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 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...
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 transferrin (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(T)/T]R[T/G]R[RGY] 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 enhance 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 1) (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 micro-arrays 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<sup>a</sup> | Sample numbers | Ref. |
|-----------------------|------------------|----------------|------|
| Higgins et al         | Normal           | 34             | (45) |
| Roth et al            | Normal           | 353            | (46) |
| Shyamsundar et al     | Normal           | 123            | (47) |
| Tabchý 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) |

<sup>a</sup>According to the Oncomine database acronym.

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^(-ΔΔCq) method (69). The primers used for PCR are listed in Table II. qPCR was performed using SYBR<sup>®</sup> 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<sub>2</sub>, 1 mM ethylene glycol-bis(β-aminopropyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM ethylene-diaminetetraacetic 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<sub>2</sub>, 1 mM EGTA, 1 mM dithiothreitol (DTT) and 1X protease inhibitor cocktail] (Sigma-Aldrich) for 30 min 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<sup>32</sup>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<sub>2</sub>]. 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...
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-Cl (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-Cl (pH 8.1) and 500 mM NaCl], a LiCl wash buffer [0.25 M LiCl, 1% (v/v) NP-40, 1% (v/v) deoxycholic acid (sodium salt), 1 mM EDTA and 10 mM Tris-Cl (pH 8.1)] and 1X Tris/EDTA [10 mM Tris-Cl (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.

| Primers          | Primer sequence (5'-3') | Amplicon size (bp) |
|------------------|-------------------------|--------------------|
| **RT-FOXAI**     | F: GGGTGGCCTCCAGGATGTTAGG | 194                |
|                  | R: GGGTCATGTTGGCGGCTCTGAG |                |
| **RT-GATA3**     | F: CAGACCACCACAACCACACTCT | 124                |
|                  | R: GGATGCTCCCTCTTTCATTGCA |                |
| **RT-PGR**       | F: CGCGCTTACCTGTCACCTC   | 121                |
|                  | R: TGAATCCGGGCCTCAGTGTTT |                |
| **RT-CMYB**      | F: GAAGGTGCAACAGGAAAGTTATCT | 224               |
|                  | R: GTAACGCTACAGGGTAGAACA |                |
| **RT-SIAH2**     | F: CCTCGGCACTGTTCTTCCTTG | 124                |
|                  | R: CCAGGACATGGGACAGGATGG |                |
| **RT-BCL2**      | F: TGTGGAAGCTAGTACCTGTC  | 116                |
|                  | R: GGACAATCAAACAGAGGCC |                |
| **RT-PS2**       | F: GAAAGGTGTATCTGCGCCTCC | 223                |
|                  | R: TTCTGGAGGAGGTCGATGG  |                |
| **RT-GAPDH**     | F: AAGATCATCAGCAATGCTCTC | 619                |
|                  | R: CTCTTCCTTGTGCTCTTG   |                |
| **FOXAI chip (FOXAI site1)** | PS2 | F: CATGTTGCCAGGCTAGTCT | 165           |
|                  | R: CATTCGGCTAGCCCTAAGCC |                |
| **FOXAI chip (FOXAI site2)** | PS2 | F: GCTTAGGCTTACAGGGAATG | 180           |
|                  | R: CTCTATCTGAGAGGCCTCTC |                |
| **PS2 chip F (ERE)** | F: TTAAGTTGATCCGGCTGTCTT | 271           |
|                  | R: CTCCGGCCAAGGTTAAATACT |                |
| **FOXAI consensus site** | F: CTTATGCAATGTGTTGTTCTACAGG |  
|                  | R: CGTGAGACACACACATTGCGAAG |                |
| **FOXAI EMSA (FOXAI site1)** | PS2 | GCCCTCCCCAAAGTGGTGGGATTACGGCGT |  
|                  | ACGCCTGAATCCACACACTTGGGAGGCC |                |
| **FOXAI EMSA (FOXAI site2)** | PS2 | CCCGCCGAGCCACTGTTGCCAGCGCCAAG |  
|                  | CTGGCCGTCGACAACACATGGCCTACAGGGG |                |
| **RT, reverse transcription; FOXAI, 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**
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                              |
| DNAI1  | 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                              |
| GFRα1  | 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                              |
| KIAA124| 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                              |
| ABCG1  | 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                               |
| BB54    | 17                               |
| C1orf28 | 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                               |
| 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                               |
| NRP1P1  | 17                               |
| PBX1    | 17                               |
| PCSK6   | 17                               |
| RAB27B  | 17                               |
| RALGPS2 | 17                               |
| RND1    | 17                               |
| SLC9A3R1| 17                               |
| SPRED2  | 17                               |
| STK32B  | 17                               |
| WWPI    | 17                               |
| ZNF703  | 17                               |

**FOXAI, 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                             |
| CAC2     | 79                              |
| GSTA3    | 79                              |
| NAT1     | 71                              |
| SLCA3A6  | 71                              |
| TCP1D9   | 71                              |
| DNAL1    | 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                              |
| SLC4A4   | 46                              |
| SLC7A8   | 46                              |
| SPDEF    | 46                              |
| BCL2     | 42                              |
| C9orf116 | 42                              |
| CACNA1D  | 42                              |
| CAC1     | 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                              |
| CYPP2B6  | 33                              |
| CYPP2B7P1| 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                              |
| NRP1     | 25                              |
| RAB17    | 25                              |
| RBM47    | 25                              |
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
ESR1 and FOXP1 co-expression genes multi-arrays (Fig. 1B). Table V presents the overlapping genes of ESR1 and FOXP1 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 FOXP1 to enhancer regions of the compact chromatin facilitates remodeling at the ESR1 binding regions (23,30,70); therefore, FOXP1 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 FOXP1 genes were represented in the overlapping, co-expressed FOXP1: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 FOXP1 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%).

| 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                   |
| DNA11I        | 54        | 67       | Axonemal dynein light intermediate polypeptide |
| FOXA1         | 100       | 67       | Forkhead box protein A1                       |
| ANXA9         | 50        | 63       | Annexin A9                                    |
| DNNAC12       | 50        | 63       | Dnal homolog subfamily C member 12            |
| GREB1         | 50        | 63       | Growth regulation by estrogen in breast cancer |
| 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          |
| 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                     |
| XBPI          | 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 family D, 2                  |
| TFF1          | 50        | 50       | Trefoil factor 1                               |
| VAV3          | 46        | 50       | Guanine nucleotide exchange factor            |
| ACADSB        | 38        | 46       | Acyl-CoA dehydrogenase, short/branched chain   |
| GFRAl         | 33        | 46       | GDNF family receptor alpha-1                  |
| INPP4B        | 46        | 46       | Inositol polyphosphate-4-phosphatase          |
| KIAA1324      | 29        | 46       | Estrogen-induced gene 12                      |
| 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            |
| CACNAID       | 33        | 42       | Calcium channel, voltage-dependent, L type, alpha 1D subunit |
| EVL           | 38        | 42       | Enah/Vasp-like                                |
| GAMT          | 33        | 42       | Guanidinoacetate N-methyltransferase          |
| GDPD1L        | 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       | Tetra-tricopeptide repeat domain 39A          |
| ZMYND10       | 33        | 42       | Zinc finger, MYND-type containing 10          |
| AFF3          | 29        | 38       | AF4/FMR2 family, member 3                     |
Table V. Continued.

| 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 16                                 |
| 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                           |
| PTPTT                 | 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 1                                                            |
| 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                                      |
| CRIPI                 | 29        | 25       | Cysteine-rich protein 1 (intestinal)                                     |
| ERBB3                 | 21        | 25       | V-Erb-B2 erythroblastic leukemia viral oncogene homolog 3 (avian)         |
| FSI1                  | 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)                                    |
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.
50% of BCs (genes demonstrated by whole 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 ESR1-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
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)
(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, methylcrotonyl-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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