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FOXA1 Represses the Molecular Phenotype of Basal Breast Cancer Cells

Gina M. Bernardo, Ph.D.[1], **Gurkan Bebek, Ph.D.**[2,6], **Charles L. Ginther, Ph.D.**[7], **Steven T. Sizemore, Ph.D.**[1], **Kristen L. Lozada, M.S.**[1], **John D. Miedler, M.D.**[8], **Lee A. Anderson, Ph.D.**[7], **Andrew K. Godwin, Ph.D.**[9], **Fadi W. Abdul-Karim, M.D.**[3,8], **Dennis J. Slamon, M.D., Ph.D.**[7], and **Ruth A. Keri, Ph.D.**[1,4,5,*]

[1]Departments of Pharmacology, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA

[2]Departments of Case Center for Proteomics and Bioinformatics, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA

[3]Departments of Pathology, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA

[4]Departments of Genetics, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA

[5]Division of General Medical Sciences-Oncology, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA

[6]Genomic Medicine Institute, Cleveland Clinic, Cleveland, OH 44106, USA

[7]Division of Hematology/Oncology, Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA 90095

[8]Department of Pathology, University Hospitals-Case Medical Center, Cleveland, OH, 44106, USA

[9]Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, Kansas City, KS, 66160, USA

Abstract

Breast cancer is a heterogeneous disease comprised of multiple subtypes. Luminal subtype tumors confer a more favorable patient prognosis, which is in part, attributed to estrogen receptor-α (ER) positivity and anti-hormone responsiveness. Expression of the forkhead box transcription factor, FOXA1, similarly correlates with the luminal subtype and patient survival, but is also present in a subset of ER-negative tumors. FOXA1 is also consistently expressed in luminal breast cancer cell

[*]**Contact:** Ruth A. Keri, Ph.D., Case Western Reserve University, School of Medicine, Department of Pharmacology, 10900 Euclid Avenue, Cleveland, OH 44106, Phone - (216) 368-3495, Fax - (216) 368-3395 keri@case.edu.

CONFLICT OF INTEREST

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lines even in the absence of ER. In contrast, breast cancer cell lines representing the basal subtype do not express FOXA1. To delineate an ER-independent role for FOXA1 in maintaining the luminal phenotype, and hence a more favorable prognosis, we performed cDNA microarray analyses on FOXA1-positive, ER-positive (MCF7, T47D) or FOXA1-positive, ER-negative (MDA-MB-453, SKBR3) luminal cell lines in the presence or absence of transient FOXA1 silencing. This resulted in three FOXA1 transcriptomes: (1) a luminal-signature (consistent across cell lines), (2) an ER-positive signature (restricted to MCF7 and T47D) and (3) an ER-negative signature (restricted to MDA-MB-453 and SKBR3). Gene Set Enrichment Analyses (GSEA) revealed FOXA1 silencing causes a partial transcriptome shift from luminal to basal gene expression signatures. FOXA1 binds to a subset of both luminal and basal genes within luminal breast cancer cells, and loss of FOXA1 increases enhancer RNA (eRNA) transcription for a representative basal gene (*CD58*). These data suggest FOXA1 directly represses basal signature genes. Functionally, FOXA1 silencing increases migration and invasion of luminal cancer cells, both characteristics of basal subtype cells. We conclude FOXA1 controls plasticity between basal and luminal breast cancer cells, not only by inducing luminal genes, but also by repressing the basal phenotype, and thus aggressiveness. Although it has been proposed that FOXA1-targeting agents may be useful for treating luminal tumors, these data suggest that this approach may promote transitions toward more aggressive cancers.

Keywords

FOXA1; luminal; basal; breast cancer

INTRODUCTION

Breast cancer is a heterogeneous disease, including at least five molecular subtypes: luminal A, luminal B, HER2, basal-like and normal-like. These subtypes were identified through unsupervised hierarchical clustering of human tumor gene expression data and are predictive of prognosis (1, 2). Specifically, luminal A tumors are estrogen receptor-α (ER) positive conferring a more favorable prognosis that is partially due to the efficacy of anti-hormone therapies (3). Conversely, patients with basal tumors have a poorer prognosis due to intrinsic chemotherapeutic resistance and lack of targeted therapies (4). While basal tumors were originally postulated to arise from the basal lineage, more recent evidence suggests that they are more similar to the normal luminal progenitor population (5, 6). Thus, either basal tumors initiate within luminal progenitors, or a tumorigenic population of luminal progenitors de-differentiates to acquire a basal-like phenotype. De-differentiation suggests that there may be significant phenotypic plasticity between basal, luminal progenitor and mature luminal cell populations in normal glands and tumors. Expression signatures similarly delineate breast cancer cell lines into luminal and basal subtypes, and classifier gene lists have been generated that can be used for examining the extent of plasticity between subtypes (7, 8).

Like *ESR1*, the gene encoding ER, *FOXA1* is specifically expressed in luminal subtype tumors (1). Tissue microarray studies revealed FOXA1 protein levels associate with breast cancer patient survival and ER expression (9–14). Furthermore, FOXA1 correlates with the

luminal subtype as defined by ER and/or PR positivity, HER2 negativity, or luminal-specific markers (*e.g.* E-cadherin, cytokeratin 18). FOXA1 and ER co-expression in human tumors has been functionally evaluated using *in vitro* analyses (reviewed in 15), which has revealed the genetic regions bound by ER are enriched for forkhead consensus motifs. In addition, FOXA1 is necessary for estrogen-induced ER binding to target genes and subsequent transcriptional regulation (16, 17). FOXA1 functions as a chromatin-remodeling factor (18–20), thus it is proposed that FOXA1 primes chromatin for subsequent ER binding. In this context, FOXA1 modulates both estrogen-induced ER transcriptional activation and repression (21, 22). In addition to being necessary for ER activity, FOXA1 also regulates ER expression in breast cancer cells, and *Foxa1* null mammary glands fail to express epithelial ER. These glands do not invade the mammary fat pad in response to pubertal hormones (23), a phenotype reminiscent of the ER knockout mice (24–26). Furthermore, FOXA1 and ER share an overlapping expression pattern throughout normal mammary morphogenesis (23).

While the positive correlation of FOXA1 and ER in breast tumors is well documented, several groups have described tumors expressing FOXA1 in the absence of ER (9–14). These data are recapitulated in the mammary gland where a sub-population of mature luminal epithelial cells expresses FOXA1 in the absence of ER (23). Moreover, *FOXA1* is also expressed in an ER-positive-like, androgen-responsive breast cancer subgroup lacking ER and Progesterone Receptor (PR) (27), and FOXA1 is required for androgen receptor (AR) binding to its target genes promoting an apocrine signature (28). FOXA1 is also implicated in other pathways including HER2/ERBB2 (29, 30) and BRCA1 (ref. 31). Combined, these data suggest that in addition to its well-known role as a modulator of estrogen regulated transcription, FOXA1 may also maintain the breast cancer luminal phenotype through ER-independent mechanisms.

Herein, we confirm FOXA1 expression in a subset of ER-negative breast tumors, and in all breast cancer cell lines classified as luminal, even those lacking ER. Utilizing transient FOXA1 silencing in ER-positive and ER-negative luminal breast cancer cells, we define three FOXA1 transcriptional signatures: ER-positive, ER-negative and luminal. Within the luminal signature, FOXA1 is not only necessary for maintaining luminal-specific gene expression, but also for repressing several of the genes specific to basal breast cancer cells. FOXA1 binds to a percentage of the luminal and basal classifier genes and the loss of FOXA1 induces enhancer RNA (eRNA) transcription of a representative basal gene indicating that FOXA1 actively represses at least a subset of basal signature genes. Functionally, FOXA1 silencing increases *in vitro* aggressiveness of luminal cells. Thus, for the first time, we reveal an ER-independent, luminal-specific function for FOXA1 in maintaining the highly differentiated characteristics of luminal breast cancer cells through transcriptional regulation of both luminal and basal genes.

RESULTS

FOXA1 correlates with the luminal subtype of breast cancer

Although previous tissue microarray analyses revealed a significant correlation between FOXA1 and ER, several groups also reported FOXA1 in a subset of ER-negative tumors (9–14). To confirm these results in an independent cohort, we investigated FOXA1 in breast

cancer specimens using immunohistochemistry (IHC). While FOXA1 is expressed in 100% of ER-positive tumors, it is also expressed in ~50% of ER-negative tumors, albeit at significantly decreased levels (Figure 1A, B). We further determined if FOXA1 correlates with the mammary epithelial phenotype using breast cancer cell lines previously classified as luminal, basal A or basal B (7). Of note, basal A cells are more characteristic of human basal-like breast cancers, while basal B cells are mesenchymal and display stem cell features (7, 8). Similar to breast tumors, FOXA1 is expressed in all luminal cell lines examined. In contrast, cells molecularly defined as luminal can lack ER [MDA-MB-453 (hereafter referred to as MB-453) and SKBR3] (Figure 1C, D). These results suggest that FOXA1 more precisely correlates with the luminal subtype than ER, and may be independently required for maintaining this phenotype.

The expression pattern we identified for FOXA1 and ER protein in these cells mirrors publicly available expression data for a larger group of breast cancer cell lines (Supplementary Figure 1) (7). These data include additional lines (AU565, HCC202, HCC2185, SUM185PE) that are luminal, but have very low *ESR1* similar to SKBR3 and MB-453 cells that lack detectable ER protein (Figure 1D). In contrast, no luminal lines examined lack FOXA1, further suggesting FOXA1 may be a critical driver of the luminal phenotype. Several of the lines that express *FOXA1* without *ESR1* over-express HER2/ERBB2 (MB-453, SKBR3, HCC202 and AU565) (7), a proto-oncogene amplified in ~25–30% of breast cancers. However, other *FOXA1* expressing, *ESR1* negative luminal cells (HCC2185, SUM185PE and 600MPE) do not (7), indicating that FOXA1 in ER deficient cells is not dependent on constitutively active HER2. The precise characteristics of cells co-expressing FOXA1 and HER2 may diverge to some extent from cells failing to over-express HER2, but all express luminal signature genes including *FOXA1*.

Generation of the FOXA1-dependent luminal transcriptome

Studies examining FOXA1 in luminal breast cancer have been primarily restricted to analyzing its participation in ER expression and activity. To identify the ER-independent function(s) of FOXA1 we utilized genome-wide expression analysis. We silenced FOXA1 in ER-positive (MCF7, T47D) and ER-negative (MB-453, SKBR3) luminal cells, and confirmed decreased FOXA1 protein levels (Figure 2A). Gene expression was quantified by microarray analysis 36 or 72 hours post-transfection (schematic in Figure 2B). Expression changes for each cell line were then compared to generate three distinct FOXA1 transcriptomes (Figure 2C): changes (1) specific to ER-positive MCF7 and T47D cells, (2) specific to ER-negative MB-453 and SKBR3 cells, and (3) observed in all four luminal lines, which are autonomous to ER expression (*i.e.* the “luminal signature”). Gene lists for each transcriptome, decreased and increased upon knockdown, are provided in Supplementary Tables 1–6.

Loss of FOXA1 induces a partial phenotypic shift from luminal to basal transcriptomes

Genes that discriminate luminal *versus* basal breast cancers (1, 2) and breast cancer cell lines (7, 8) have been reported. Our analysis of FOXA1-associated transcriptomes reveals that loss of FOXA1 in ER-positive (MCF7, T47D) and ER-negative (MB-453, SKBR3) luminal cells significantly induces a number of previously defined basal A and basal B breast cancer

cell line classifier genes, while concomitantly reducing a number of luminal classifier genes (Table 1). Notably, none of the basal A classifying genes are significantly decreased, and only one luminal gene is increased consistently in all four cell lines (data not shown). Ordered heat maps depicting expression changes of classifiers for luminal and basal cells are shown in Figure 3, Supplementary Figure 2 and Supplementary Figure 3.

Silencing FOXA1 decreases expression of several luminal, and increases multiple basal genes in all four luminal cell lines. Using a large subset of the classifier genes defined by Neve *et al.* (RN) (7) that discriminates basal A, basal B, and luminal subtypes (Supplementary Table 7), Gene Set Enrichment Analysis (GSEA) revealed a significant shift from the luminal to the basal A signature. As shown in Table 2, loss of FOXA1 decreases enrichment of luminal gene expression, while concomitantly increasing enrichment of basal A gene expression [False Discovery Rate (FDR) $q < 0.05$]. In contrast, there is no enrichment of basal B genes. A combined gene set of basal A and B genes is also significantly enriched (FDR $q < 0.05$), indicating the core enrichment power of basal A genes. These data confirmed our observation that loss of FOXA1 increases basal A more so than basal B signature genes (Table 1). We further confirmed these findings by performing GSEA on a subset of the classifying genes defined by Charafe-Jauffret *et al.* (ECJ) (8) (Supplementary Table 8). This classifier discriminates luminal [luminal (B)] and basal subtypes, without subdivision into basal A or B. It also delineates luminal [luminal (M)] and mesenchymal subtypes. The mesenchymal gene set is descriptive of a phenotype similar to the Neve *et al.* (7) basal B cells (32). Loss of FOXA1 decreases enrichment of luminal (B) genes, and increases enrichment of basal genes (FDR $q < 0.05$). No change in enrichment of luminal (M) or mesenchymal gene sets is observed (Table 2). Enrichment plots are shown in Figure 3 and Supplementary Figure 4.

To confirm expression microarray data, changes in a subset of the significantly altered luminal and basal genes from either the Neve *et al.* (7) or Charafe-Jaufrett *et al.* (8) classifiers were quantified using real-time RT-PCR. The increase in basal gene expression upon FOXA1 silencing was confirmed in each luminal cell line (Figure 4). Similar results were obtained in MCF7 cells using an independent siRNA targeting FOXA1 (siA1#1) (Supplementary Figure 5). Of note, expression of luminal genes is decreased to a greater extent in ER-positive cell lines (MCF7, T47D), likely because these genes are also responsive to estradiol (33, 34). The increase in basal gene expression was also confirmed at the protein level, where Annexin 1 is increased in each cell line with FOXA1 silencing (Figure 4; Supplementary Figure 5).

FOXA1 transcriptionally regulates luminal and basal genes in luminal breast cancer cells

To determine if FOXA1 may bind and directly repress basal gene expression independent of ER function, we examined publically available ChIP-chip and ChIP-seq datasets of FOXA1 chromosomal binding locations in MCF7 and MB-453 cells, respectively (21, 35). Each basal A gene that was commonly induced with FOXA1 silencing and luminal gene that was commonly repressed with FOXA1 silencing (Table 1) was investigated *in silico* for potential FOXA1 binding sites upstream (50kb), intragenically, or downstream (50kb). These analyses revealed FOXA1 binds either within, or adjacent to 67% of these basal A genes and

100% of these luminal genes in MCF7 and/or MB-453 cells with several binding sites being shared between the two cell lines (Table 3). For genes not bound within the MCF7 dataset, we also interrogated the proximal promoter (−1000bp) for FOXA1 consensus binding elements using the Transcription Element Search System (TESS) (36). To directly assess whether FOXA1 binds to these predictive regions, gene-specific ChIP was performed in ER-positive (MCF7) and ER-negative (MB-453) luminal breast cancer cells (Figure 5A–C). We also analyzed binding to three representative luminal genes (*ERBB3, SPDEF, XBP1*). FOXA1 binds to each basal gene tested in MCF7 cells, including a site near *LYN* that was only reported in the MB-453 ChIP-seq dataset. FOXA1 binding in MB-453 cells was comparable to MCF7 cells for sites that were commonly identified in both ChIP-chip and ChIP-seq datasets. However, FOXA1 failed to bind to a number of the sites reported exclusively in MCF7 cells when examined in MB-453 cells. This is exemplified with *CD58* where FOXA1 is bound in MB-453 cells to a commonly identified site, but does not bind to a site identified exclusively in MCF7 cells. The *ANXA1* proximal promoter has two predicted FOXA1 consensus motifs. FOXA1 binds to one of these in MCF7 cells, while binding is minimal to either sequence in MB-453 cells.

The ability of FOXA1 to bind to several of the luminal and basal A genes independently of ER co-expression, suggested that FOXA1 may directly repress basal signature genes as well as maintain luminal gene expression. Since FOXA1 binding to gene enhancer regions correlates with DNA hypomethylation (37), we postulated that loss of FOXA1 could alter methylation of basal gene promoters and increase their activity. To test this, methylated DNA was immunoprecipitated following FOXA1 silencing and analyzed on a methylation-specific promoter microarray (Agilent Human CpG Island Array). Duplicate experiments were performed in ER-positive (MCF7) and ER-negative (SKBR3) cells, and cells were harvested 72 hours post-transfection. FOXA1 knockdown did not affect genome wide promoter methylation, or the methylation status of the basal gene promoters binding FOXA1 in Figure 5 (data not shown). These data indicate that FOXA1 does not repress basal gene expression by altering proximal promoter methylation status. However, unlike conventional transcription factors, FOXA1 predominantly binds to enhancer regions distal to transcriptional start sites (21, 38). To determine if FOXA1 can directly repress basal gene expression distally, we examined enhancer RNA (eRNA) synthesis, a highly specific marker of enhancer activation, from a FOXA1 binding site in a representative basal A gene (39, 40). We investigated a region bound by FOXA1 downstream of *CD58* (common binding site observed in Figure 5A–C; schematic shown in Supplemental Figure 6). Since loss of FOXA1 significantly induces *CD58* expression (Figure 4), we hypothesized that upon silencing there should be a concomitant upregulation of eRNA(s) at this site. Indeed, loss of FOXA1 induces eRNA synthesis bidirectionally in MCF7 cells and unidirectionally in MB-453 cells (Figure 5D). Importantly, the extent of eRNA synthesis is correlated with changes in *CD58* expression, but not expression of the adjacent gene (*C1orf203*) in any of the cell lines we tested (data not shown). Hence, the FOXA1 binding site appears to have a direct and specific effect on *CD58* expression. Combined, these data indicate that FOXA1 actively maintains at least a subset of basal genes in a transcriptionally repressed state in luminal breast cancer cell lines.

FOXA1 represses breast cancer cell aggressiveness

The ability of FOXA1 to repress a subset of basal genes suggested that its loss in luminal cells may lead to acquisition of the more aggressive phenotypes associated with basal breast cancer cells. To test this, both ER-positive (MCF7) and ER-negative (MB-453) luminal breast cancer cells were transiently transfected with FOXA1-targeted siRNAs and assessed for changes in migration and invasion, two hallmarks of highly aggressive breast cancer cells. Both MCF7 and MB-453 cells exhibit increased migration following FOXA1 silencing compared to the non-targeting control (Figure 6A). Cellular invasion was also increased in MCF7 cells (Figure 6B). These results were not secondary to an increase in cell number (Figure 6C). In fact, FOXA1 silencing significantly decreased MCF7 cell number, which is consistent with previous reports (17, 30, 38). Overall, these results suggest that FOXA1 repression of basal gene expression decreases *in vitro* cellular aggressiveness, and does so independently of ER.

DISCUSSION

FOXA1 represses basal breast cancer genes

FOXA1 is necessary for estrogen-induced ER binding and subsequent transcriptional activation of luminal genes in breast cancer cells (16, 17, 21, 22, 38). This property of FOXA1 was identified in ER-positive cell lines in the presence or absence of estrogens. The co-modulatory role between FOXA1 and ER has also been reported for estrogen-mediated transcriptional repression, specifically in the repression of the *RPRM* gene (41). In this context, FOXA1 and ER form a tripartite complex with Histone Deacetylase 7 (HDAC7), and silence *RPRM* independently of HDAC activity. Interestingly, FOXA1 does not bind to the *RPRM* promoter when ER is silenced, but does bind to HDAC7 in an estrogen-independent fashion. FOXA1/ER/HDAC7 are likely required for the estrogen-induced repression of additional target genes, but it is unknown whether FOXA1/HDAC7 represses gene transcription in cells lacking ER.

Herein, we report that FOXA1 represses a subset of basal breast cancer cell line classifier genes in ER-positive and ER-negative luminal breast cancer cells. Since FOXA1 is not known to have intrinsic repressor activity, it likely recruits co-repressors to these chromosomal locations. HDAC7 is a possible candidate given its role as just described. Since our data indicate that FOXA1 represses basal gene expression in an ER-independent fashion, FOXA1 and HDAC7 may similarly inhibit gene expression in the absence of ER. It is also possible that FOXA1 represses basal genes in complex with HDAC7 only in ER-positive cells, and interacts with different co-repressors in ER-negative cells. Interestingly, loss of BRCA1 in T47D cells induces gene expression associated with the basal subtype, where a BRCA1/c-MYC complex mediates repression by directly binding to respective promoters (42). FOXA1 synergizes with BRCA1 to regulate the $p27^{KIP1}$ promoter (31); thus, it is tempting to speculate that FOXA1 and BRCA1 may cooperate to repress basal gene expression.

Although loss of FOXA1 induces a subset of basal genes, not all basal genes are increased upon FOXA1 silencing. Similarly, not all luminal signature genes are decreased. The

simplest explanation for these results is that FOXA1 expression was transiently suppressed. The basal genes that are not directly regulated by FOXA1, or those that encode mRNAs with long half-lives may not change by 72 hours after silencing. FOXA1 knockdown was also not 100% effective, possibly resulting in an incomplete phenotypic change. Other than technical limitations, it is also possible that additional factors control the other basal genes. It is also likely that a group of transcriptional activators are necessary to express the full complement of basal genes, and simply reducing FOXA1 is not sufficient to activate all basal genes. Even if transient FOXA1 silencing induces expression of these activators, the experimental time course may have been insufficient to detect alterations in target gene expression. Stable knockdown of FOXA1 will be necessary to ultimately determine if FOXA1 can repress the entire basal gene set, and hence, cause a complete phenotypic conversion. However, our preliminary studies suggest that stable suppression of FOXA1 causes cell cycle arrest, limiting the ability to evaluate the consequences of extended FOXA1 silencing in these cells. Lastly, loss of FOXA1 can induce redistribution of genome-wide nuclear receptor binding (40, 43). Thus, while our data suggests that FOXA1 plays an active role in the repression of basal genes, FOXA1 silencing may de-repress these genes indirectly through several mechanisms including loss of a transcriptional repressor.

FOXA1 maintains a less aggressive phenotype in breast cancer cells

Transient knockdown of FOXA1 in luminal breast cancer cells induces a molecular signature with a more basal pattern. Breast cancer phenotypes are proposed to reflect the lineage progression of normal mammary epithelial cells, with basal tumors arising from luminal progenitors and luminal tumors arising from mature luminal epithelium (5, 6). The degree of plasticity between these states is not yet known. However, our studies suggest that they may be highly dynamic wherein more differentiated breast cancer cells can acquire phenotypic characteristics of less differentiated, or basal cells and that cellular state is controlled by FOXA1. It will be important to determine whether FOXA1 functions similarly during developmental specification of the mammary epithelium. If so, this would indicate that a major function of FOXA1 in breast development and cancer is lineage commitment. Another luminal subtype-specific transcription factor, GATA3, has been proposed to play a similar role as FOXA1. It is also necessary for normal mammary morphogenesis (44, 45) and its forced expression in tumors induces differentiation and prevents disease dissemination (46). However, the impact of GATA3 on the expression of basal signature genes has not yet been reported.

Paralleling the molecular transition towards a basal phenotype, transient FOXA1 silencing similarly increases *in vitro* aggressiveness of luminal breast cancer cells. Importantly, changes in migration and invasion are not secondary to increased proliferation. Indeed, loss of FOXA1 in MCF7 cells significantly decreased cell number. These data are consistent with previous reports that FOXA1 silencing blocks estrogen-mediated cell cycle progression (17, 38). FOXA1 knockdown similarly reduces cell number under hormone-replete conditions (30). While the combined phenotype of increased aggressiveness and decreased cell number appears counterintuitive, heterogeneity within breast cancer cell lines may explain this dichotomy (47). Specifically, loss of FOXA1 may lead to growth arrest of a subpopulation of differentiated cells (*i.e.* mature luminal), while the remaining cells may

have greater plasticity to de-differentiate towards the basal phenotype. MCF7 cells, in particular, have a side-population with tumor initiating capabilities (47). FOXA1 silencing may enrich this population resulting in the observed shift toward the basal subtype. Supporting the role of FOXA1 in maintaining decreased aggressiveness of breast cancer cells, stable overexpression of FOXA1 in a basal B breast cancer cell line (MDA-MB-231) induces E-cadherin expression, and decreases the migratory capacity of these aggressive, highly metastatic cells (48). Thus, FOXA1 is both necessary and sufficient to lessen tumor aggressiveness.

Targeting FOXA1 as an approach for breast cancer therapy

Although we did not identify ER-positive tumors that lack FOXA1 within our cohort, others have reported such tumors (9–14). There are currently no breast cancer cell lines that recapitulate the FOXA1-negative, ER-positive molecular phenotype. Future studies should delineate their molecular and phenotypic differences from FOXA1-positive, ER-positive cancers to determine if and how ER functions in the absence of FOXA1. ER transcriptional regulation in these tumors may recapitulate ER activity in other cancer types (*e.g.* osteosarcoma, ovarian) that do not co-express FOXA1 (22, 49).

The observation that FOXA1 controls a subset of luminal and basal breast cancer cell line classifier genes further supports the prognostic value of FOXA1; its expression is not only correlative, but is functionally important in maintaining a differentiated, less aggressive state. It has been suggested that targeting FOXA1 in patients with hormone receptor positive disease would abrogate ER signaling and possibly aid in the efficacy of tamoxifen (50). Our analyses dispute this postulate because FOXA1 silencing may expand a more aggressive, basal-like population of tumor cells, or cause de-differentiation of existing cells. For example, we found that FOXA1 silencing substantially induces Annexin 1 (*ANXA1*), a protein required for basal breast cancer metastasis (51). These results, in combination with the increased *in vitro* aggressiveness that occurs in response to FOXA1 silencing, indicate that FOXA1 directly maintains the more favorable luminal phenotype, even in the absence of ER. While pharmacologic reduction of FOXA1 in luminal tumors may be contraindicated, elevating expression of FOXA1 in basal breast cancers may prove useful for inducing luminal differentiation and acquisition of hormone responsiveness. Such differentiation approaches may uncover novel therapeutic approaches for treating recalcitrant basal tumors.

MATERIALS AND METHODS

cDNA microarrays

MCF7, T47D, SKBR3 and MB-453 cells were harvested at 36 or 72 hours post-transfection with siRNA. Three biological replicates were performed per cell line at the 72-hour time point. Total RNA was isolated using TRIzol Reagent (Invitrogen) and treated with DNAse I (DNA-*free*, Ambion). RNA quantity was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) and by separation via capillary electrophoresis using the Agilent 2000 Bioanalyzer (Agilent Technologies). Characterization of individual transcripts was performed by comparison of control (non-targeting siRNA)

with FOXA1 knockdown (siRNA to FOXA1 #4) samples labeled with Cy5-UTP or Cy3-UTP using the Agilent Quick Amp Labeling Kit (Agilent Technologies). Equal amounts of labeled treated and untreated RNA were mixed and hybridized to Agilent Human 44K microarray slides at 65°C for 24 hours. After washing, microarray slides were read using an Agilent Scanner, and Agilent Feature Extraction software v7.5 was used to calculate gene expression values. The feature extracted files were imported into Rosetta Resolver v7.1 for data analysis (Rosetta Biosoftware). The intensity ratios between the cell line sample and mixed reference calculated for each sequence were computed according to the Agilent error model. A sequence was considered differentially expressed if the calculated p-value was $p \leq 0.05$. Expression data has been submitted to Gene Expression Omnibus (Accession #GSE31003). Biological replicates were further combined using a ratio-error weighted ANOVA. Common gene expression changes between the four cell lines were compared using Venn diagrams with a threshold at $p \leq 0.001$.

Gene Set Enrichment Analysis (GSEA)

GSEA determines whether expression measurements for a set of genes shows statistically significant concordant differences between two biological states (*e.g.* phenotypes) (52). Since the Agilent Human 44K microarray reported log-ratio values of non-targeting *versus* FOXA1 knockdown expression data, log-intensities of the array measurements instead of log-ratios were calculated. Utilizing individual channel analysis of the two-color Agilent microarrays using R-package limma (53), an average microarray measurement across all cell lines (MCF7, T47D, MB-453, SKBR3) was generated. Background correction and normalization was done on the two-color microarray data, and a linear model was fit to the data to acquire average values for each biological replicate. First, the raw image files from the instrument were read using limma. The intensities were global loess normalized. Within each array, the M-value variance was stabilized as a function of probe intensity. Next, across all arrays, the A-values of the probes were quantile normalized so that all probe intensities have the same distribution across all arrays. The two-color target frame was converted to single channel data format, and linear models were fitted for every probe. Averages were determined for three biological replicates plus a technical dye reversal replicate for each cell line. Cell line averages were then combined to obtain a common average value (see Supplementary methods for more detail). Due to differences between the microarray platform used herein and those used for classifier development, GSEA was conducted on subsets of the classifier gene lists that were identified in Neve *et al.* (RN) (7) and Charafe-Jauffret *et al.* (ECJ) (8) (Supplementary Tables 7 and 8, respectively). Enrichment of each classifier gene list was then determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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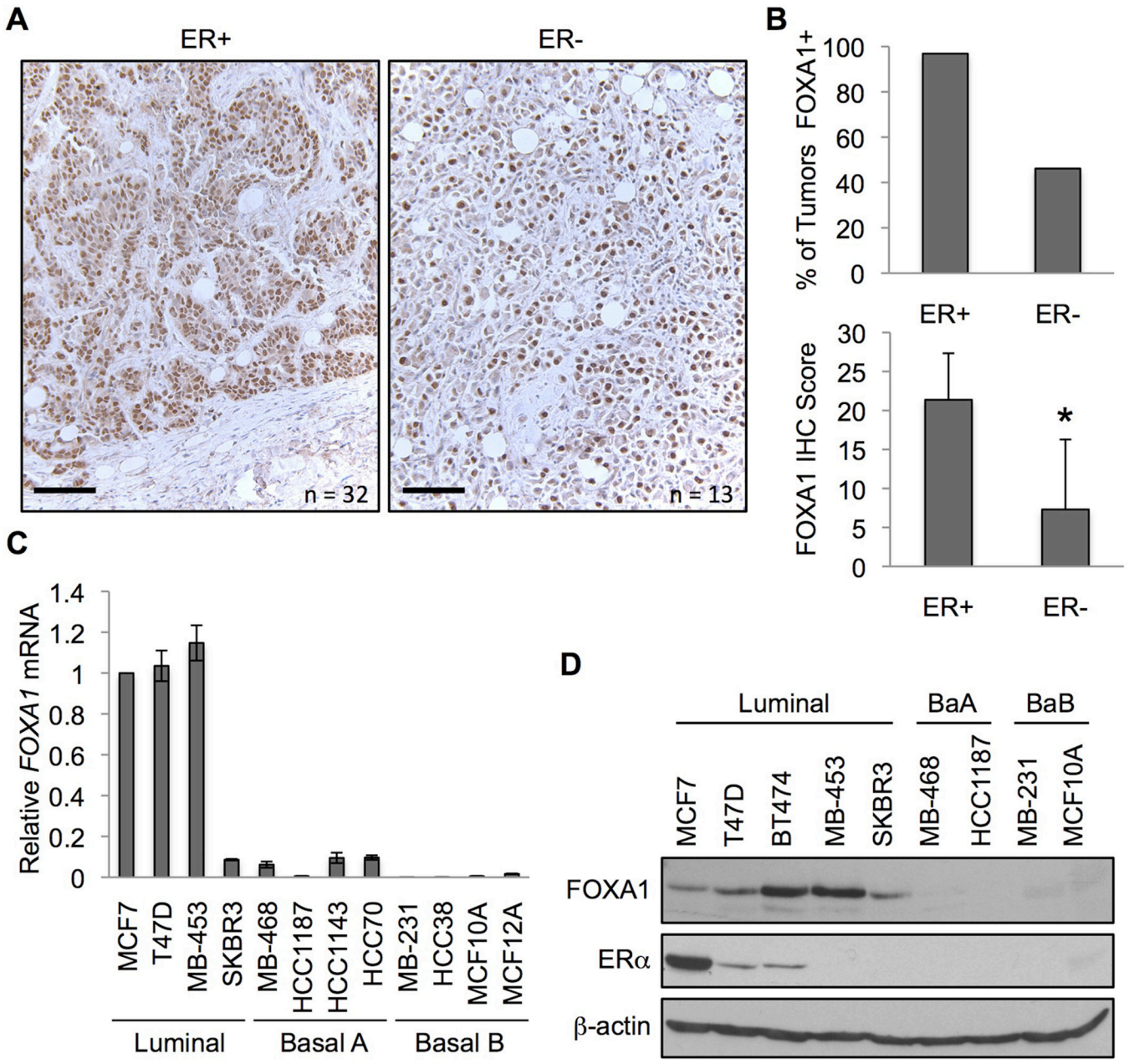

Figure 1. FOXA1 is expressed in the absence of ER in breast tumors and luminal cell lines

(A) Representative FOXA1 IHC of ER-positive (n=32) and ER-negative (n=13) breast tumor sections. FOXA1 expression (brown) is counterstained with hematoxylin. Scale bars = 100 μm. (B) FOXA1 is expressed in all ER-positive and ~50% of ER-negative tumors. ER-negative tumors express significantly less FOXA1 than ER-positive tumors (*p < 1×10−6). Scores were computed by multiplying signal intensity (1=lowest; 3=highest) by the percentage of positive cells (1=10%, 2=20%, etc.) (10). (C) Quantitative real-time PCR of *FOXA1* mRNA in a diverse group of breast cancer cell lines. Bars represent the mean of three experiments (cells harvested on separate occasions) ± s.e.m. relative to *GAPDH*. (D)

Immunoblot analysis of a cohort of breast cancer cell lines for FOXA1 and ER (BaA=Basal A; BaB=Basal B).

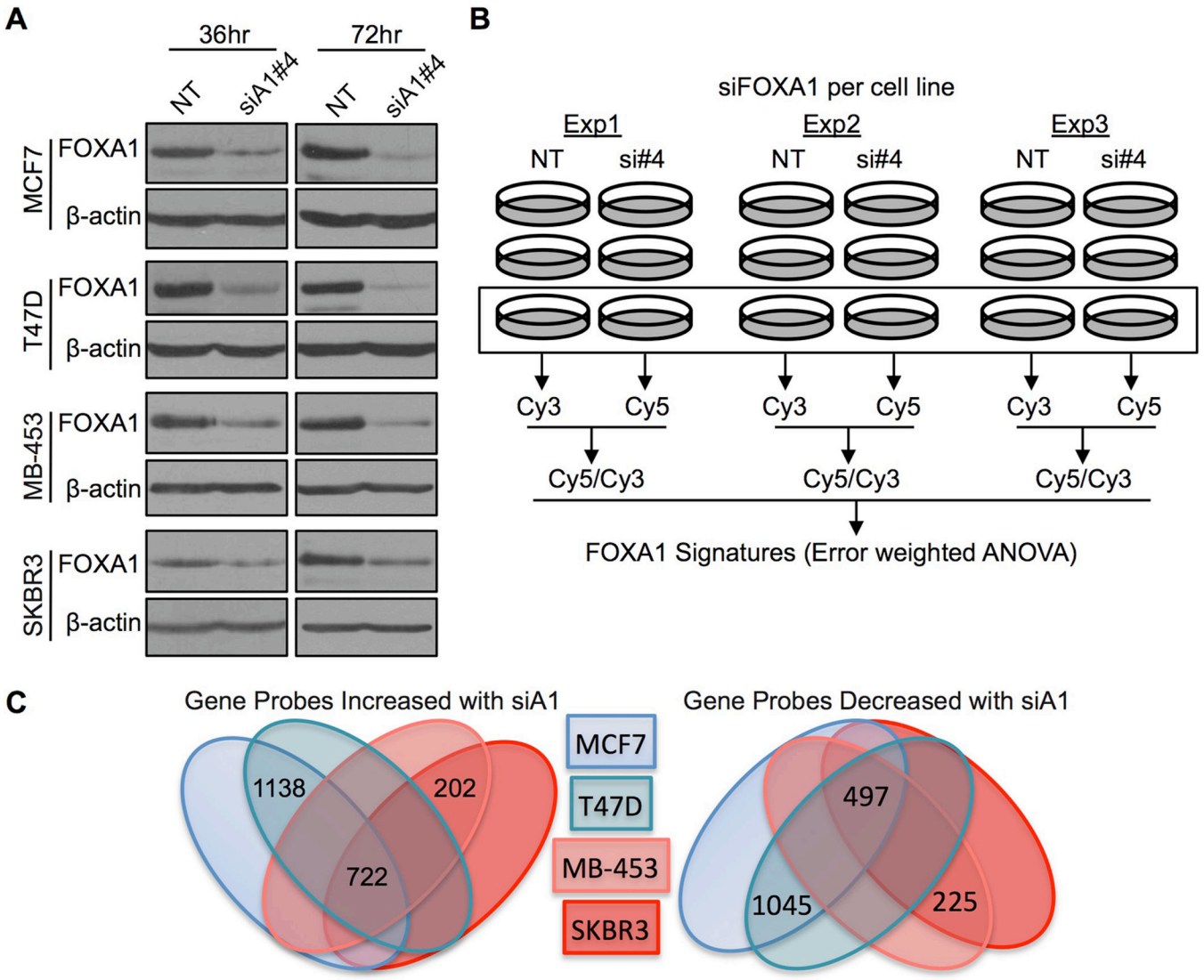

Figure 2. Identification of a FOXA1-dependent luminal transcriptome

(A) Representative immunoblots confirming FOXA1 knockdown (~60%–87%) with siA1#4 at 36 (n=1) and 72 hours (n=3) post-transfection. NT = non-targeting siRNA. (B) Schematic of the experimental design for each cell line. Technical replicates from each experiment were performed in triplicate and were processed for microarray analysis. Biological replicates were combined via error-weighted ANOVA. (C) Venn diagrams of commonly changed gene probes at 72 hours post-transfection ($p < 0.001$).

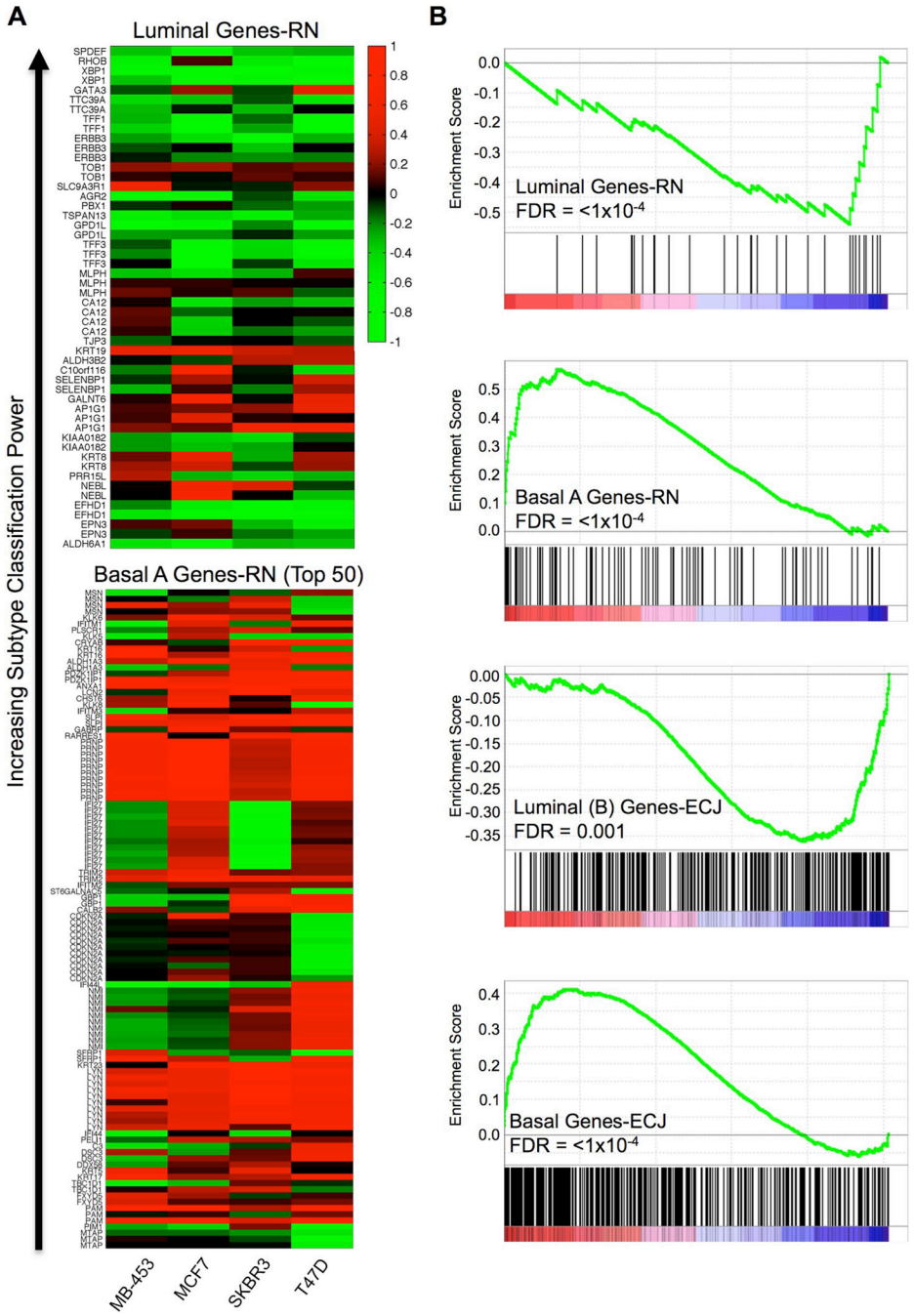

Figure 3. Loss of FOXA1 decreases enrichment for luminal genes, while increasing enrichment for basal genes

(A) Heat maps depicting expression changes of the genes within the Neve *et al.* (RN) (7) luminal and basal A classifier lists upon knockdown of FOXA1 (siA1#4) at 72 hours post-transfection. Genes are ordered from highest to lowest classification power. A propensity of red or green is indicative of a directional shift in global expression of the gene classifier. (B) GSEA enrichment plots utilizing a subset of the luminal and basal discriminatory gene sets generated by Neve *et al.* (RN) (7) and Charafe-Jauffret *et al.* (ECJ) (8). Vertical lines represent individual genes of the respective classifier that contribute to the enrichment score.

Genes are ranked by signal to noise ratio: left (most positive) to right (most negative). Values below 0 indicate reduced enrichment of a signature gene set, while values above 0 indicate a gain in enrichment.

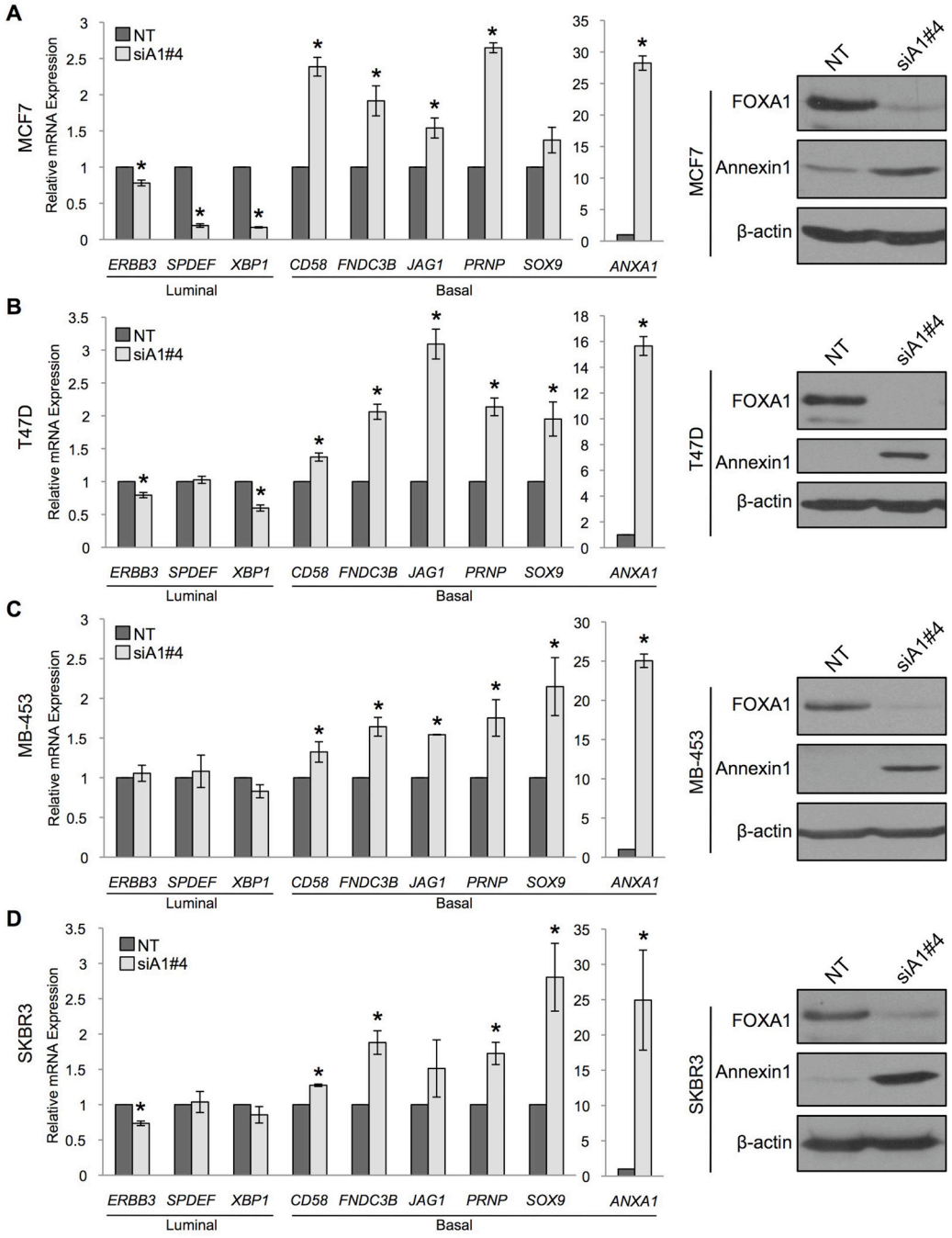

Figure 4. Loss of FOXA1 induces basal mRNA and protein expression

(A) MCF7, (B) T47D, (C) MB-453, and (D) SKBR3 cells were transiently transfected with non-targeting (NT) or siRNA targeting *FOXA1* (siA1#4). (Left) Quantitation of mRNA changes for a subset of differentially expressed luminal and basal classifying genes at 72 hours post-transfection using real time RT-PCR. Bars represent the mean of three experiments ± s.e.m. relative to *GAPDH* (*$p < 0.05$). (Right) Representative immunoblots at 72 hours post-transfection showing induction of the basal protein, Annexin 1, in response to FOXA1 silencing (n=3).

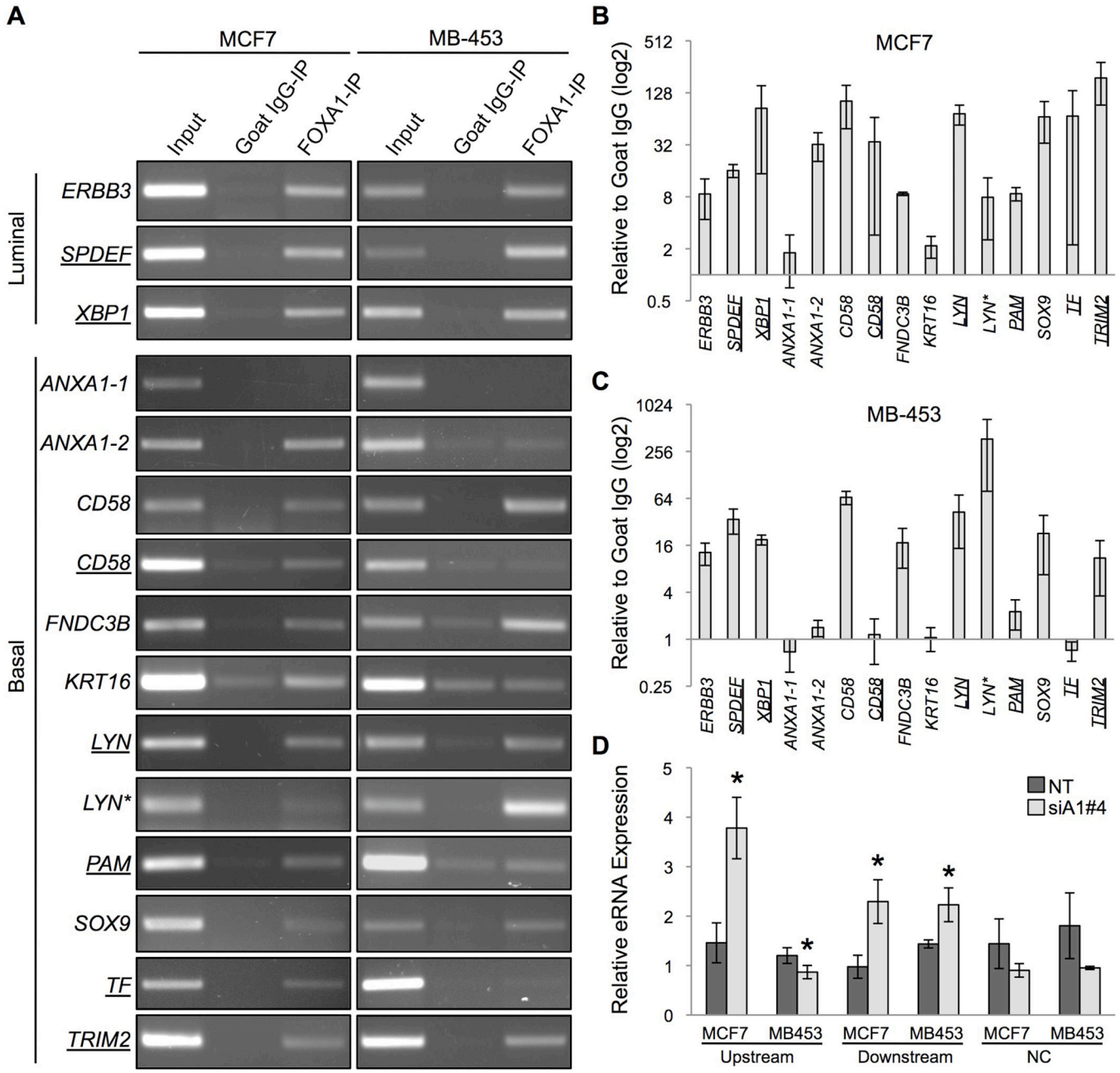

Figure 5. FOXA1 transcriptionally regulates luminal and basal gene expression in luminal breast cancer cells

(A–C) FOXA1 ChIP of a subset of basal and luminal genes in MCF7 and MB-453 cells. (A) Representative images and (B–C) quantitation of FOXA1 ChIP. Bars represent the fold change in binding relative to normal goat IgG ± s.e.m. (n=3) where the Y-axis is log2. Genes underlined represent sites predicted to bind FOXA1 in a previously published MCF7 ChIP-chip dataset. The asterisk represents a site predicted in a previously published MB-453 ChIP-seq dataset. The remaining sites were either predicted to bind in both MCF7 and MB-453 datasets, or are regions surrounding consensus elements within the gene promoter (see Table 3). Genes listed twice represent independent binding locations for that gene. (D)

Quantitation of eRNA transcripts generated upstream and downstream of the common FOXA1 binding site in *CD58* at 72 hours post-transfection with non-targeting (NT) or siRNA targeting *FOXA1* (siA1#4). A region ~10 kilobases upstream of *CD58* not bound by FOXA1 in previously published datasets was used as a negative control (NC). See Supplementary Figure 6 for a schematic of the associated binding sites and primer locations for *CD58*. Bars represent the mean of three experiments ± s.e.m. relative to *GAPDH* (*$p < 0.05$).

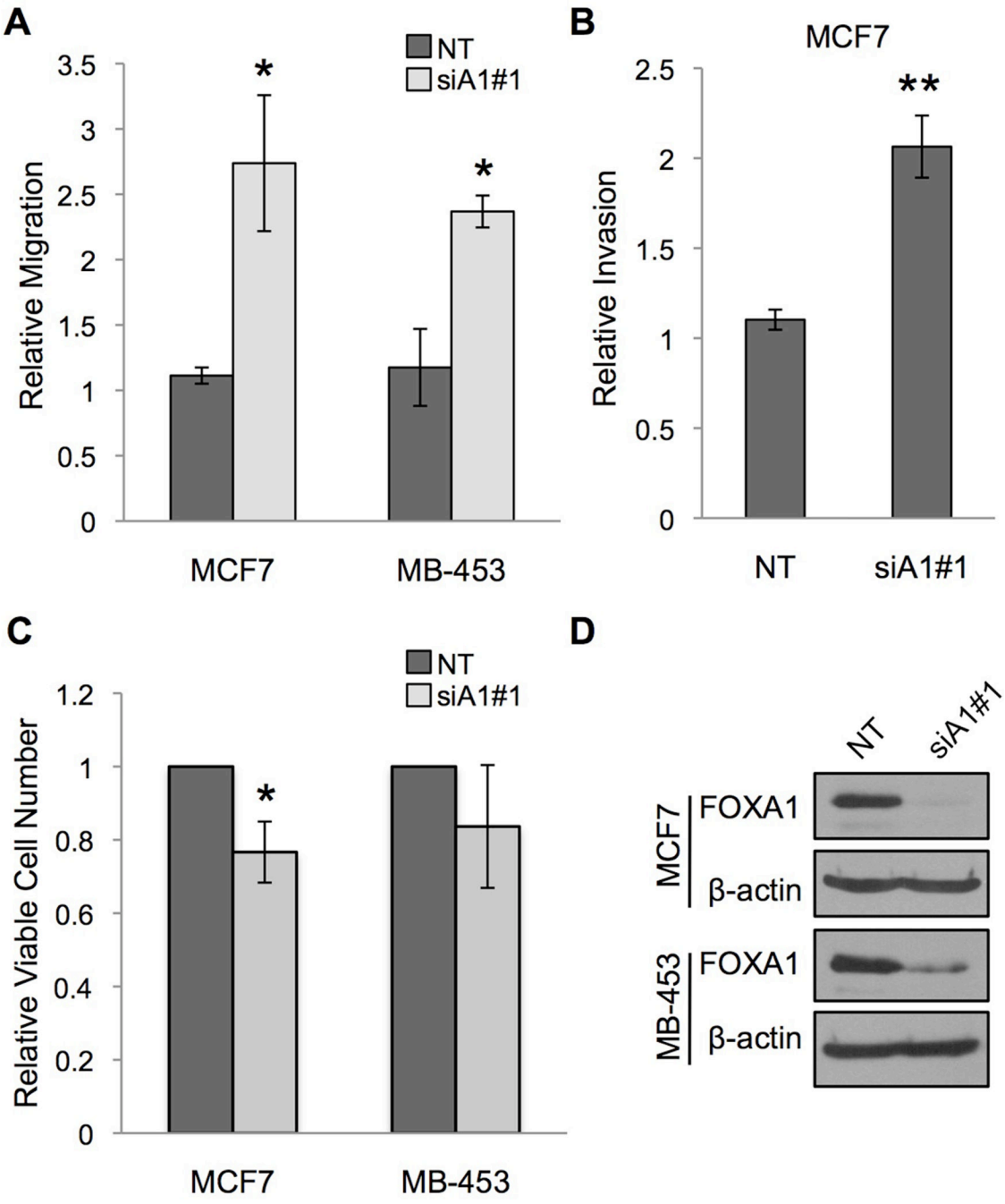

Figure 6. Loss of FOXA1 increases migration and invasion of luminal breast cancer cells

(A–D) MCF7 and MB-453 cells were transiently transfected with non-targeting (NT) or siRNA targeting *FOXA1* (siA1#1). At 48 hours post-transfection, cells were plated in modified Boyden chambers to analyze (A) migration at 24 hours or (B) invasion at 48 hours. (C) Number of viable cells (trypan blue excluded) at 48 hours post-transfection. Bars in A–C represent the mean of three experiments ± s.e.m. relative to NT (*$p < 0.05$; **$p < 0.01$). (D)

Representative immunoblots confirming knockdown of FOXA1 with siA1#1 at 72 hours post-transfection (MCF7, n=3; MB-453, n=2).

Table 1

Basal A, Basal B and Luminal classifier genes whose expression is changed upon knockdown of FOXA1 in MCF7, T47D, MB-453 and SKBR3 cells ($p<0.05$).

Basal A	**Basal B**	**Luminal**
Increased in all four lines	**Increased in all four lines**	**Decreased in all four lines**
ANXA1	*DSE*	*ALDH6A1*
ARHGEF9	*ELK3*	*EFHD1*
CD58	*FSTL1*	*ERBB3*
FNDC3B	*GLS*	*SPDEF*
JAG1	*LHFP*	*TFF3*
KRT16	*MALT1*	*XBP1*
LYN	*PALM2*	**20.7% of total Lum**
MGP	**10.1% of total BasB**	
PAM		
PRNP		
S100A2		
SLPI		
SOX9		
TF		
TRIM2		
16.3% of total BasA		

Classifier genes based on Neve *et al.* (2006) (ref. 7).

Table 2

GSEA of classifier gene lists that are discriminatory of luminal *v.* basal breast cancer molecular subtypes.

	Lum-RN	BasA-RN	BasB-RN	BasAB-RN	Lum(B)-ECJ	Bas-ECJ	Lum(M)-ECJ	Mes-ECJ
Enrichment Score	−0.54	0.57	0.27	0.43	−0.36	0.41	−0.18	0.28
Normalized ES	−1.75	2.18	0.99	1.82	−1.75	1.9	−0.89	1.27
NOM p-value	0.002	0	0.473	0	0	0	0.896	0.026
FDR q-value	**$<1\times10^{-4}$**	**$<1\times10^{-4}$**	0.467	**$<1\times10^{-4}$**	**0.001**	**$<1\times10^{-4}$**	0.787	0.074
FWER p-value	0.001	0	0.942	0	0.001	0	0.921	0.286

Classifier gene lists based on Neve *et al.* (2006) (RN) (ref. 7) and Charafe-Jauffret *et al.* (2006) (ECJ) (ref. 8) (Supplementary Tables 7 & 8). ES = Enrichment Score, NOM = nominal, FDR = false discovery rate, FWER = familywise-error rate.

Table 3

Potential FOXA1 binding sites in basal and luminal signature genes regulated by FOXA1

	# of *in silico* ChIP sites*		# of consensus motifs□
Basal A	**MCF7**	**MB-453**	
ANXA1	none	none	2
ARHGEF9	none	none	none
CD58	4**	1**	ND
FNDC3B	10**	3**	ND
JAG1	2	none	ND
KRT16	none	1	1
LYN	1	1	ND
MGP	none	none	3
PAM	3	none	ND
PRNP	none	none	1
S100A2	none	1	none
SLPI	none	none	1
SOX9	1**	1**	ND
TF	1	none	ND
TRIM2	1	none	ND
Luminal	MCF7	MB-453	
ALDH6A1	1**	1**	ND
EFHD1	5	none	ND
ERBB3	2**	2**	ND
SPDEF	3	none	ND
TFF3	5**	1**	ND
XBP1	5**	3**	ND

*FOXA1 is bound <50kb upstream, intragenically, or <50kb downstream in previously published MCF7 ChIP-chip (ref. 21) or MB-453 ChIP-seq (ref. 35) datasets

□FOXA1 Consensus motif identified in the proximal (−1000bp) promoter by TESS. ND = not determined.

**Common site(s) in MCF7 and MB-453 cells

Shaded grey = Confirmed by ChIP-PCR in Figure 5