *FOXA1* were searched and analyzed in the multi-arrays (Table I). The first 500 highly co-expressed genes (exhibiting both significantly low and high expression) with a cut-off frequency of ≥ 4 ($\geq 16\%$) studies in each microarray were selected (Tables III and IV). Approximately 16-20% of genes were observed to overlap with each other when the co-expressed genes of *ESR1* and *FOXA1* were combined (Fig. 1A and B). Under higher stringent conditions with a cut-off frequency of ≥ 5 ($\geq 20\%$), ~115 genes overlapped in

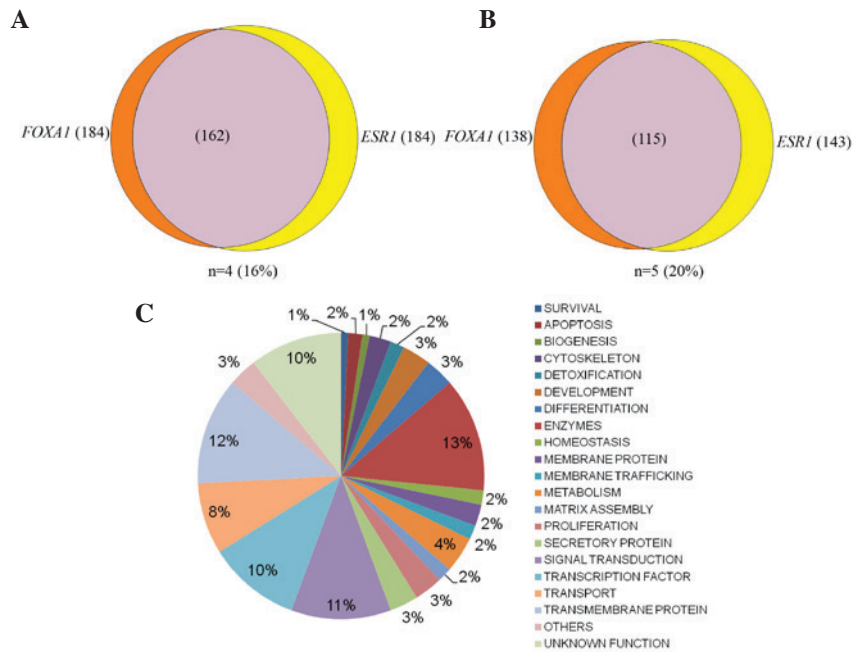


Figure 1. Analysis of overlapping *FOXA1* and *ESR1* co-expression genes. Venn diagrams depicting genes overlapping with *FOXA1* and *ESR1* with a cut-off frequency of (A) 4 (~16%) and (B) 5 (~20%) by meta-analysis with Oncomine™. (C) Pie chart of functional categories for *FOXA1:ESR1* overlapping genes with a cut-off frequency of ≥5 studies. The Oncomine™ data analyzed consisted of 4 normal and 20 breast cancer microarrays data sets. *FOXA1*, forkhead box protein A1; *ESR1*, estrogen receptor 1.

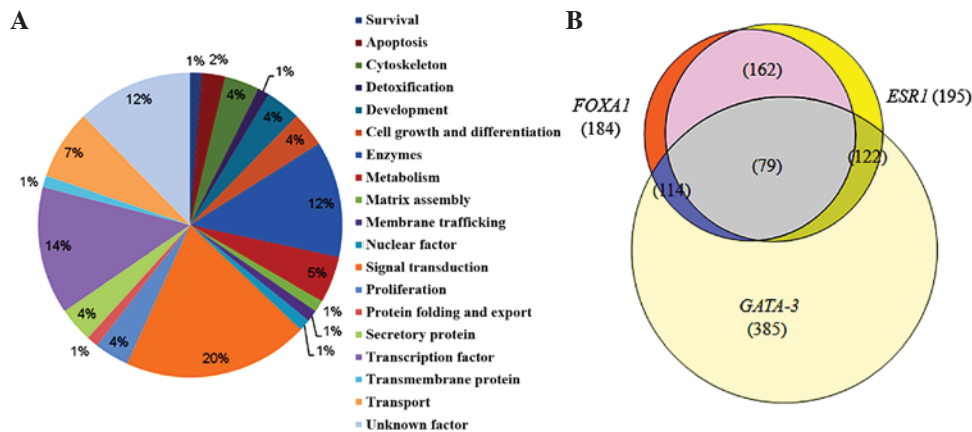


Figure 2. Analysis of overlapping *FOXA1*, *ESR1* and *GATA3* co-expression genes. (A) Pie chart of *FOXA1*, *ESR1* and *GATA3* overlapping genes with a cut-off frequency of 4. (B) Pathway pie chart of *FOXA1*, *ESR1* and *GATA3* overlapping genes with a cut-off frequency of 4. The *FOXA1* and *ESR1* Oncomine™ microarray analysis consisted of 4 normal and 20 breast cancer microarray data. The *GATA3* microarray data was extracted from published data by Wilson and Giguère (31). *ESR1*, estrogen receptor 1; *FOXA1*, forkhead box protein A1; *GATA3*, GATA binding protein 3.

ESR1 and *FOXA1* co-expression genes multi-arrays (Fig. 1B). Table V presents the overlapping genes of *ESR1* and *FOXA1* identified in the aforementioned multi-arrays.

The transcription factor ESR is overexpressed in 70% of BCs, and is a major target for endocrine therapies for luminal A BC patients (13). Dimeric ESR binds to promoter and distant enhancer regions of E2-sensitive genes to regulate their expression. The binding of FOXA1 to enhancer regions of the compact chromatin facilitates remodeling at the ESR1 binding regions (23,30,70); therefore, FOXA1 is also known as 'pioneer' transcription factor (20). When the 115 overlapping genes from microarrays (cut-off frequency of 5) were compared with ESR1-stimulated genes (71), ~22% of *ESR1* and 17% of *FOXA1* genes were represented in the

overlapping, co-expressed *FOXA1:ESR1* microarray gene cluster (Table VI). Furthermore, comparisons were performed only for 51 of the ESR1-upregulated genes identified by Tozlu *et al* (71), but these 51 genes were not classified as such if they were regulated classically or in a non-genomic manner by ESR1 protein.

GATA3 is required for mammary gland morphogenesis and luminal cell differentiation, and is implicated in BC metastasis and progression (38,72). Additionally, *GATA3* is also closely associated with *ESR1* expression status, and its expression indicates favorable BC pathological outcome (73). Since *GATA3* expression together with *ESR1* and *FOXA1* expression correlates strongly with luminal BC subtypes (33,74), *GATA3* (43) was also observed to be overlapped with the

Table V. Overlapping meta-analysis of *ESR1* and *FOXA1* with a cut-off frequency of 5 (20%).

Overlap of <i>ESR1</i> and <i>FOXA1</i> (≥ 5 studies, <i>ESR1</i> =143, <i>FOXA1</i> =138, overlapping genes=115)			
Gene	<i>FOXA1</i> (%)	<i>ESR1</i> (%)	Function
<i>ESR1</i>	67	100	Estrogen receptor 1
<i>CA12</i>	63	79	Carbonic anhydrase 12
<i>GATA3</i>	67	79	GATA binding protein 3
<i>NAT1</i>	58	71	NAT1 N-acetyltransferase 1
<i>SLC39A6</i>	58	71	Zinc transporter ZIP6
<i>TBC1D9</i>	58	71	TBC1 domain family member 9
<i>DNALI1</i>	54	67	Axonemal dynein light intermediate polypeptide 1
<i>FOXA1</i>	100	67	Forkhead box protein A1
<i>ANXA9</i>	50	63	Annexin A9
<i>DNAJC12</i>	50	63	DnaJ homolog subfamily C member 12
<i>GREB1</i>	50	63	Growth regulation by estrogen in breast cancer 1
<i>MAPT</i>	50	63	Microtubule-associated protein tau
<i>NPDC1</i>	25	63	Neural proliferation differentiation and control protein 1
<i>ABAT</i>	42	58	4-aminobutyrate aminotransferase
<i>SCUBE2</i>	46	58	Signal peptide, CUB domain, EGF-like 2
<i>TFF3</i>	63	58	Trefoil factor 3
<i>ERBB4</i>	38	54	Receptor tyrosine-protein kinase erbB-4
<i>KDM4B</i>	46	54	Lysine (K)-specific demethylase 4B
<i>MLPH</i>	67	54	Melanophilin
<i>MYB</i>	50	54	Myb proto-oncogene protein
<i>XPB1</i>	63	54	X-box binding protein 1
<i>AGR2</i>	63	50	Anterior gradient homolog 2
<i>DACH1</i>	33	50	Dachshund homolog 1
<i>FBP1</i>	50	50	Fructose-1,6-bisphosphatase 1
<i>IL6ST</i>	42	50	Glycoprotein 130
<i>MAGED2</i>	50	50	Melanoma antigen family D, 2
<i>TFF1</i>	50	50	Trefoil factor 1
<i>VAV3</i>	46	50	Guanine nucleotide exchange factor
<i>ACADSB</i>	38	46	Acyl-CoA dehydrogenase, short/branched chain
<i>GFRA1</i>	33	46	GDNF family receptor alpha-1
<i>INPP4B</i>	46	46	Inositol polyphosphate-4-phosphatase
<i>KIAA1324</i>	29	46	Estrogen-induced gene 121
<i>PGR</i>	29	46	Progesterone receptor
<i>SCNN1A</i>	54	46	Sodium channel, non-voltage-gated 1 alpha subunit
<i>SLC44A4</i>	54	46	Choline transporter-like protein 4
<i>SLC7A8</i>	33	46	Solute carrier family 7 (amino acid transporter light chain, L system)
<i>SPDEF</i>	54	46	SAM pointed domain-containing ETS transcription factor
<i>BCL2</i>	42	42	B-cell lymphoma 2
<i>C9orf116</i>	29	42	Chromosome 9 open reading frame 116
<i>CACNA1D</i>	33	42	Calcium channel, voltage-dependent, L type, alpha 1D subunit
<i>EVL</i>	38	42	Enah/Vasp-like
<i>GAMT</i>	33	42	Guanidinoacetate N-methyltransferase
<i>GPD1L</i>	42	42	Glycerol-3-phosphate dehydrogenase 1-like
<i>NME5</i>	38	42	NME/NM23 family member 5
<i>SERPINA5</i>	29	42	Serpin peptidase inhibitor, clade A (alpha-1 antitrypsin, antitrypsin), member 5
<i>STC2</i>	33	42	Stanniocalcin-related protein
<i>SYBU</i>	38	42	Syntaxin (syntaxin-interacting)
<i>TTC39A</i>	42	42	Tetratricopeptide repeat domain 39A
<i>ZMYND10</i>	33	42	Zinc finger, MYND-type containing 10
<i>AFF3</i>	29	38	AF4/FMR2 family, member 3

Table V. Continued.

Overlap of <i>ESR1</i> and <i>FOXAI</i> (≥ 5 studies, <i>ESR1</i> =143, <i>FOXAI</i> =138, overlapping genes=115)			
Gene	<i>FOXAI</i> (%)	<i>ESR1</i> (%)	Function
<i>AGR3</i>	25	38	Anterior gradient 3 homolog (xenopus laevis)
<i>AR</i>	46	38	Androgen receptor
<i>FAM174B</i>	46	38	Family with sequence similarity 174, member B
<i>SIDT1</i>	46	38	SID1 transmembrane family, member 1
<i>THSD4</i>	25	38	Thrombospondin, type I, domain containing 4
<i>TSPAN1</i>	54	38	Tetraspanin 1
<i>CLSTN2</i>	21	33	Calsyntenin 2
<i>CYP2B6</i>	21	33	Cytochrome P450, family 2, subfamily B, polypeptide 6
<i>CYP2B7P1</i>	25	33	Cytochrome P450, family 2, subfamily B, polypeptide 7 pseudogene 1
<i>ELOVL5</i>	29	33	ELOVL fatty acid elongase 5
<i>FAM134B</i>	25	33	Family with sequence similarity 134, member B
<i>KCNK15</i>	29	33	Potassium channel, subfamily K, member 15
<i>RERG</i>	21	33	RAS-like, estrogen-regulated, growth inhibitor
<i>RHOB</i>	42	33	Ras homolog family member B
<i>SLC16A6</i>	33	33	Solute carrier family 16, member 6 (monocarboxylic acid transporter 7)
<i>SLC22A5</i>	21	33	Solute carrier family 22 (organic cation/carnitine transporter), member 5
<i>UGCG</i>	25	33	UDP-glucose ceramide glucosyltransferase
<i>ZNF552</i>	38	33	Zinc finger protein 552
<i>ABCC8</i>	21	29	ATP-binding cassette transporter sub-family C member 8
<i>C5orf30</i>	21	29	Chromosome 5 open reading frame 30
<i>C6orf97</i>	21	29	Chromosome 6 open reading frame 97
<i>CYB5A</i>	29	29	Cytochrome B5 type A (microsomal)
<i>DYNLRB2</i>	21	29	Dynein, light chain, roadblock-type 2
<i>GSTM3</i>	25	29	Glutathione S-transferase mu 3 (brain)
<i>IRS1</i>	21	29	Insulin Receptor Substrate 1
<i>MAST4</i>	25	29	Microtubule associated serine/threonine kinase family member 4
<i>MCCC2</i>	29	29	Methylcrotonoyl-CoA carboxylase 2 (beta)
<i>MTL5</i>	29	29	Metallothionein-like 5, testis-specific (tesmin)
<i>PNPLA4</i>	25	29	Patatin-like phospholipase domain containing 4
<i>PTPRT</i>	21	29	Protein tyrosine phosphatase, receptor type, T
<i>RABEP1</i>	25	29	Rabaptin, RAB GTPase binding effector protein 1
<i>SEMA3B</i>	25	29	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B
<i>SIAH2</i>	29	29	Siah E3 ubiquitin protein ligase 2
<i>SYT17</i>	25	29	Synaptotagmin XVII
<i>TSPAN13</i>	33	29	Tetraspanin 13
<i>ABLIM3</i>	21	25	Actin binding LIM protein family, member 3
<i>AKR7A3</i>	29	25	Aldo-keto reductase family 7, member a3 (aflatoxin aldehyde reductase)
<i>C10orf116</i>	29	25	Chromosome 10 open reading frame 116
<i>CRIP1</i>	29	25	Cysteine-rich protein 1 (intestinal)
<i>ERBB3</i>	21	25	V-Erb-B2 erythroblastic leukemia viral oncogene homolog 3 (avian)
<i>FSIP1</i>	21	25	Fibrous sheath interacting protein 1
<i>GALNT6</i>	33	25	Polypeptide N-acetylgalactosaminyltransferase 6
<i>INPP5J</i>	25	25	Inositol polyphosphate-5-phosphatase J
<i>KCTD3</i>	21	25	Potassium channel tetramerisation domain containing 3
<i>KIF5C</i>	25	25	Kinesin family member 5C
<i>MED13L</i>	25	25	Mediator complex subunit 13-like
<i>RAB17</i>	33	25	Ras-related protein Rab-17
<i>RBM47</i>	33	25	RNA binding motif protein 47
<i>SCCPDH</i>	25	25	Saccharopine dehydrogenase (putative)

Table V. Continued.

Overlap of <i>ESR1</i> and <i>FOXA1</i> (≥ 5 studies, <i>ESR1</i> =143, <i>FOXA1</i> =138, overlapping genes=115)			
Gene	<i>FOXA1</i> (%)	<i>ESR1</i> (%)	Function
<i>SEMA3F</i>	25	25	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3F
<i>SLC2A10</i>	29	25	Solute carrier family 2 (facilitated glucose transporter), member 10
<i>TBX3</i>	21	25	T-box protein 3
<i>TOX3</i>	38	25	TOX high mobility group box family Member 3
<i>WFS1</i>	21	25	Wolfram syndrome 1 (wolframin)
<i>CAMK2N1</i>	25	21	Calcium/calmodulin-dependent protein kinase II inhibitor 1
<i>EFHC1</i>	21	21	EF-hand domain (C-terminal) containing 1
<i>FAAH</i>	21	21	Fatty acid amide hydrolase
<i>GALNT7</i>	29	21	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 7 (GalNAc-T7)
<i>KIAA0040</i>	21	21	Uncharacterized protein KIAA0040
<i>LASS6</i>	29	21	LAG1 homolog, ceramide synthase 6
<i>LRBA</i>	21	21	LPS-responsive vesicle trafficking, beach and anchor containing
<i>METRN</i>	21	21	Meteorin, glial cell differentiation regulator
<i>MREG</i>	21	21	Melanoregulin
<i>RAB26</i>	29	21	RAB26, member RAS oncogene family
<i>RNF103</i>	21	21	Ring finger protein 103

FOXA1, forkhead box protein A1; *ESR1*, estrogen receptor 1.

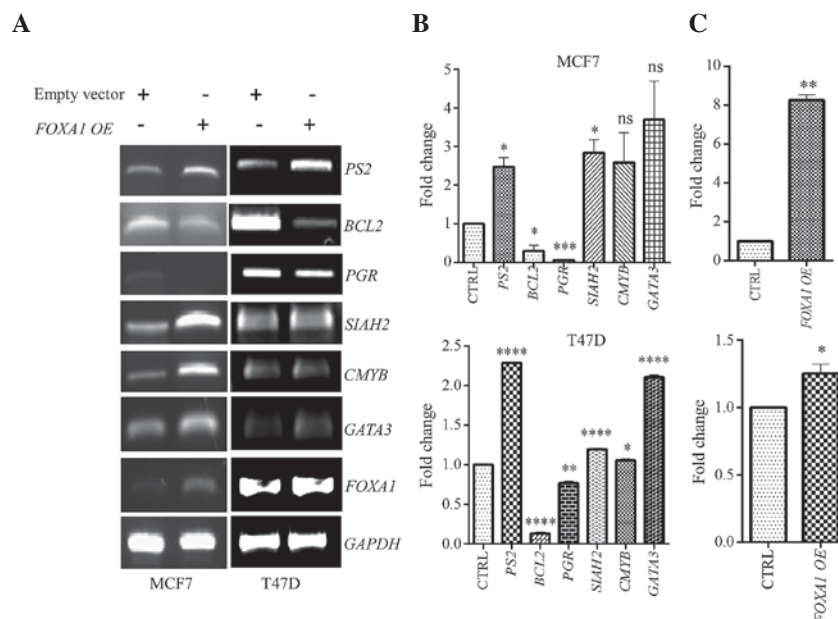


Figure 3. Gene expression analysis using RT-PCR and RT-qPCR. Several identified genes from the *FOXA1:ESR1* overlapping cluster were examined following ectopic *FOXA1* expression in *ESR1*-positive MCF7 and T47D cell lines at 24 h post-transfection. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control. (A) Gene expression of *FOXA1:ESR1* overlapping genes using RT-PCR. (B) Gene expression of *FOXA1:ESR1* overlapping genes using RT-qPCR. (C) *FOXA1* overexpression following *FOXA1* ectopic expression, as determined by RT-qPCR. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, vs. the control. ns, not significant; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; *BCL2*, B-cell lymphoma 2; *PGR*, progesterone receptor; *GATA3*, GATA binding protein 3; *FOXA1*, forkhead box protein A1; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; CTRL, control; OE, overexpression; *PS2*, trefoil factor 1; *SIAH2*, seven in absentia homolog 2; *CMYB*, cellular myeloblastosis viral oncogene homolog.

ESR1:FOXA1 gene cluster. Approximately 79 genes were co-expressed in all the three microarrays: *ESR1*, *FOXA1* and *GATA3*. Notably, in both co-expression overlaps

(*FOXA1:ESR1* and *FOXA1:ESR1:GATA3*), the majority of genes were involved in signal transduction (Figs. 1C and 2A), thus suggesting a prominent role of these genes in BC

Table VI. Comparison of *ESR1* and *FOXA1* co-expression Oncomine™ analysis with 51 estrogen-upregulated genes reported by Tozlu *et al* (71).

<i>ESR1</i>	Co-expression Oncomine™	<i>FOXA1</i>	Co-expression Oncomine™
<i>ESR1</i>	+	<i>FOXA1</i>	+
<i>CA12</i>	+	<i>ESR1</i>	+
<i>GATA3</i>	+	<i>GATA3</i>	+
<i>NAT1</i>	+	<i>MLPH</i>	
<i>SLC39A6</i>	+	<i>AGR2</i>	
<i>TBC1D9</i>		<i>CA12</i>	+
<i>DNALI1</i>		<i>TFF3</i>	+
<i>FOXA1</i>	+	<i>XBP1</i>	+
<i>ANXA9</i>		<i>NAT1</i>	+
<i>DNAJC12</i>	+	<i>SLC39A6</i>	+
<i>TFF3</i>	+	<i>SPDEF</i>	
<i>ERBB4</i>	+	<i>TSPAN1</i>	
<i>MLPH</i>		<i>DNAJC12</i>	+
<i>MYB</i>	+	<i>FBP1</i>	
<i>XBP1</i>	+	<i>GREB1</i>	
<i>FBP1</i>		<i>MYB</i>	+
<i>IL6ST</i>	+	<i>TFF1</i>	+
<i>MAGED2</i>		<i>AR</i>	+
<i>TFF1</i>	+	<i>FAM174B</i>	
<i>ACADSB</i>	+	<i>KDM4B</i>	
<i>PGR</i>	+	<i>ABAT</i>	
<i>SCNN1A</i>		<i>BCL2</i>	+
<i>SLC7A8</i>		<i>IL6ST</i>	+
<i>BCL2</i>	+	<i>TTC39A</i>	
<i>C9orf116</i>		<i>ACADSB</i>	+
<i>CACNA1D</i>		<i>ERBB4</i>	+
<i>STC2</i>	+	<i>CACNA1D</i>	
<i>AR</i>	+	<i>RBM47</i>	
<i>THSD4</i>		<i>STC2</i>	+
<i>CYP2B6</i>	+	<i>AFF3</i>	
<i>RERG</i>	+	<i>CYB5A</i>	
<i>C6orf97</i>		<i>PGR</i>	+
<i>PTPRT</i>	+	<i>GPR160</i>	
<i>RABEP1</i>	+	<i>GSTM3</i>	
<i>SEMA3B</i>	+	<i>INPP5J</i>	
<i>AKR7A3</i>		<i>RABEP1</i>	+
<i>CACNA2D2</i>		<i>SEMA3B</i>	+
<i>RAB17</i>		<i>CYP2B6</i>	+
<i>CAMK2N1</i>		<i>KRT18</i>	+
<i>CCDC74B</i>		<i>LRBA</i>	+
<i>FAAH</i>		<i>PTPRT</i>	+
<i>KIAA0040</i>		<i>RERG</i>	+
<i>LRBA</i>	+	<i>SLC19A2</i>	
<i>HPN</i>	+	<i>EEF1A2</i>	
<i>MYO5C</i>		<i>HPN</i>	+

FOXA1, forkhead box protein A1; *ESR1*, estrogen receptor 1.

Table VII. Comparison of *ESR1* and forkhead box protein A1 co-expression Oncomine™ analysis with the direct targets of *ESR1* (39).

Genes	Expression pattern
<i>STC2</i>	↑
<i>GREB1</i>	↑
<i>SIAH2</i>	↑
<i>PGR</i>	↑
<i>IL6ST</i>	↑
<i>NRIP1</i>	↑
<i>ADCY9</i>	↑
<i>CCNG2</i>	↓

ESR1, estrogen receptor 1; ↑, upregulation; ↓, downregulation.

tumorigenesis. Lin *et al* demonstrated by whole-genome microarray analysis that 137 genes were regulated by *ESR1* out of the ~19,000 genes surveyed (75). However, only 89 of the 137 *ESR1*-regulated genes were direct targets of *ESR1*. When the overlapping co-expression gene clusters (*FOXA1:ESR1* or *FOXA1:ESR1:GATA3*) were compared with the Lin *et al* data (74), only 8 genes were observed to be direct target genes (Table VII). One of the possible reasons for such low detection of *ESR*-responsive genes may be the absence of a responsive DNA element or non-genomic binding through specificity protein 1, activator protein 1 or specificity protein 3 (76-78). The pie chart and Venn diagram based on pathways of overlapping co-expression cluster genes of *FOXA1:ESR1* and *FOXA1:ESR1:GATA3* are shown in Fig. 1A-C and Fig. 2A and B, respectively.

FOXA1, also known as hepatocyte nuclear factor 3 α , is a member of the forkhead class of DNA-binding proteins, and is co-expressed with *ESR1* in BC luminal subtype A (49,79). Importantly, it has been previously reported that *FOXA1*-mediated chromatin changes were not influenced by E2 treatment, but contributed to the recruitment of *ESR* to chromatin by creating optimal binding conditions (70). The co-expression of *ESR1* and *FOXA1* is also associated with the luminal subtype of breast tumors and patient survival (33). Approximately 50% of *ESR*-E2 responsive genes require prior *FOXA1* binding for their optimal expression (32,33). As illustrated in luminal A BC cells MCF7, there is a reduced E2-dependent gene expression and proliferation during *FOXA1* depletion in the cells (30,31). In addition, RNA interference-mediated depletion of *FOXA1* in MCF7 cells leads to a decreased expression of the *PS2*, *BCL2*, *SIAH2* and *CMYB* genes (25). By contrast, in the present study, ectopic *FOXA1* expression was able to regulate the *ESR1* target genes *PS2*, *BCL2*, *PGR*, *SIAH2*, *CMYB* and *GATA3* in both MCF7 and T47D BC cells (Fig. 3A and B). The ectopic expression of *FOXA1* is shown in Fig. 3A and C.

The secretory protein trefoil factor (TFF) 1 or *PS2* is abnormally expressed in ~50% of BCs (80). In mammary carcinoma, forced *PS2* expression resulted in increased cell proliferation and survival in mammary carcinoma cells with anchorage-independent growth, migration and invasion in a xenograft model (81). The present study identified that the *PS2*

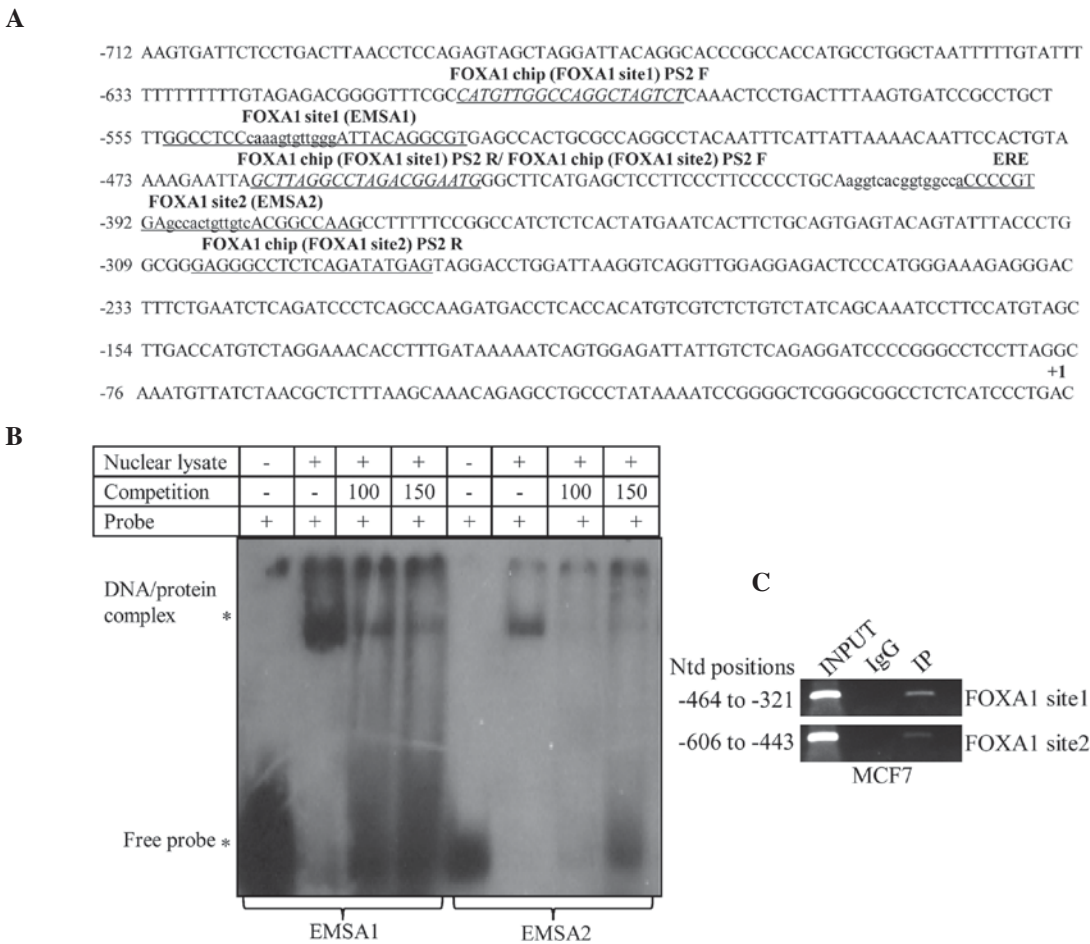


Figure 4. Schematic representation of the PS2 promoter. (A) Schematic diagram showing the presence of a functional estrogen response element (-407 nucleotide position) and two putative FOXA1 binding sites at -384 and -539 nucleotide positions, respectively. (B) *In vitro* binding assay. A total of 30 bp oligonucleotides containing FOXA1 binding sites were labeled with γ^{32} P radioisotope and incubated with nuclear lysate extracted from MCF7 cells. An unlabeled FOXA1 (cold probe) consensus sequence was used for competition at 100 and 150-fold molar excess. The reactions were subjected to electrophoresis in a 6% polyacrylamide gel at 180 V in 0.5X Tris/borate/ethylenediaminetetraacetic acid for ~1 h, and subsequently, the gel was dried and autoradiographed. (C) *In vivo* ChIP assay was performed for FOXA1 binding sites using an anti-FOXA1 antibody. The DNA elute from ChIP was subjected to polymerase chain reaction analysis from -321 to -464 and from -443 to -606 nucleotide positions for site 1 and site 2, respectively. FOXA1, forkhead box protein A1; ERE, estrogen response element; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; IP, immunoprecipitation; IgG, immunoglobulin G; Ntd, nucleotide; PS2, trefoil factor 1.

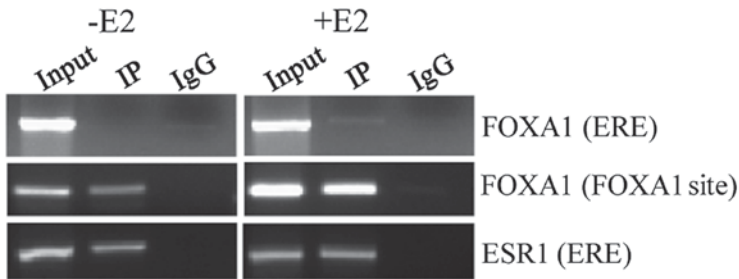


Figure 5. Effect of FOXA1 and ESR1 on the PS2 promoter. For ChIP assay, MCF7 cells in the absence or presence of estradiol stimulation were sonicated, lysed and pre-cleared. ChIP with specific antibodies against FOXA1 and ESR1 along with corresponding control Ig G was performed. The nucleotide positions -573 to -315 and -506 to -344 represent the ERE and FOXA1 binding sequences, respectively, in the PS2 promoter. The eluted ChIP DNA samples were subjected to PCR analysis using ERE or FOXA1 site-specific primers. The PCR samples were electrophoresed in a 2% agarose gel. E2, estradiol; Ntd, nucleotide; IP, immunoprecipitation; IgG, immunoglobulin G; FOXA1, forkhead box protein A1; ERE, estrogen response element; ESR1, estrogen receptor 1; ChIP, chromatin immunoprecipitation; PCR, polymerase chain reaction; PS2, trefoil factor 1.

gene co-expresses with ESR1 and FOXA1, but the molecular pathway involved is not clearly understood. Bioinformatic analysis of the PS2 promoter indicated the presence of two FOXA1 binding sites at 8 bp downstream and 132 bp upstream, respectively, of a molecularly characterized ERE site in the PS2 promoter (Fig. 4A). EMSA confirmed that FOXA1 binds to the PS2 promoter at FOXA1 site 1 (-546 to 534 nucleotide position) and FOXA1 site 2 (-390 to -378 nucleotide position)

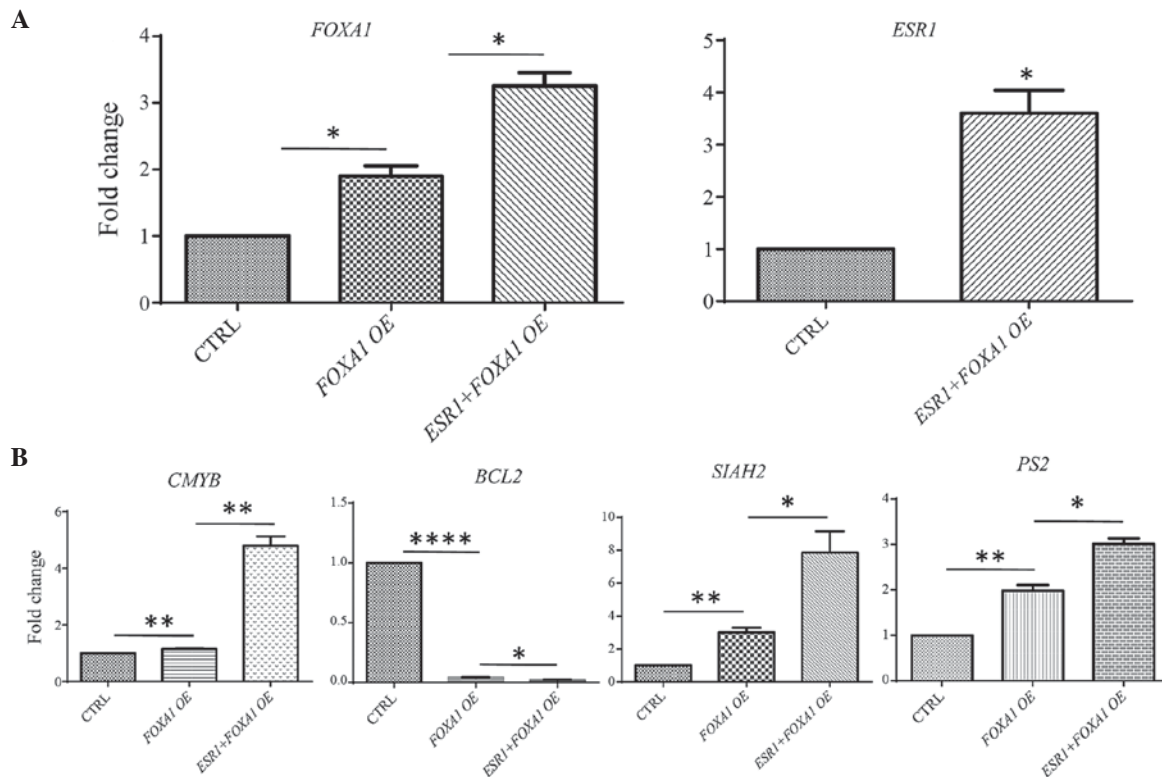


Figure 6. Quantification of target genes regulated by *FOXA1* and *FOXA1/ESR1* extracted from multi-array analysis. RT-qPCR was performed from *FOXA1* and *FOXA1/ESR1*-transfected samples in the T47D cell line. (A) Overexpression of *FOXA1* and *ESR1* was confirmed by RT-qPCR in *FOXA1* and *FOXA1/ESR1*-co-transfected cells. (B) Effect of *FOXA1* and *FOXA1/ESR1* transfection on the target genes (*CMYB*, B-cell lymphoma 2, *SIAH2* and *PS2*) at 24 h post-transient transfection in T47D cells. The bar diagram represents data derived from triplicate experiments. * $P \leq 0.05$, ** $P \leq 0.001$, *** $P \leq 0.001$, **** $P \leq 0.0001$. CTRL, control; *FOXA1*, forkhead box protein A1; *ESR1*, estrogen receptor 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; OE, overexpression; *PS2*, trefoil factor 1; *SIAH2*, seven in absentia homolog 2; *CMYB*, cellular myeloblastosis viral oncogene homolog.

(Fig. 4B and C). To confirm the specificity of EMSA binding, cold probe (non-radioactively labeled) competition with FOXA1 consensus sequence was performed for both EMSA1 and EMSA2 sequences. With increasing concentrations of cold probe (100-150-fold) there was a clear indication of cold probe competition, as observed by the decreased protein-DNA complex (Fig. 4B). *In vivo* ChIP assay also confirmed FOXA1 binding in both sites using an anti-FOXA1 antibody (Fig. 4C). A similar *in vitro* assay for the ERE site in *PS2* was not performed, as it was confirmed previously by Amiry *et al* (81). Notably, an enhanced recruitment of FOXA1 to its site was also observed during E2 stimulation. Subsequently, enhanced FOXA1 recruitment to the FOXA1 site also resulted in elevated levels of ESR1 recruitment to the ERE site of the *PS2* gene. In addition, there was also a slight recruitment of FOXA1 to the ERE site during E2 stimulation (Fig. 5). To understand the effect of *ESR1* and *FOXA1* co-expression on the *PS2* gene and other *FOXA1/ESR1* co-regulated genes, transient transfection was performed in ESR1⁺ T47D BC cells. *PS2* along with *CMYB*, *BCL2* and *SIAH2* were significantly regulated by *FOXA1*, and co-transfection with *ESR1* expression plasmid suggested an interaction between these genes. Importantly, the regulation was significantly enhanced during *ESR1* and *FOXA1* co-transfection compared with only *FOXA1*-transfected cells (Fig. 6). For example, the target genes *CMYB*, *SIAH2* and *PS2* were significantly upregulated upon co-transfection with *ESR1/FOXA1* expression plasmids, thus suggesting a co-regulatory function of *ESR1/FOXA1* on

the above target genes. In the case of the *PS2* gene, *FOXA1* and *ESR1* responsive elements were observed to be separated by ~122 nucleotides (Fig. 4A). Therefore, one of the probable reasons for enhanced *PS2* transcription during *FOXA1/ESR1* co-transfection may be the recruitment of *ESR1* and *FOXA1* to their respective responsive sites, thereby causing a synergistic effect. However, the presence of FOXA1 sites adjacent to ERE in the promoter of other target genes remains to be determined. In addition to *PS2*, the established target gene of *ESR1*, other genes such as *BCL2*, *PGR*, *SIAH2* and *CMYB* were also detected in both the co-expression overlapping genes and in individual microarrays with *ESR1* and *FOXA1*, which suggests the validity of the present meta-analysis.

In addition to extrapolating highly correlated overlapping genes, the present study also enabled the comparison of genes that may not always have high correlation coefficient values, and provide an advantage in clustering co-expression overlapping genes based on their pathway (Figs. 1C and 2B). In addition to the ESR-established pathway genes (*GATA3*, growth regulation by estrogen in breast cancer 1, *TFF1*, *TFF3*, epidermal growth factor receptor 4, *MYB*, *PGR* and *BCL2*), novel pathways can be proposed according to the results of the present study, including protein folding (DnaJ heat shock protein family 40 member C12), development and differentiation (neural proliferation, differentiation and control 1, anterior gradient 2, metallothionein-like 5, semaphorin 3B, actin-binding LIM protein 3, chromosome 10 open reading frame 116, T-box 3 and meteorin) and metabolism (solute

carrier family 39, member 6, 4-aminobutyrate aminotransferase, elongation of very long chain fatty acids protein 5, methylcrotonoyl-CoA carboxylase 2 and cytochrome P450 2B6), which have a direct and indirect influence during tumorigenesis.

In the present study, co-expression analysis has been used to depict overlapping co-regulatory genes in known pathways; however, this analysis has certain caveats. First, the overlapping genes were clustered based on gene ontology data. Second, the clustered meta-analysis genes are only a predictive hypothesis, which requires experimental validation. Third, it may be possible that a number of true *FOXA1:ESR1* pathways interacting partners are lost due to the stringency used in the analysis. However, the present analysis provides novel pathways for assessing the *FOXA1:ESR1* and *FOXA1:ESR1:GATA3* signaling pathway axes, particularly in breast tumorigenesis.

In conclusion, Oncomine™ co-expression meta-analysis provided a cluster of genes with definitive pathways based on stronger co-expression co-efficient analysis using different microarrays, which may be of higher significance than a single microarray. To the best of our knowledge, the present is the first study to provide insight into *FOXA1:ESR1* and *FOXA1:ESR1:GATA3* co-expressed genes involved in BC tumorigenesis. The microarray analysis also provides information on novel intricate pathways, including protein folding, metabolism, development and differentiation. To understand the role of these predictive pathways, a future experimental model is required to further validate the present findings.

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