The Forkhead Box M1 Protein Regulates the Transcription of the Estrogen Receptor \( \alpha \) in Breast Cancer Cells*§

Patricia A. Madureira1, Rana Varshochi1, Demetra Constantinidou1, Richard E. Francis2, R. Charles Coombes3, Kwok-Ming Yao‡, and Eric W.-F. Lam*†

From the *Cancer Research—United Kingdom Laboratories, Department of Oncology, MRC Cyclotron Building, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0NN, United Kingdom, ‡Department of Biochemistry, The University of Hong Kong, 3/F Laboratory Block, The Faculty of Medicine Building, 21 Sassoon Road, Pokfulam, Hong Kong, China

In this study, we have identified the Forkhead transcription factor FoxM1 as a physiological regulator of estrogen receptor \( \alpha \) (ER\( \alpha \)) expression in breast carcinoma cells. Our survey of a panel of 16 different breast cell lines showed a good correlation (13/16) between FoxM1 expression and expression of ER\( \alpha \) at both protein and mRNA levels. We have also demonstrated that ectopic expression of FoxM1 in two different estrogen receptor-positive breast cancer cell lines, MCF-7 and ZR-75–30, led to up-regulation of ER\( \alpha \) expression at protein and transcript levels. Furthermore, treatment of MCF-7 cells with the MEK inhibitor U0126, which blocks ERK1/2-dependent activation of FoxM1, also repressed ER\( \alpha \) expression. Consistent with this, silencing of FoxM1 expression in MCF-7 cells using small interfering RNA resulted in the almost complete abrogation of ER\( \alpha \) expression. We also went on to show that FoxM1 can activate the transcriptional activity of human ER\( \alpha \) promoter primarily through two closely located Forkhead response elements located at the proximal region of the ER\( \alpha \) promoter. Chromatin immunoprecipitation and biotinylated oligonucleotide pulldown assays have allowed us to confirm these Forkhead response elements as important for FoxM1 binding. Further co-immunoprecipitation experiments showed that FoxO3a and FoxM1 interact in vivo. Together with the chromatin immunoprecipitation and biotinylated oligonucleotide pulldown data, the co-immunoprecipitation results also suggest the possibility that FoxM1 and FoxO3a cooperate to regulate ER\( \alpha \) gene transcription.

The biological effects of estrogen are primarily mediated through two nuclear steroid receptors, estrogen receptors \( \alpha \) and \( \beta \) (ER\( \alpha \)s and ER\( \beta \))2 (1–3). Estrogen receptors play a major role in regulating the growth, survival, and differentiation of normal and malignant breast epithelial cells. In normal mammary epithelium, ER\( \alpha \)s are rarely expressed in a large proportion of cells or at high levels (4, 5). Approximately 60–80% of all breast cancers overexpress ER\( \alpha \), and ~70% of those respond to endocrine treatment, with further increased risk of breast cancer development in benign mammary tissue. These findings suggest that ER\( \alpha \)s play a major role in breast cancer initiation and progression (4). ER\( \beta \) appears to have an opposing function to ER\( \alpha \)s in tumor growth (6), and low levels of ER\( \alpha \) predict resistance to Tamoxifen therapy in breast cancer (7).

ER\( \alpha \)s functions as a classical transcription factor as well as a signal transducer. Estrogen binding activates ER\( \alpha \)s through phosphorylation, dissociates it from chaperonin proteins, and alters its conformation. Several kinases in the mitogenic signaling pathways can also phosphorylate and consequently activate ER\( \alpha \)s through a ligand-independent manner. Hormone-bound ER\( \alpha \)s dimerize and bind to estrogen response elements present in the promoter regions of target genes, including c-fos, c-jun, c-myc, TGF-\( \alpha \), retinoic acid receptor \( \alpha 1 \), and progesterone receptor A, etc., to activate gene transcription (8–12). ER\( \alpha \)s has also been shown to modulate gene transcription through alternative regulatory DNA sequences, such as AP-1, NF-\( \kappa \)B, and Sp-1 binding sites (13). Many of these ER-regulated genes, including IGFR1, cyclin D1, c-myc, and the anti-apoptotic gene bcl-2, are important for cell proliferation and survival (14–17). Recent studies also suggest that a pool of ERs are located in the plasma membrane and cytoplasm. Direct binding of ER\( \alpha \)s to a diversity of membrane/cytoplasmic signaling molecules have been observed, and they include the p85 regulatory subunit of class I phosphoinositide 3-kinase, Src tyrosine kinase, and the insulin-like growth factor 1 (13). Activation of these pathways by estrogen through the ER initiates cell survival and proliferation signals via phosphorylation and activation of Akt and MAPK. Additionally, these signaling molecules are able to phosphorylate the ER and its co-regulators to augment nuclear ER signaling (13). In summary, the genomic and non-genomic actions of ER\( \alpha \)s play a crucial role in breast epithelial cell proliferation and survival.

Endocrine therapies aimed at blocking the action of estrogens have been the most effective and widely used methods for treating ER\( \alpha \)-positive breast cancers. These therapeutic
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approaches involve blocking estrogen binding to ER using anti-estrogens such as tamoxifen, inhibiting estrogen synthesis using aromatase inhibitors such as exemestane, and reducing ER protein levels using “pure” anti-estrogens such as fluvastatin (ICI 182,780 or Faslodex) (13). Nevertheless, a proportion of ERα-positive tumors do not respond to hormone treatment at all (de novo resistance), and the majority of those that initially respond eventually become resistant (acquired resistance). Most resistant tumors remain ERα-positive, indicating a continued role for ERα in breast cancer cell survival and proliferation (18, 19).

A recent study suggested that ERα transcription is regulated by FoxO3a (Forkhead box class O, 3a), a member of the Forkhead family of transcription factors (20). FoxO3a expression is negatively regulated by the phosphoinositide 3-kinase signaling pathway. Activation of phosphoinositide 3-kinase by growth factors leads to phosphorylation and inactivation of FoxO3a by Akt (also termed PKB [protein kinase B]) (21). When activated, FoxO3a up-regulates p27^{kip1}, p130, and bim and down-regulates cyclin D1/2 and bcl-XL expression to mediate cell cycle arrest and apoptosis (22–25). Because ERα expression is associated with breast cancer initiation and progression and FoxO3a activation with cell cycle arrest and/or apoptosis, it is implausible that FoxO3a would be the principal positive regulator of ERα transcription. Because all members of the Forkhead transcription factors share a similar DNA binding domain, we reasoned that other family member(s) could be the main physiological regulator(s) of ERα transcription.

Forkhead box (Fox) M1 is a transcription factor ubiquitously expressed in proliferating cells and a key regulator of both G1/S and G2/M phases of the cell cycle (26–30). FoxM1 is localized mainly in the cytoplasm in late G1 and S phases; nuclear translocation occurs during entry into the G2/M phase and is associated with FoxM1 phosphorylation by ERK1/2 (31). In this study, we have examined the possibility that FoxM1 regulates ERα expression and explored the mechanism involved.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, and Cell Lines—The human breast carcinoma cell lines MCF-7, ZR-75–1, ZR-75–30, 734 B, MDA-MB-175, CAMA1, MDA-MB-231, MDA-MB-453, MDA-MB-469, HMT 3552, HBL-100, SKBR-3, SKBR-7, BT-474, BT-549, T47-D, and CAL-51 were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin in a humidified incubator in an atmosphere of 10% CO₂ at 37 °C. MCF-7 Tet-on FoxM1 cells were selected and maintained in the presence of 500 μg/ml of zeocin (Invitrogen).

Plasmids—The FoxO3a expression vector pLPC-FoxO3a(wt) and pLPC-FoxO3a(A3) and the FoxM1 expression vector pcDNA3-FoxM1 have previously been described (22, 32). The ERα promoter constructs pGL3-proA and pGL3-proB were kindly provided by Prof. Shin-Ichi Hayashi (Saitama Cancer Center Research Institute, Saitama, Japan) (33). The psiRNA-FoxO3a expression vector was generated by cloning small synthetic oligonucleotides encoding two complementary sequences of 19 nucleotides, TCACTGCATAGTCGATTCA, into the Invivogen psiRNA plasmid (Autogen Bioclear, Wiltshire, UK). The pTER-FoxM1 siRNA expression vector was generated by cloning the two complementary hairpin sequences of CACGCAAGTAGTGGCCATC into the BglII and HindIII sites of the pTER vector provided by Dr. Marc van de Wetering (Department of Immunology, University Medical Center, Utrecht, The Netherlands) (34). The respective control RNA interference vectors were generated from nucleotide sequences with the middle 2 bases mutated (i.e. FoxM1-TCACTGCCCAGTCGATTCA and FoxO3a-TCACTGCAC-CGTGATTCA). The pcDNA4-FoxM1 vector used for the establishment of MCF-7 Tet-on FoxM1 MCF-7 cells was generated by PCR amplification of the human FoxM1 cDNA (Gen-Bank™ accession number U83113) using a high-fidelity polymerase (Clontech) and subcloning into the HindIII/XbaI restriction sites of pcDNA4TM4T/O (Invitrogen) mammalian expression plasmid.

Western Blotting and Antibodies—Western blotting was performed on whole cell extracts prepared by lysing cells with a 2× packed cell volume of Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 10 mM NaF, 1 mM sodium orthovanadate, and protease inhibitors (“Complete” protease inhibitor mixture), as instructed by the manufacturer (Roche Applied Science). Protein concentration was determined by Bio-Rad Dc protein assay. 25 μg of protein was size-fractionated using SDS-PAGE and electrotransferred onto Protran nitrocellulose membranes (Schleicher & Schuell). Antibodies recognizing FoxO3a phosphorylated at Thr-32 and total FoxO3a (06–951) were purchased from Upstate (Dundee, UK). Mouse monoclonal antibody against FoxO3a (F-1304) was purchased from Sigma. Antibodies against p27^{kip1} (C-19), Bim (H-191), Cdc25b, Cdc25c (C-20), cyclin B1 (H-433), Pfk (F-8), actin (I-19), FoxM1 (C-20), FoxM1 (H-300), and ERα (F-10) were purchased from Santa Cruz Biotechnology. Antibodies against phospho-p44/42 MAPK (Thr-202/Tyr-204), total MAPK, phospho-Akt (Ser-473), and total Akt were purchased from Cell Signaling Technologies (Hitchin, UK). Primary antibodies were detected using horseradish peroxidase-linked anti-mouse, anti-goat, or anti-rabbit conjugates (DAKO, Ely, UK), as appropriate, and visualized using the ECL detection system (Amersham Biosciences).

Immunoprecipitation Assay—Cells were washed in phosphate-buffered saline and lysed with 500 μl of Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris, pH 7.4, 10 mM sodium molybdate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and Complete Mini protease inhibitors (Roche Applied Science)) for 15 min on ice. The cell lysates were precleared for 1 h with protein G-Sepharose, incubated with specific antibodies for 1 h, and then with 50% slurry of protein G-Sepharose for 1 h. The beads were washed five times with 500 μl of lysis buffer and analyzed by Western blotting.

Transfection and Luciferase Reporter Promoter Assay—MCF-7 cells were cultured in 96-well plates until 60% confluent. The cells were transfected using FuGENE 6 reagent (Roche Applied Science) according to the manufacturer’s instructions for 24 h, washed twice in phosphate-buffered saline, and then harvested.
for firefly/\textit{Renilla} luciferase assays using the Dual-Luciferase reporter assay system (Promega, Southampton, UK).

\textit{Mutagenesis}—Mutagenesis of putative Forkhead binding sites of the ERα promoter was carried out using the QuikChange\textsuperscript{\textregistered} site-directed mutagenesis kit (Stratagene, Cambridge, UK). The sequences for primers used for creating point mutations and deletions are essentially those described for the DNA pulldown assays plus flanking regions to give oligonucleotides of 42 bases.

\textbf{Real-time Quantitative PCR (RT-qPCR)}—Total RNA was isolated using the RNeasy kit (Qiagen, Crawley, UK). 1 \(\mu\)g of total RNA was reverse-transcribed using the Superscript first strand synthesis system for RT-qPCR (Invitrogen), and the total RNA was reverse-transcribed using the Superscript first strand cDNA was used as the template in the real-time quantitative PCR analysis. All measurements were performed in triplicate. The mRNAs analyzed were FoxO3a, FoxM1, ERα, and GAPDH, which served as an internal control and was used to normalize for variances in input cDNA. The following gene-specific primer pairs were designed using the ABI Primer Express software: FoxO3a-sense, 5'-TGCACTATGCACTGAGGTGTTG-3' and FoxM1-sense, 5'-GGAGGATCTGATCAGTATACTC-3' and FoxM1-antisense, 5'-GGAGGATGTCAGTATCCAAGA-3'.

\textbf{ChIP assay}—Total RNA was isolated using the RNaseasy kit (Qiagen, Crawley, UK). 1 \(\mu\)g of total RNA was reverse-transcribed using the Superscript first strand synthesis system for RT-qPCR (Invitrogen), and the total RNA was reverse-transcribed using the Superscript first strand cDNA was used as the template in the real-time quantitative PCR analysis. All measurements were performed in triplicate. The mRNAs analyzed were FoxO3a, FoxM1, ERα, and GAPDH, which served as an internal control and was used to normalize for variances in input cDNA. The following gene-specific primer pairs were designed using the ABI Primer Express software: FoxO3a-sense, 5'-TGCACTATGCACTGAGGTGTTG-3' and FoxM1-sense, 5'-GGAGGATCTGATCAGTATACTC-3' and FoxM1-antisense, 5'-GGAGGATGTCAGTATCCAAGA-3'.

\textbf{RESULTS}---
\textit{FoxM1, but Not FoxO3a, Expression Correlates with ERα Expression at the Transcriptional Level in Breast Cancer Cell Lines}---To explore the possibility that FoxM1 regulates ERα expression, we first analyzed the correlation between the levels of expression of FoxM1 or FoxO3a and ERα in a panel of 16 different breast cell lines by Western blotting. As shown in Fig. 1A, 13 of the 16 breast carcinoma cell lines showed good correlations between the levels of FoxM1 and ERα expression. In contrast, only 6 of the 16 cell lines demonstrated positive correlation between the levels of FoxO3a and ERα expression. We next examined the mRNA levels of ER\(\alpha\) in these cells by RT-qPCR (Fig. 1B). The result showed that, although the expression level of FoxM1 protein correlated with that of ER\(\alpha\) mRNA in 13 of the 16 cell lines, only 6 of the 16 cell lines analyzed showed significant correlations between FoxO3a and ER\(\alpha\).
mRNA. Because the activity of FoxO3a is negatively regulated by Akt phosphorylation, the phosphorylation status of Akt was also studied using specific antibodies against phosphorylated and total Akt. The result again failed to account for the lack of correlation between FoxO3a and ERα/H9251 expression. These data suggest that FoxM1, rather than FoxO3a, is the physiological regulator of ERα/H9251 transcription in breast cancer cells.

**Ectopic Expression of FoxM1, but Not FoxO3a, Results in Up-regulation of ERα Transcription**—To test the hypothesis that FoxM1, but not FoxO3a, regulates ERα expression, we transiently transfected the ERα/H9251-positive breast cancer cell line ZR-75–30 with different amounts of expression plasmids encoding for FoxM1 and a constitutively active FoxO3a(A3) and analyzed the expression levels of ERα and known FoxM1 and FoxO3a targets at 48 h after transfection by Western blotting. These results showed that ectopic expression of FoxM1 increased the expression levels of the FoxM1 targets Plk and Cdc25c (Fig. 2A). We also observed a moderate but significant increase in ERα expression in the ZR-75–30 cells overexpressing FoxM1 but not those overexpressing FoxO3a(A3).

To further investigate whether FoxM1 was regulating the transcription of ERα, we analyzed these cells by RT-qPCR. These results showed an ~2-fold increase in ERα transcription when FoxM1 was overexpressed, which was not observed when FoxO3a(A3) was overexpressed (Fig. 2A).

**Induction of FoxM1 Expression in an MCF-7 Tet-on System Results in an Increase in the Levels of ERα Expression/Transcription**—To further confirm that FoxM1 regulates ERα expression, we generated an MCF-7 cell line (MCF-7 Tet-on FoxM1) stably transfected with the pTet-On (Clontech) and pcDNA4-FoxM1 plasmids in which the FoxM1 expression was inducible by the addition of doxycycline. As shown in Fig. 2B, treatment of this cell line with 2 μg/ml doxycycline increased the expression of FoxM1 with time, and this was accompanied by an increase in the expression of ERα and known FoxM1 targets such as Cdc25c and Plk. There was a modest down-regulation of FoxM1 expression at 24 h, and this was probably because of the negative cell proliferative effect of doxycycline. Nevertheless, this was accompanied by a similar decline in ERα, Cdc25c, and Plk expression, further confirming our hypothesis that FoxM1 regulates ERα expression. Notably, the level of FoxO3a expression remained constant up to 48 h and declined moderately at 72 h after doxycycline treatment, suggesting that the induction of ERα by FoxM1 is not mediated through modulating FoxO3a expression. It is notable that the level of ERα induction was lower in the ZR-75–1 cells compared with the MCF7 cells. The reason is unclear, but it is likely to be due to the fact that not all of the ZR-75–1 cells would be expressing FoxM1 at high levels during transient transfection, whereas the MCF7 (Tet-on FoxM1) is a clonal cell line in which all of the cells would express FOXM1 at high levels following induction. Moreover, the low level of ERα expression in the ZR-75–30 cells might also be the result of post-transcriptional regulation. To further address whether the regulation...
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Inhibition of FoxM1 Activity Leads to Down-regulation of ERα Expression—We next studied whether inhibition of FoxM1 activity would lead to down-regulation of ERα expression. To this end, we treated MCF-7 cells with 10 μM U0126 and performed a time course experiment as indicated in Fig. 3A. Western blot analysis showed down-regulation of the known FoxM1 targets Cdc25c and Cyclin B1 as early as 1 h after treatment. This was accompanied by a mobility shift and decrease in FoxM1 levels. The faster migration species of FoxM1 observed after treatment with U0126 probably reflects the dephosphorylated and inactive form of this protein. We also observed a similar down-regulation of ERα expression but only at 3 h after treatment. This probably highlights the fact that the half-life of ERα protein is longer than those of Cdc25c and cyclin B1. The levels of expression of FoxO3a and the FoxO3a targets Bim and p27Kip1 were constant throughout the time course, indicating that there was no change in FoxO3a activity. These results provide further evidence that FoxM1 is a physiological regulator of ERα expression in breast cancer cells. To extend these observations, we next studied the effects of FoxM1 and FoxO3a silencing on the expression of ERα in MCF-7 cells. To this end, we transfected siRNA vectors targeting FoxM1 or FoxO3a into MCF-7 cells and analyzed the protein expression at 72 h post-transfection by Western blotting. The results showed that abolishing FoxM1 expression in MCF-7 cells led to the abrogation of expression of ERα as well as FoxM1 targets, such as Cdc25c, and Cdc25c (Fig. 3B). Silencing of FoxO3a in MCF-7 cells resulted in the down-regulation of the known FoxO3a targets Bim and p27Kip1. Surprisingly, we also detected a moderate down-regulation of ERα and FoxM1 expression in these cells when compared with mock and control siRNA-transfected MCF-7 cells. The down-regulation of ERα in cells transfected with FoxO3a siRNA could be mediated through down-regulation of FoxM1 expression. The results also indicated that there were no significant changes in the expression and activity of ERK1/2 and Akt, which are upstream regulators of FoxM1 and FoxO3a, respec-
activity of promoter B was low when compared with A, suggesting that promoter A is the predominant promoter in these cell lines. Sequence analysis of the ERα promoters A and B identified a number of consensus Forkhead-responsive elements (FHREs), including sites S1, S2, and S4 on promoter B, previously identified by Guo and Sonenshein (20) to be inducible by FoxO3a (Fig. 4A). To then map the transcriptional elements responsible for mediating the induction by FoxM1, a series of deletion reporter constructs of the ERα promoters A and B were co-transfected with FoxM1 expression vector into COS cells and analyzed for transcriptional activity 24 h after transfection. Deletion of the region of the ERα promoter B (−4542 to −3038) containing the previously defined FHREs S1 and S2 reduced significantly the activation by FoxM1 from 1.3- to 1.1-fold. The average induction of the longest wild-type ERα promoter A (−1168/+190) after FoxM1 transfection was 3.3-fold, and the comparably low levels of induction by FoxM1 probably reflects the fact that the FoxM1 expression vector is not expressing a constitutively active form of FoxM1. Deletion of the 5′ distal region (−1168/−513) only marginally reduced the ability of FoxM1 to induce the promoter activity. The fold of activation by FoxM1 reduced significantly to background level when the region (−513/+190) of the promoter containing three putative FoxM1 binding sites (S6, S7, and S8) was deleted. Similarly, a decrease in the level of induction was also observed when any one of the FHREs of the full-length ERα promoter A was mutated, suggesting that these FHRE sites are responsible for FOXM1 transactivation. Mutation of all three putative FHREs rendered the ERα promoter insensitive to FoxM1 induction. However, our transfection results also indicated that S8 is a weak FHRE compared with S6 and S7. Together, these transfection results suggested that the proximal ERα promoter FHREs S6, S7, and S8 primarily mediate the response to FoxM1 of the promoter.

**FoxM1 and FoxO3a Bind to the Same Responsive Elements on the ERα Promoter**—To further characterize these putative FHREs identified by promoter analysis and to demonstrate physical interaction of FoxM1 and FoxO3a with these sites, we used biotinylated oligonucleotides coupled to streptavidin-agarose beads to “pulldown” proteins interacting with different FHREs in MCF-7 cells and then analyzed the bound proteins by Western blotting. The result showed that FoxM1 bound to the S1/S2, S4, S6/S7, and S8 FHREs containing oligonucleotides but not to similar mutant oligonucleotides with the FHREs mutated, consistent with the transfection results. Surprisingly, FoxO3a was also shown to be interacting with the same FHREs. Similar to the deletion analysis results, we failed to demonstrate binding of FoxM1 or FoxO3a to the S5 FHRE. These interactions depend on the FHREs, as both the FoxM1 and FoxO3a binding could be competed off using increasing amounts of oligonucleotides containing the FHRE but not similar oligonucleotides with the FHRE mutated (Fig. 5A). These results together suggest that both FoxM1 and FoxO3a are recruited directly to the FHREs.

**FoxM1 and FoxO3a Associate with the ERα Promoter in Vivo**—We next performed ChIP assays to examine the in vivo occupancy of the ERα promoter by FoxM1 and FoxO3a. As demonstrated in Fig. 5B, both the anti-FoxM1 and anti-FoxO3a antibodies, but not the control antibodies (IgGs), precipitated the ERα pro-
moter regions containing the S1/S2, S4, and S6/S7 FHREs. Consistent with the oligo pulldown result, the ChIP assay also illustrated that the anti-FoxM1 and anti-FoxO3a antibodies failed to precipitate promoter fragments containing the S5 site. Interestingly, although the pulldown experiment showed that the S8

**FIGURE 4.** Characterization of the human ERα promoter and its activity in response to FoxM1 and FoxO3a expression. Schematic representation of the human ERα promoter showing the putative FHREs (top panel). A, comparison of the ability of FoxO3a and FoxM1 to transactivate the human ERα promoters A and B. MCF-7 and COS cells were transiently transfected with the 10-μg human ERα promoter A or B constructs together with increasing amounts (0, 2, 10, 20, and 50 μg) of pLPCFoxO3a(wt), A3, or pcDNA3FoxM1. Cells were harvested 24 h after transfection and assayed for luciferase activity. Values are corrected for co-transfected Renilla activity. All data shown represent the average of three independent experiments, and the error bars show the standard deviation. B, cycling COS cells were transfected with different human ERα promoter-luciferase reporter constructs as indicated together with 20 μg of pcDNA3FoxM1. The COS cells were harvested 24 h and assayed for luciferase activity. All values were corrected for co-transfected Renilla activity. The folds of induction by FoxM1 are shown on the right.

th could bind FoxM1 and FoxO3a, the ChIP assay failed to show either FoxM1 or FoxO3a binding to the promoter region containing S8 in vivo (Fig. 6). In general, the ChIP result confirms the findings from the co-transfection and pulldown experiments.

_FoxO3a Interacts with FoxM1 in Vivo_—With the chromatin immunoprecipitation and biotinylated oligonucleotide pulldown data indicating that FoxM1 and FoxO3a bind to the same elements on the ERα promoter, we next investigated whether these two proteins could interact in vivo. To this end, we performed immunoprecipitation assays using antibodies specific for FoxM1 or FoxO3a. The co-precipitated proteins were then identified by Western blotting. These results showed that the endogenous FoxM1 and FoxO3a proteins co-precipitated with one another in MCF-7 cells, thus confirming in vivo interaction between FoxM1 and FoxO3a.

**DISCUSSION**

Estrogens are powerful mitogens essential for the initiation and progression of human breast and other gynecological cancers. Because ERα mediates the effects of estrogens, endocrine agents, such as Tamoxifen, Raloxifene, and Fulvestrant (ICI 182,780), are frequently used to block their synthesis or their activities in breast cancer treatment regimes. The successes of endocrine therapies for breast cancer treatments are often hampered by the development of resistance to hormonal treatments. Although enhanced growth factor signaling (which induces both genomic and non-genomic activities of ERα) is the principal mechanism of acquired resistance, the major reason for de novo resistance to endocrine therapy is loss of ERα expression. Thus, unraveling the molecular mechanisms by which ERα transcription is regulated might provide vital information not only for the development of novel therapeutic strategies against endocrine-resistant ERα-positive tumors but also for understanding the loss of ERα in de novo resistant diseases.

In this study, we have identified FoxM1 protein as a physio-
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Our survey of a panel of 16 different breast cancer cell lines showed a good correlation (13/16) between FoxM1 expression and expression of ERα at both the protein and mRNA levels. We have also demonstrated that ectopic expression of FoxM1 in two different ER-positive breast cancer cell lines, MCF-7 and ZR-75–30, led to up-regulation of ERα expression at the protein and mRNA levels. Moreover, treatment of MCF-7 cells with the MEK inhibitor U0126, which blocks ERK1/2-dependent activation of FoxM1, also repressed ERα expression. Furthermore, silencing of FoxM1 expression in MCF-7 cells using siRNA resulted in the almost complete abrogation of ERα expression. We have also shown that FoxM1 can activate the transcriptional activity of human ERα promoter primarily through three closely located FHREs located at the proximal region of the ERα promoter.

FoxO3a has previously been reported to regulate ERα transcription (20). Although we have confirmed in vitro and in vivo binding of FoxO3a to the ERα promoter and shown that overexpression of a constitutively active FoxO3a is able to transactivate region B of the ERα promoter in co-transfection studies, the results of our functional studies do not totally support the idea that FoxO3a is the primary physiological regulator of ERα transcription. We observed a poor correlation of 6/16 between FoxO3a expression levels and ERα expression in a larger panel of breast carcinoma cells compared with the previous study. Moreover, although ectopic expression of FoxM1 resulted in a significant increase in the level of ERα transcription in the ZR-75–30 breast cancer cell line, overexpression of the constitutively active FoxO3a did not lead to an up-regulation of ERα transcription in the same cell system. Although we did observe a moderate down-regulation of ERα expression in MCF-7 cells transfected with siRNA against FoxO3a, the decline in ERα expression could be a result of the down-regulation of FoxM1 expression as the level of FoxM1 expression was also significantly lowered in the same cells. Furthermore, given that FoxO transcription factors

FIGURE 5. In vitro and in vivo binding of FoxM1 and FoxO3a to the putative Forkhead-responsive elements on the ERα promoter. Shown is a schematic representation of the human ERα promoter showing the putative FHREs as well as the regions corresponding to sequences used for DNA pulldown and chromatin immunoprecipitation PCR analyses (top panel). A, oligonucleotides containing different putative FHREs of the human ERα promoter were generated with 5′ ends either free or conjugated to biotin molecules. DNA-binding nuclear extracts prepared from cycling MCF-7 cells were incubated with 1 μg of biotin-conjugated wild-type (wt) and mutant (mut) oligonucleotides; see the first two lanes and wt in all other lanes. In lanes 3–5 and lanes 6–8, increasing amounts (1, 5, and 10× molar excess) of competing non-conjugated oligos were incubated with the mix. Proteins binding to the biotinylated oligonucleotides were pulled down using streptavidine-agarose beads, and the washed lysates were analyzed using SDS-PAGE followed by immunoblotting using specific antibodies against FoxO3a and FoxM1. B, protein-DNA complexes from cycling MCF-7 cells were formaldehyde cross-linked in vivo. Chromatin fragments from these cells were subjected to immunoprecipitation with antibodies against IgG (nonspecific), FoxM1, or FoxO3a as indicated. After cross-link reversal, the co-immunoprecipitated DNA was amplified by PCR using the indicated primers and resolved in 2% agarose gels.
mediate cell cycle arrest and apoptosis whereas expression of ERα is associated with breast cancer cell proliferation, it is unlikely that FoxO3a is the primary physiological activator of ERα expression in breast cancer cells. However, paradoxically, a number of previous studies have shown that ERα and the proliferation marker Ki67 nuclear antigen are not usually expressed in the same breast tumor cells, leading to the suggestion that ERα regulates breast epithelial cell proliferation indirectly (35). Despite these earlier studies, more recent data demonstrates that ERα is expressed in proliferating breast epithelial cells and co-expresses with proliferative markers, including Ki67, MYC, cyclin D1, and stromal cell-derived factor (SDF)-1 (36). The failure in these earlier studies to demonstrate the co-expression of ERα and Ki67 in breast epithelial cells has been proposed to be due to the fact that the low mitotic index in these early samples and the short half-life and narrow expression window of Ki67 during the cell cycle have made the detection of transient co-expression of Ki67 and ERα difficult (36).

In exploring the mechanism by which FoxM1 regulates ERα expression, we performed mutation and deletion analyses of the ERα promoter and identified two FHREs located at the proximal region of the ERα promoter A to be primarily responsible for the transactivation of the ERα promoter by FoxM1. Further, our chromatin immunoprecipitation and biotinylated oligonucleotide pull-down and co-immunoprecipitation results all suggest the possibility that FoxM1 and FoxO3a cooperate to regulate ERα gene transcription. Thus, despite an evident role of FoxM1 in ERα gene regulation, the function and mechanism of FoxO3a in regulating ERα expression in breast epithelial cells are still unclear and warrant further investigation.

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