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 significantly 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 *GATA*3 co-expressed genes that may be involved in breast tumorigenesis.

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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

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 Oncomine™ 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 Oncomine™ 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 Oncomine™ 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 Oncomine™ 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

Oncomine™ analysis. Oncomine™ is an integrated cancer microarray database and web-based data-mining platform (44). Oncomine™ analysis was performed as previously described (42,43). The co-expressed genes correlated with *FOXA1* and *ESR1* were searched for in the Oncomine™ 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 microarray used for the analysis.

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

[a]According 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% CO_2 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 $2^{-\Delta\Delta Cq}$ 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 $MgCl_2$, 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 $MgCl_2$, 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 γ^{32}P 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 $MgCl_2$]. 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-*FOXA1*	F: GGGTGGCTCCAGGATGTTAGG	194
	R: GGGTCATGTTGCCGCTCGTAG	
RT-*GATA3*	F: CAGACCACCACAACCACACTCT	124
	R: GGATGCCTCCTTCTTCATAGTCA	
RT-*PGR*	F: CGCGCTCTACCCTGCACTC	121
	R: TGAATCCGGCCTCAGGTAGTT	
RT-*CMYB*	F: GAAGGTCGAACAGGAAGGTTATCT	224
	R: GTAACGCTACAGGGTATGGAACA	
RT-*SIAH2*	F: CCTCGGCAGTCCTGTTTCCCTGT	124
	R: CCAGGACATGGGCAGGAGTAGGG	
RT-*BCL2*	F: TGTGGATGACTGAGTACCTG	116
	R: GGAGAAATCAAACAGAGGCC	
RT-*PS2*	F: GAACAAGGTGATCTGCGCCC	223
	R: TTCTGGAGGGACGTCGATGG	
RT-*GAPDH*	F: AAGATCATCAGCAATGCCTC	619
	R: CTCTTCCTCTTGTGCTCTTG	
FOXA1 chip (*FOXA1* site1) *PS2*	F: CATGTTGGCCAGGCTAGTCT	165
	R: CATTCCGTCTAGGCCTAAGC	
FOXA1 chip (*FOXA1* site2) *PS2*	F: GCTTAGGCCTAGACGGAATG	180
	R: CTCATATCTGAGAGGCCCTC	
PS2 chip F (ERE)	F: TTAAGTGATCCGCCTGCTTT	271
	R: CTCCCGCCAGGGTAAATACT	
FOXA1 consensus site	F: CTTATGCAATGTGTTGGTCTCACG	
	R: CGTGAGACCAACACATTGCATAAG	
FOXA1 EMSA (*FOXA1* site1) *PS2*	GGCCTCCCAAAGTGTTGGGATTACAGGCGT	
	ACGCCTGTAATCCCAACACTTTGGGAGGCC	
FOXA1 EMSA (*FOXA1* site2) *PS2*	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-FOXA1 (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 (%)
FOXA1	100
ESR1	67
GATA3	67
MLPH	67
AGR2	63
CA12	63
TFF3	63
XBP1	63
NAT1	58
SLC39A6	58
TBC1D9	58
DNALI1	54
SCNN1A	54
SLC44A4	54
SPDEF	54
TSPAN1	54
ANXA9	50
DNAJC12	50
FBP1	50
GREB1	50
MAGED2	50
MAPT	50
MYB	50
TFF1	50
AR	46
FAM174B	46
INPP4B	46
KDM4B	46
SCUBE2	46
SIDT1	46
VAV3	46
ABAT	42
BCL2	42
GPD1L	42
IL6ST	42
RHOB	42
TTC39A	42
ACADSB	38
ERBB4	38
EVL	38
NME5	38
SYBU	38
TOX3	38
ZNF552	38
CACNA1D	33
DACH1	33
GALNT6	33
GAMT	33
GFRA1	33
RAB17	33
RBM47	33
SLC16A6	33

Table III. Continued.

Gene	Percentage of co-expression (%)
SLC7A8	33
STC2	33
TSPAN13	33
ZMYND10	33
AFF3	29
AKR7A3	29
C10orf116	29
C9orf116	29
CRIP1	29
CYB5A	29
ELOVL5	29
GALNT7	29
KCNK15	29
KIAA1324	29
LASS6	29
MCCC2	29
MTL5	29
PGR	29
RAB26	29
SERPINA5	29
SIAH2	29
SLC2A10	29
AGR3	25
CAMK2N1	25
CYP2B7P1	25
FAM134B	25
GPR160	25
GSTM3	25
INPP5J	25
KIF5C	25
MAST4	25
MED13L	25
NPDC1	25
PNPLA4	25
PP14571	25
RABEP1	25
SCCPDH	25
SEMA3B	25
SEMA3F	25
STARD10	25
SYT17	25
THSD4	25
UGCG	25
ABCC8	21
ABLIM3	21
BCAS1	21
C5orf30	21
C6orf97	21
C9orf152	21
CLSTN2	21
CYP2B6	21
DHCR24	21

Table III. Continued.

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

Table III. Continued.

Gene	Percentage of co-expression (%)
CYP4B1	17
DEGS2	17
EEF1A2	17
FAM110C	17
FUT8	17
HHAT	17
HPN	17
IGF1R	17
KIAA0232	17
KIAA1244	17
KRT8	17
LRIG1	17
MEIS3P1	17
MKL2	17
MYST4	17
NBEA	17
NPNT	17
NRIP1	17
PBX1	17
PCSK6	17
RAB27B	17
RALGPS2	17
RND1	17
SLC9A3R1	17
SPRED2	17
STK32B	17
WWP1	17
ZNF703	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 ± 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 (%)
ESR1	100
CA12	79
GATA3	79
NAT1	71
SLC39A6	71
TBC1D9	71
DNALI1	67
FOXA1	67
ANXA9	63
DNAJC12	63
GREB1	63
MAPT	63
ABAT	58
SCUBE2	58
TFF3	58
ERBB4	54
KDM4B	54
MLPH	54
MYB	54
XBP1	54
AGR2	50
DACH1	50
FBP1	50
IL6ST	50
MAGED2	50
TFF1	50
VAV3	50
ACADSB	46
GFRA1	46
INPP4B	46
KIAA1324	46
PGR	46
SCNN1A	46
SLC44A4	46
SLC7A8	46
SPDEF	46
BCL2	42
C9orf116	42
CACNA1D	42
EVL	42
GAMT	42
GPD1L	42
NME5	42
SERPINA5	42
STC2	42
SYBU	42
TTC39A	42
ZMYND10	42
AFF3	38
AGR3	38
AR	38
FAM174B	38

Table IV. Continued.

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

Table IV. Continued.

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

Table IV. Continued.

Gene	Percentage of co-expression (%)
DHCR24	17
GDF15	17
HPN	17
KIAA0232	17
LONRF2	17
MKL2	17
MYO5C	17
MYST4	17
NKAIN1	17
PARD6B	17
PBX1	17
PCP2	17
PCSK6	17
PECI	17
PLAT	17
PLCD4	17
PP14571	17
PP1R3C	17
PREX1	17
PRLR	17
RALGPS2	17
RARA	17
REEP1	17
REEP6	17
SEC14L2	17
SEMA3C	17
SERPINA3	17
SLC19A2	17
SLC22A18	17
SLC27A2	17
SSH3	17
STARD10	17
SYTL2	17
TCEAL1	17
TMEM25	17
TMEM30B	17
TP53TG1	17
TPRG1	17
WNK4	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 (≥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 (≥20%), ~115 genes overlapped in

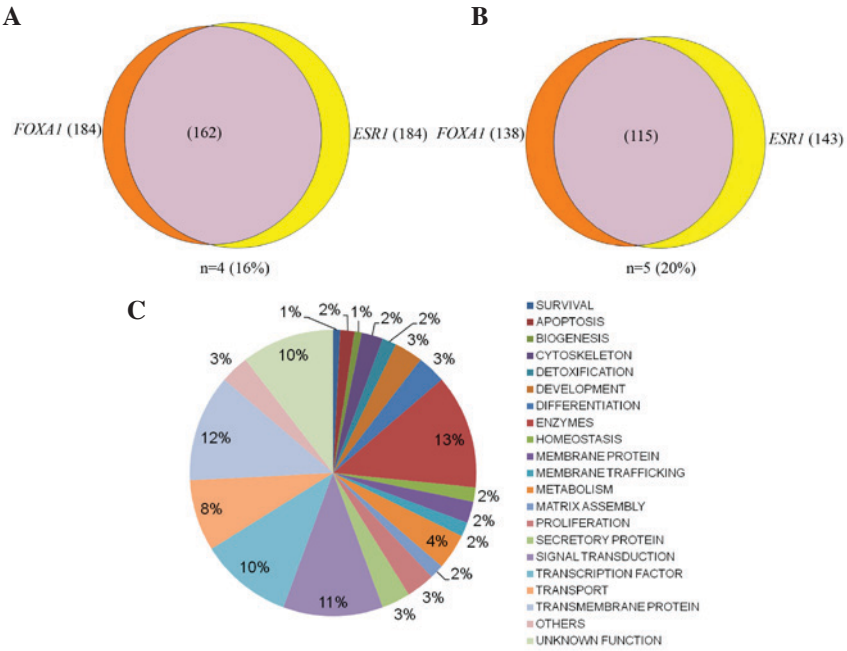

Figure 1. Analysis of overlapping *FOXA1* and *ESR1* co-expression genes. Venn diagrams depicting genes overlapping with *FOXA1* and *ESR1* with a cut-off frequency of (A) 4 (~16%) and (B) 5 (~20%) by meta-analysis with Oncomine™. (C) Pie chart of functional categories for *FOXA1:ESR1* overlapping genes 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.

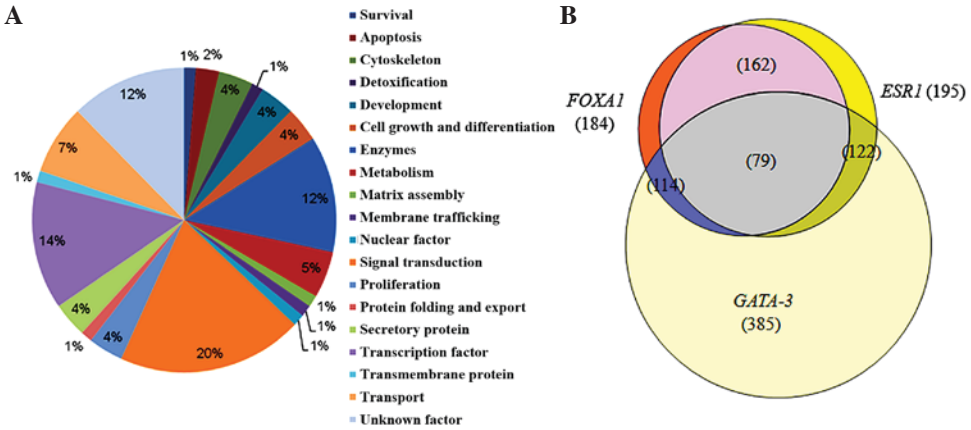

Figure 2. Analysis of overlapping *FOXA1*, *ESR1* and *GATA*3 co-expression genes. (A) Pie chart of *FOXA1, ESR1* and *GATA3* overlapping genes with a cut-off frequency of 4. (B) Pathway pie chart of *FOXA1, ESR1* and *GATA3* overlapping genes with a cut-off frequency of 4. The *FOXA1* and *ESR1* Oncomine™ microarray analysis consisted of 4 normal and 20 breast cancer microarray data. The *GATA*3 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 *ESR1* and *FOXA1* (≥5 studies, *ESR1*=143, *FOXA1*=138, overlapping genes=115)		
Gene	*FOXA1 (%)*	*ESR1 (%)*	Function
ESR1	67	100	Estrogen receptor 1
CA12	63	79	Carbonic anhydrase 12
GATA3	67	79	GATA binding protein 3
NAT1	58	71	NAT1 N-acetyltransferase 1
SLC39A6	58	71	Zinc transporter ZIP6
TBC1D9	58	71	TBC1 domain family member 9
DNALI1	54	67	Axonemal dynein light intermediate polypeptide 1
FOXA1	100	67	Forkhead box protein A1
ANXA9	50	63	Annexin A9
DNAJC12	50	63	DnaJ homolog subfamily C member 12
GREB1	50	63	Growth regulation by estrogen in breast cancer 1
MAPT	50	63	Microtubule-associated protein tau
NPDC1	25	63	Neural proliferation differentiation and control protein 1
ABAT	42	58	4-aminobutyrate aminotransferase
SCUBE2	46	58	Signal peptide, CUB domain, EGF-like 2
TFF3	63	58	Trefoil factor 3
ERBB4	38	54	Receptor tyrosine-protein kinase erbB-4
KDM4B	46	54	Lysine (K)-specific demethylase 4B
MLPH	67	54	Melanophilin
MYB	50	54	Myb proto-oncogene protein
XBP1	63	54	X-box binding protein 1
AGR2	63	50	Anterior gradient homolog 2
DACH1	33	50	Dachshund homolog 1
FBP1	50	50	Fructose-1,6-bisphosphatase 1
IL6ST	42	50	Glycoprotein 130
MAGED2	50	50	Melanoma antigen fmily D, 2
TFF1	50	50	Trefoil factor 1
VAV3	46	50	Guanine nucleotide exchange factor
ACADSB	38	46	Acyl-CoA dehydrogenase, short/branched chain
GFRA1	33	46	GDNF family receptor alpha-1
INPP4B	46	46	Inositol polyphosphate-4-phosphatase
KIAA1324	29	46	Estrogen-induced gene 121
PGR	29	46	Progesterone receptor
SCNN1A	54	46	Sodium channel, non-voltage-gated 1 alpha subunit
SLC44A4	54	46	Choline transporter-like protein 4
SLC7A8	33	46	Solute carrier family 7 (amino acid transporter light chain, L system)
SPDEF	54	46	SAM pointed domain-containing ETS transcription factor
BCL2	42	42	B-cell lymphoma 2
C9orf116	29	42	Chromosome 9 open reading frame 116
CACNA1D	33	42	Calcium channel, voltage-dependent, L type, alpha 1D subunit
EVL	38	42	Enah/Vasp-like
GAMT	33	42	Guanidinoacetate N-methyltransferase
GPD1L	42	42	Glycerol-3-phosphate dehydrogenase 1-like
NME5	38	42	NME/NM23 family member 5
SERPINA5	29	42	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5
STC2	33	42	Stanniocalcin-related protein
SYBU	38	42	Syntabulin (syntaxin-interacting)
TTC39A	42	42	Tetratricopeptide repeat domain 39A
ZMYND10	33	42	Zinc finger, MYND-type containing 10
AFF3	29	38	AF4/FMR2 family, member 3

Table V. Continued.

	Overlap of *ESR1* and *FOXA1* (≥5 studies, *ESR1*=143, *FOXA1*=138, overlapping genes=115)		
Gene	*FOXA1 (%)*	*ESR1 (%)*	Function
AGR3	25	38	Anterior gradient 3 homolog (xenopus laevis)
AR	46	38	Androgen receptor
FAM174B	46	38	Family with sequence similarity 174, member B
SIDT1	46	38	SID1 transmembrane family, member 1
THSD4	25	38	Thrombospondin, type I, domain containing 4
TSPAN1	54	38	Tetraspanin 1
CLSTN2	21	33	Calsyntenin 2
CYP2B6	21	33	Cytochrome P450, family 2, subfamily B, polypeptide 6
CYP2B7P1	25	33	Cytochrome P450, family 2, subfamily B, polypeptide 7 pseudogene 1
ELOVL5	29	33	ELOVL fatty acid elongase 5
FAM134B	25	33	Family with sequence similarity 134, member B
KCNK15	29	33	Potassium channel, subfamily K, member 15
RERG	21	33	RAS-like, estrogen-regulated, growth inhibitor
RHOB	42	33	Ras homolog family member B
SLC16A6	33	33	Solute carrier family 16, member 6 (monocarboxylic acid transporter 7)
SLC22A5	21	33	Solute carrier family 22 (organic cation/carnitine transporter), member 5
UGCG	25	33	UDP-glucose ceramide glucosyltransferase
ZNF552	38	33	Zinc finger protein 552
ABCC8	21	29	ATP-binding cassette transporter sub-family C member 8
C5orf30	21	29	Chromosome 5 open reading frame 30
C6orf97	21	29	Chromosome 6 open reading frame 97
CYB5A	29	29	Cytochrome B5 type A (microsomal)
DYNLRB2	21	29	Dynein, light chain, roadblock-type 2
GSTM3	25	29	Glutathione S-transferase mu 3 (brain)
IRS1	21	29	Insulin Receptor Substrate 1
MAST4	25	29	Microtubule associated serine/threonine kinase family member 4
MCCC2	29	29	Methylcrotonoyl-CoA carboxylase 2 (beta)
MTL5	29	29	Metallothionein-like 5, testis-specific (tesmin)
PNPLA4	25	29	Patatin-like phospholipase domain containing 4
PTPRT	21	29	Protein tyrosine phosphatase, receptor type, T
RABEP1	25	29	Rabaptin, RAB GTPase binding effector protein 1
SEMA3B	25	29	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B
SIAH2	29	29	Siah E3 ubiquitin protein ligase 2
SYT17	25	29	Synaptotagmin XVII
TSPAN13	33	29	Tetraspanin 13
ABLIM3	21	25	Actin binding LIM protein family, member 3
AKR7A3	29	25	Aldo-keto reductase family 7, member a3 (aflatoxin aldehyde reductase)
C10orf116	29	25	Chromosome 10 open reading frame 116
CRIP1	29	25	Cysteine-rich protein 1 (intestinal)
ERBB3	21	25	V-Erb-B2 erythroblastic leukemia viral oncogene homolog 3 (avian)
FSIP1	21	25	Fibrous sheath interacting protein 1
GALNT6	33	25	Polypeptide N-acetylgalactosaminyltransferase 6
INPP5J	25	25	Inositol polyphosphate-5-phosphatase J
KCTD3	21	25	Potassium channel tetramerisation domain containing 3
KIF5C	25	25	Kinesin family member 5C
MED13L	25	25	Mediator complex subunit 13-like
RAB17	33	25	Ras-related protein Rab-17
RBM47	33	25	RNA binding motif protein 47
SCCPDH	25	25	Saccharopine dehydrogenase (putative)

Table V. Continued.

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

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

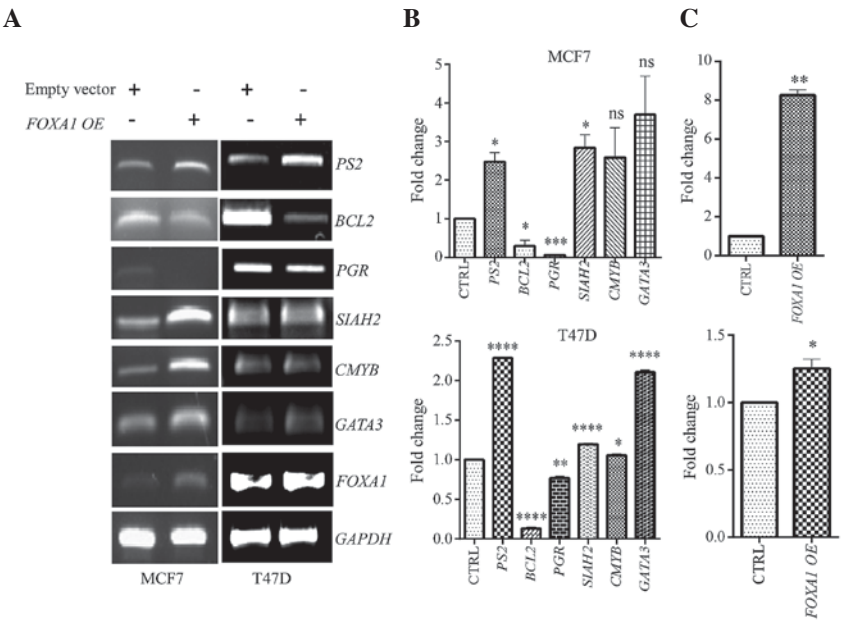

Figure 3. Gene expression analysis using RT-PCR and RT-qPCR. Several identified genes from the *FOXA1:ESR1* overlapping cluster were examined following ectopic *FOXA1* expression in *ESR*-positive MCF7 and T47D cell lines at 24 h post-transfection. Glyceraldehyde 3-phosphate dehydrogenase was used as 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≤0.05, **P≤0.01, ***P≤0.001, ****P≤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).

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

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
STC2	↑
GREB1	↑
SIAH2	↑
PGR	↑
IL6ST	↑
NRIP1	↑
ADCY9	↑
CCNG2	↓

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 3α, 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*

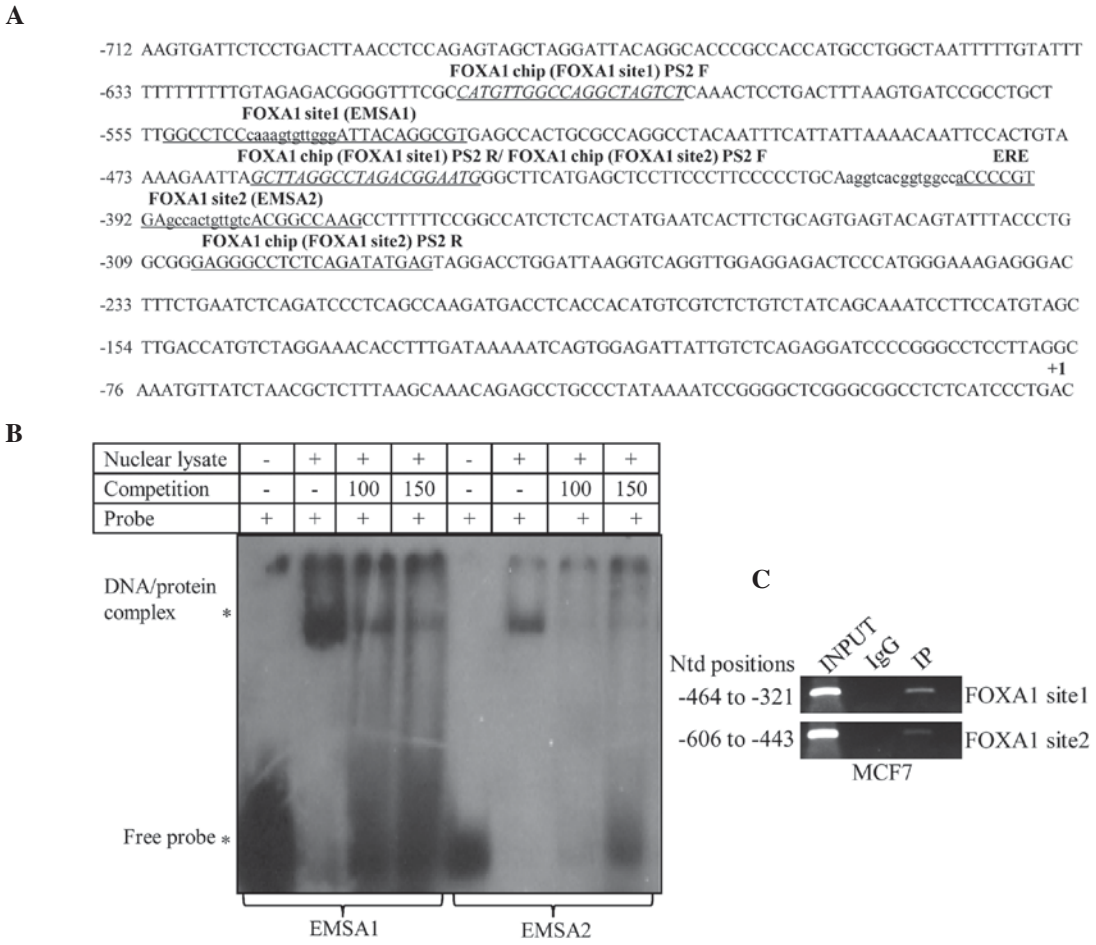

Figure 4. Schematic representation of the *PS2* promoter. (A) Schematic diagram showing the presence of a functional estrogen response element (-407 nucleotide position) and two putative *FOXA1* binding sites at -384 and -539 nucleotide positions, respectively. (B) *In vitro* binding assay. A total of 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.

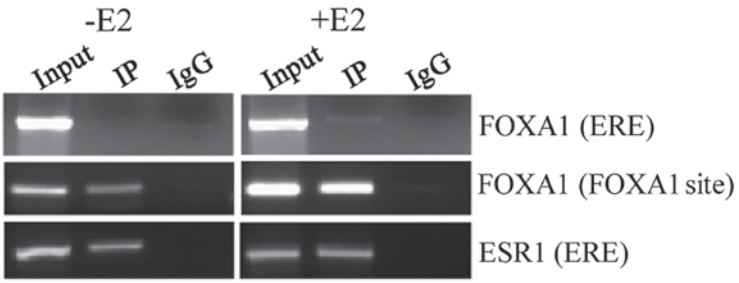

Figure 5. Effect of *FOXA1* and *ESR1* on the *PS2* promoter. For ChIP assay, MCF7 cells in the absence or presence of estradiol stimulation were sonicated, lysed and pre-cleared. ChIP with specific antibodies against FOXA1 and ESR1 along with corresponding control Ig G was performed. The nucleotide 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)

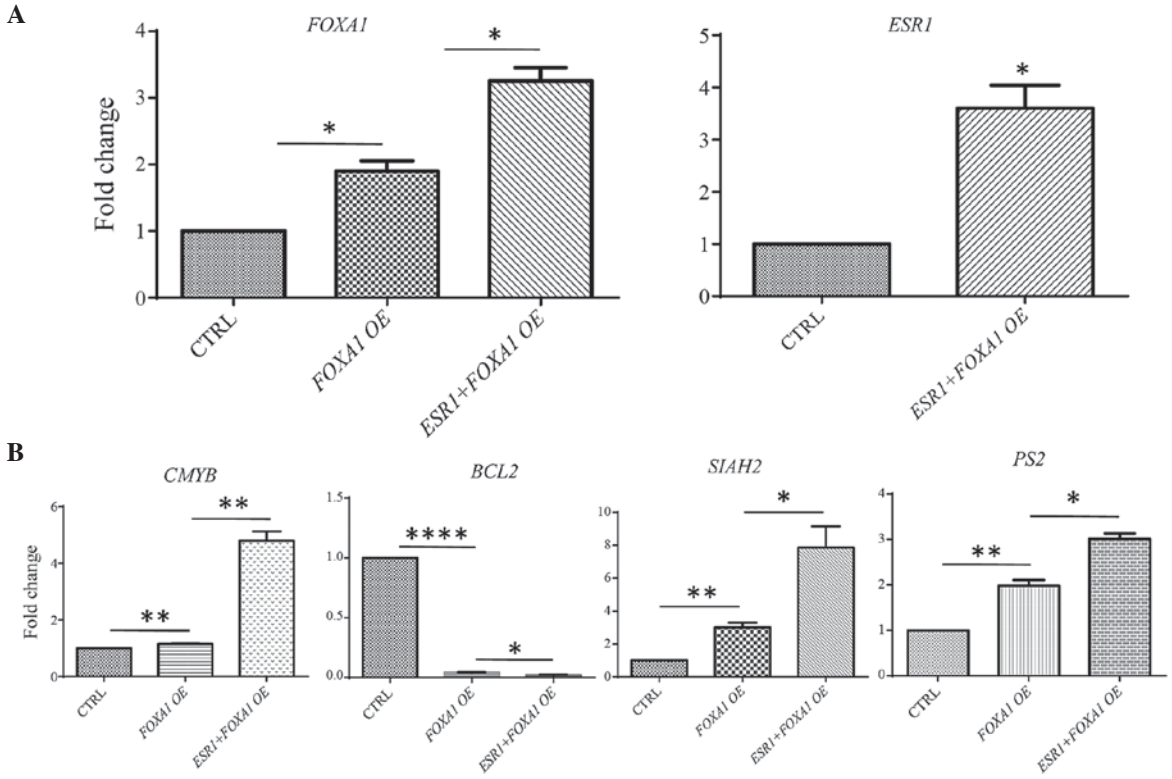

Figure 6. Quantification of target genes regulated by *FOXA1* and *FOXA*/*ESR1* extracted from multi-array analysis. RT-qPCR was performed from *FOXA1* and *FOXA1*/*ESR1*-transfected samples in the T47D cell line. (A) Overexpression of *FOXA1* and *ESR1* was confirmed by RT-qPCR in *FOXA1* and *FOXA1*/*ESR1*-co-transfected 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≤0.05, **P≤0.001, ***P≤0.001, ****P≤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 *FOXA*/*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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