Upstream Determinants of Estrogen Receptor-α Regulation of Metastatic Tumor Antigen 3 Pathway*

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Abstract

Although recent studies have shown a role of estrogen receptor-α (ER) in the regulation of epithelial-to-mesenchymal transition via MTA3, the role of upstream determinants of ER regulation of MTA3 and the underlying molecular mechanism remains unknown. Here we show that MTA3 gene regulation by ER is influenced by dynamic changes in levels of nuclear coregulators. MTA3 promoter has a functional ER element half-site with which MTA1 and HDACs interact under basal conditions. Upon estrogen stimulation, these corepressors are derecruited with concomitant recruitment of ER, leading to increased MTA3 transcription and expression. Genetic inactivation of MTA1 pathway promotes the ability of ER to up-regulate MTA3 expression, whereas knockdown of ER enhances MTA1 association with MTA3 gene. Modulation of ER functions, by corepressors (i.e. MTA1 and MTA1s) or coactivators (i.e. AIB1 and PELP1/MNAR), alters ER recruitment to MTA3 chromatin, MTA3 transcription, and expression of downstream epithelial-to-mesenchymal transition components. These studies provide novel insights into the transregulation of the MTA3 gene and reveal novel roles of upstream determinants in modifying the outcome of MTA3 axis and cell differentiation.

The development of human breast cancer is promoted by estrogen stimulation of mammary epithelial cell growth. Estrogen receptor-α (ER) is the major estrogen receptor in the human mammary epithelium. The binding of estrogen to ER triggers conformational changes that allow ER to bind to the 13-base-pair palindromic estrogen response element (ERE) in the target gene promoters and stimulates gene transcription thereby promoting the growth of breast cancer cells. The transcriptional activity of ER is affected by a number of regulatory cofactors including chromatin-remodeling complexes, coactivators, and corepressors (1–3).

Recent findings have demonstrated that the NuRD-70 polypeptide of the nucleosome-remodeling complex is identical to metastatic tumor antigen 1 (MTA1) (4,5) and that MTA1 physically interacts with HDAC1/2 (6,7). The MTA1 gene is shown to correlate well with the metastatic potential of several human cell lines and cancers, including breast cancers (8–11).

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1The abbreviations used are: ER, estrogen receptor-α; MTA, metastatic tumor antigen; mAb, monoclonal antibody; ERE, estrogen response element; EMT, epithelial-to-mesenchymal; ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA; PR, progesterone receptor; HDAC, histone deacetylase.
Using in vitro models, Mazumdar et al. (12) have shown that MTA1 interacts with ER and represses ER transcription by recruiting HDAC to the ERE-containing target gene chromatin in breast cancer cells. MTA1-overexpressing breast cancer cells exhibit aggressive phenotypes (13). MTA1s, another family member, is a naturally occurring variant of MTA1 that contains a novel sequence of 33 amino acids with one potential nuclear receptor binding motif, LRILL. MTA1s inhibits ER nuclear signaling by sequestering ER in the cytoplasm but enhances ER cytoplasmic signaling and thus promotes tumorigenesis (14).

One of the principal phenotypic changes in breast cancer metastasis is the increased tendency of the cancer cells to undergo epithelial-to-mesenchymal (EMT) transition that is characterized by reduced expression and consequently, functions of cell-adhesion components such as E-cadherin (15–17). The zinc finger transcriptional repressor, Snail, mediates the repression of E-cadherin expression and leads to the inhibition of Snail function in epithelial cells, thus, restoring the expression of E-cadherin as well as cell-to-cell junctions (18,19). Recently, Fujita et al. (20) identified MTA3 as an ER-regulated gene and showed that MTA3 up-regulation prevents EMT by directly repressing Snail and thereby up-regulating E-cadherin. Although these observations highlight the significance of EMT in breast cancer invasiveness and suggest a complex role for MTA family members in modifying ER functions in breast cancer cells, the precise mechanism by which ER regulates MTA3 expression and the putative nature of upstream determinants of MTA3 expression remain poorly understood.

Here we show that MTA3 contains an ER element half-site and that both ER and MTA1 are recruited to the same site in the MTA3 promoter chromatin. Further, estrogen stimulates MTA3 promoter activity in a corepressor-sensitive manner. In addition, modulation of ER functions by corepressors (i.e. MTA1 and MTA1s) or coactivators (i.e. AIB1 and PELP1/MNAR) results in the suppression or stimulation of ER recruitment to the MTA3 chromatin and consequently affects the expression of EMT components. Together, these studies reveal that the dynamic changes in the levels of ER coregulators influence ER regulation of MTA3 and reveal a novel role for nuclear coregulators in modifying the outcome of MTA3-mediated EMT.

**EXPERIMENTAL PROCEDURES**

### Cell Lines and Reagents

MCF-7 human breast cancer cells and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium-F12 (1:1) supplemented with 10% fetal calf serum. HeLa cervical cancer cells were obtained from the American Type Culture Collection (Manassas, VA). MCF-7 clones stably expressing MTA1, MTA1s, and PELP1 have been described earlier (12,14,21). Steroid hormone E2, tamoxifen, and charcoal-stripped serum (N,N′-dicyclohexylcarbodiimide serum) was purchased from Sigma. ICI-182, 780 was purchased from Tocris, Ellisville, MO. Antibodies against MTA1 were purchased from Santa Cruz Inc. (Santa Cruz, CA) and T7 monoclonal antibodies (mAb) was procured from Novagen (Milwaukee, WI). Antibodies for E-Cadherin were obtained from Zymed Laboratories Inc., and ER antibody was purchased from UBI. The Snail antibody used here has been described before (22).

Rabbit anti-peptide against MTA3 was generated against the mouse MTA3 amino acids 420–438 (sequence SDEEKPSPTAEDPRASH). The MTA3-GST fusion protein was run on SDS-PAGE and transferred to nitrocellulose filter. The blot was stained with Ponceau S, and the region of the blot with the GST-MTA3 fusion protein was cut. The GST-MTA3 blots were incubated in 10 ml of 1% bovine serum albumin for 3–4 h at 4 °C to block nonspecific sites. The bovine serum albumin-treated nitrocellulose pieces were incubated with diluted rabbit antiserum in cold overnight. Subsequently, the nitrocellulose pieces were washed 4× in Tris-buffered saline with 0.01% (v/v) Tween 20 for 5 min each and transferred to Eppendorf tubes.
and the bound MTA3 antibody was eluted by sequential incubation with 300 μl of monoclonal antibody purification system buffer, pH 3.0, for 5 min. Immediately after elution, 200 μl of 1 M Tris, pH 9.5, was added to prevent denaturation of the antibody. The eluates were pooled and stored −20 °C.

**Cloning of MTA3 Promoter and MTA3 cDNA**

To clone the MTA3 promoter, we first identified the BAC clone (number RP11-314A20) containing the MTA3 genomic region using human genome sequence information. We then purchased the BAC clone from BACPAC Resources (Children’s Hospital Oakland Research Institute, Oakland, CA). Two fragments (1078 and 735 bp) of the MTA3 promoter region were amplified by PCR. The amplified products were cloned into the PGL3 luciferase reporter vector (Promega Corp., Madison, WI) using SacI and XhoI sites. The sequence of the construct was verified by comparing its sequence with that in the human genome data base. PCR based cloning was used to generate the deletion construct and point mutation of the 1078 bp MTA3 promoter. PCR product of MTA3 cDNA was cloned at pcDNA3.1A using KpnI and XhoI sites (see Table I for primer sequences).

**Reporter Assays**

For the reporter gene transient transfections, cells were cultured for 24 h in minimal essential medium without phenol red containing 5% N,N′-dicyclohexylcarbodiimide serum. The MTA3-luciferase reporter constructs were transfected using FuGENE 6 according to the manufacturer’s instructions (Roche Applied Science). Twenty-four hours later, the cells were treated with E2 for 16 h. The cells were then lysed with a passive lysis buffer, and the luciferase assay was performed using a luciferase reporter assay kit (Promega). The total amount of DNA used in the transfections was kept constant by adding a parental vector. Each transfection was carried out in six-well plates in triplicate wells.

**Chromatin Immunoprecipitation (ChIP) Assay**

Approximately 10⁶ cells were treated with 1% formaldehyde (final concentration, v/v) for 10 min at 37 °C to cross-link histones to DNA. The cells were washed twice with phosphate-buffered saline, pH 7.4, containing protease inhibitor mixture (Roche Applied Science). The ChIP assay was performed as described previously (12). An ER-α-specific antibody, MTA1 mAb, or T7 mAb were used for the immunoprecipitation of protein-bound chromatin, and precipitated DNA was amplified by PCR using primers flanking the proximal half-ERE site (see Table I for primer sequences). The amplified fragment was sequence-verified.

**Gene Knockdown by Small Interfering RNA (siRNA)**

ER-α-specific siRNA and control nonspecific siRNA were purchased from Dharmacon. For MTA1 knockdown, 4-for-Silencing siRNA Duplexes were designed using a Qiagen program and synthesized at Qiagen. siRNA transfections were carried out using 20 μM pooled siRNA duplexes and by using 4 μl of Oligofectamine (Invitrogen) according to the manufacturer’s protocol in six-well plates. After 72 h, cells were prepared for ChIP assay or Western blotting.

**Immunofluorescence and Confocal Studies**

The cellular location of proteins was determined using indirect immunofluorescence, as described previously (14). In brief, MCF-7 cells were plated on glass coverslips in six-well culture plates. When the cells were 50% confluent, cells were rinsed with phosphate-buffered saline, fixed in cold methanol for 6 min, and then processed for immunofluorescence staining of endogenous MTA3, E-Cadherin, or Snail. Cells were counterstained with ToPro3 to visualize the nucleus. Slides were further processed for imaging and confocal analysis using a Zeiss LSM 510 microscope and a 40× objective.
RESULTS AND DISCUSSION

Cloning and Regulation of MTA3 Promoter by Estrogen

To delineate the mechanism of ER regulation of MTA3 expression, we first analyzed the sequence of the putative MTA3 promoter region (GenBank™ genomic sequence accession number NT_022184) for the presence of ER-responsive elements using Matinspector (Genomatix). This program did not reveal any consensus 13-bp ERE sites. However, we found that the MTA3 promoter contains three potential ERE half-sites (TGACC) (Fig. 1A). Interestingly, all the three ERE half-sites were localized in the vicinity of AP1 binding sites. Because a number of ER-responsive genes have been shown to be regulated via ERE half-sites in conjunction with either AP1 or SP1 sites (23), we examined the potential involvement of these ERE half-sites in the regulation of MTA3 expression by ER.

To explore the recruitment of ER to the endogenous MTA3 promoter, MCF-7 breast cancer cells were treated with or without estrogen and subjected to ChIP assays using an anti-ER antibody. We found that ligand-activated ER is recruited to the endogenous MTA3 promoter at the ER element half-site from positions −1256 to −1245 but not to the other two potential sites (Fig. 1B). To verify these results, we next designed a pair of primers that encompass proximal as well as the middle potential ERE half-sites. Results showed estrogen-induced recruitment of ER to the MTA3 promoter region of expected 714 bp (Fig. 1B, right panel). To ascertain that the detected 239-bp band indeed represents the regulatory region corresponding to the proximal ERE half-site, the PCR-amplified DNA fragment was cloned into a TOPO vector and confirmed by sequencing (data not shown). Estrogen-induced recruitment of ER to the MTA3 promoter chromatin was effectively blocked by the inclusion of anti-estrogen ICI-182780 suggesting that estrogen-activated recruitment of ER to the MTA3 gene was specific. Because ER recruitment to the MTA3 promoter was also inhibited by the protein-synthesis inhibitor cycloheximide (Fig. 1C), it appears that this event requires new protein synthesis, and the noted effects could be mediated via an indirect mechanism. This appears to be consistent with the notion that ER action through an ERE half-site requires an associated factor and that direct interaction with DNA may not be involved (21). Given that it has been demonstrated previously that estrogen up-regulates both its own receptor, ER, as well as ER coactivators expression (21,24), it seemed likely that sufficient quantities of ER and its coactivators were necessary for up-regulation of MTA3 expression by estrogen. To test this hypothesis, we looked at the levels of ER under the same experimental conditions and did not find changes in ER amounts in the cell for any of the conditions.

To further study the regulation of the MTA3 promoter via the ERE half-site, a MTA3 promoter fragment was amplified from the BAC clone and DNA fragments of desired sizes, 1078 and 735 bp, were obtained. The MTA3 promoter fragments were cloned into a pGL3-luciferase reporter system. The functionality of MTA3-luciferase vectors was tested in MCF-7 and HeLa cells (Fig. 2, A and B). Estrogen treatment of the cells stimulated the MTA3-regulatory element-driven reporter activity from the 1078-bp fragment (−1528 to −450, contains two ERE half-sites, at −1459 and at −1241) but not from the 735-bp fragment (−1185 to −450, lacks −1241-bp ERE half-site) (Fig. 2B), and therefore, the MTA3 1078-bp luciferase construct was used in the subsequent studies. Estrogen-stimulated MTA3 promoter activity was effectively blocked by tamoxifen, an estrogen antagonist, which also had a modest inhibitory effect on basal MTA3 promoter activity (Fig. 2, C and D). To validate the proximal ERE half-site (at −1241) in the estrogen stimulation of MTA3 promoter, we created a point mutation up to −1241 and deletion at −1450 of the 1078-bp MTA3 promoter. As shown in Fig. 2, E and F, the point mutation at the proximal ERE half-site abolished the estrogen stimulation, whereas deletion of the middle ERE half-site did not affect estrogen stimulation as compared with the 1078 bp fragment. Together, these findings confirm that the observed recruitment of ligand-activated ER to the MTA3 promoter chromatin is accompanied by increased MTA3 promoter activity.
Regulation of MTA3 Expression and EMT by MTA1

Because MTA1 and MTA1s are natural inhibitors of ER functions (12,14,25), we next tested whether the deregulation of these proteins may influence MTA3 expression and functions. Using breast cancer cells stably expressing T7-MTA1 (12) or T7-MTA1s (14), we found that the repression of ER function by MTA1 or MTA1s abolished the ability of estrogen to promote the recruitment of ER to the MTA3 gene chromatin (Fig. 3, A and B). Because both MTA1 and MTA1s had similar effects, we used only MTA1 to repress the functions of ER in subsequent studies. Overexpression of MTA1 in breast cancer cells also resulted in the inability of estrogen to induce MTA3 mRNA (Fig. 3C). The observed inhibition of MTA3 expression by MTA1 was at the level of transcription, as coexpression of MTA1 inhibited both the basal and estrogen-induced stimulation of MTA3 promoter activity (Fig. 3D). In MTA1 overexpressing conditions without estrogen stimulation, expression of the MTA3 protein was not inhibited, whereas MTA3 promoter activity did show repression. Based on these results, one could speculate that there could be additional regulatory elements that may be involved in MTA3 basal expression in the physiological setting. It could also be possible that the basal repression observed in the promoter-reporter analysis is due to an inherent limitation of the assay system, because it reflects the regulation of the promoter area largely in isolation and under artificial conditions. Results from confocal scanning microscopy also demonstrated that MTA1 deregulation leads to a significant reduction in the levels of nuclear MTA3, up-regulation of Snail and consequent down-regulation of E-cadherin as compared with the levels of MTA3, Snail, and E-cadherin in control vector-transfected cells (Fig. 4). All of the above findings suggest that the inhibition of ER transactivation functions by MTA1 could impair the ability of ER to up-regulate MTA3 expression.

Modulation of MTA3 Chromatin by ER Coregulators

Because MTA1 prevented the recruitment of ligand-activated ER to the MTA3 promoter, we next investigated the possibility of MTA1 recruitment to the MTA3 promoter chromatin. MCF-7 cells were treated with or without estrogen, and chromatin lysates were immunoprecipitated with anti-MTA1 antibody. We found that MTA1 associates with the MTA3 promoter in unstimulated MCF-7 cells but not in estrogen-stimulated cells (Fig. 5A). MTA1 association with the MTA3 promoter was found to be at the proximal ERE half-site, which was also immunoprecipitated in the ER ChIP assay. These observations suggest that MTA1 associates with the basal MTA3 promoter chromatin and upon estrogen treatment is derecruited with simultaneous recruitment of ER to the ER element half-site in the MTA3 promoter chromatin. Because MTA1 has been shown to physically interact with HDAC1 and HDAC2 (6), we next examined whether the HDACs also interact with the basal MTA3 promoter. Results from ChIP studies using anti-HDAC antibodies show the association of the HDACs with the MTA3 promoter that, like MTA1, were also derecruited from the MTA3 promoter upon estrogen treatment (Fig. 5B). These findings raise possibility that the basal MTA1 association with the MTA3 promoter might influence the status of EMT components presumably because of the inhibitory effect of MTA1 on the ER transactivation functions. To test this notion, we silenced endogenous MTA1 expression using siRNA and examined the status of MTA3 target genes, as well as ER target genes in MCF-7 cells. Results indicated that inhibition of MTA1 expression leads to the down-regulation of Snail and the up-regulation of E-cadherin (Fig. 5C). Interestingly, there was also significant up-regulation of progesterone receptors PR-A and PR-B, well established ER target gene products, thus suggesting enhanced ER transactivation functions due to reduced levels of MTA1 and, consequently, loss of corepressor functions of MTA1. Consistent with these findings, a reduction in the levels of MTA1 by siRNA was accompanied both by an increased basal expression of MTA3 and the enhanced ability of estrogen to up-regulate MTA3 expression (Fig. 5D). Interestingly, there was also increased estrogen-induced stimulation in the levels of ER in MTA1 knockdown cells

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Regulation of MTA3 Gene by the Endogenous MTA1

To determine whether the above changes in the levels of MTA3 and its targets in MTA1 knockdown MCF-7 cells were because of a modification of the MTA3 chromatin in the vicinity of the ER element half-site, we next performed ChIP analysis of ER recruitment on the MTA3 chromatin under conditions of MTA1 knockdown (Fig. 6A). We found that the suppression of endogenous MTA1 expression leads to detectable basal association of ER with the MTA3 promoter, which as expected was further enhanced upon estrogen signaling. The above result was corroborated in a luciferase assay system where the knockdown of MTA1 resulted in a significant increase in both basal and estrogen-induced MTA3-luciferase activity (Fig. 6B). Consistent with these results, silencing of the endogenous MTA1 in MCF-7 cells resulted in a substantial decrease in the recruitment of HDACs to the MTA3 promoter segment, and estrogen stimulation led to complete dissociation of the HDACs from the MTA3 promoter (Fig. 6C). To further support the notion that MTA1 and ER might be competing for the ER element half-site in the MTA3 promoter, we next knocked down the endogenous ER in MCF-7 cells by siRNA, as assessed by the levels of ER and the ER target gene products, PR-A and PR-B (Fig. 6D). As before, cells were also treated with a control siRNA. Next we performed a ChIP assay to analyze the status of MTA1 recruitment on the MTA3 promoter under the conditions of ER knockdown. We found that ER silencing indeed leads to a significantly increased association of endogenous MTA1 with the basal MTA3 chromatin. Results also suggest that estrogen stimulation was unable to trigger derecruitment of MTA1 from the MTA3 promoter region (Fig. 6E). Together, these experiments establish that the MTA3 promoter is a target of MTA1 and that manipulation in the levels of MTA1 expression influences the recruitment pattern of ER to the MTA3 promoter chromatin in a significant manner.

In conformity with a recent report (20), we found that estrogen signaling increases MTA3 protein levels (Fig. 7B) in a dose-dependent manner. Fig. 7A shows Western blot analysis carried out to characterize the MTA3 antibody and indicates the abundant amounts of MTA3 protein in MCF-7 cells. Because the ER-mediated activation of transcription involves recruitment of coactivators to the promoter area, we next examined the potential role of ER coactivators in the regulation of MTA3 expression. Using previously characterized MCF-7 cells stably expressing T7-PELP1 (also known as MNAR) (26), we showed that estrogen stimulation leads to enhanced recruitment of PELP1/MNAR to the MTA3 promoter chromatin (Fig. 7C), implying a role of coactivators in the regulation of MTA3 expression by ER. To determine the impact of MTA1 in the recruitment of coactivators to the MTA3 promoter chromatin, we next used MCF-7 cells expressing T7-MTA1 and examined the ability of estrogen to recruit AIB1, another ER-coactivator (27,28), to the MTA3 promoter chromatin. As illustrated in Fig. 7D, upon estrogen stimulation, we found significant recruitment of endogenous AIB1 to the MTA3 promoter chromatin in MCF-7/vector cells. Deregulation of MTA1 completely abolished the noticed AIB1 interaction with the MTA3 promoter chromatin. To further understand the mechanistic participation of ER coactivators in the regulation of MTA3 expression, we next examined the ability of PELP1 and/or AIB1 to stimulate MTA3 transcription using promoter assays (Fig. 7E). We observed a substantial induction of the MTA3 promoter activity by PELP1/MNAR or AIB1 in comparison with the vector-cotransfected cells. Coexpression of both PELP1/MNAR and AIB1 further augmented transcriptional activity of the MTA3 promoter as compared with expression of the individual coactivators by themselves. Interestingly, overexpression of MTA1 completely inhibited the ability of PELP1/MNAR and/or AIB1 to stimulate MTA3 transcription.
In brief, these observations suggest that the MTA3 chromatin segment, containing the \textit{bona fide} ER recruitment site, represents a highly dynamic surface for ER as well as ER interacting coactivators and corepressors. The resulting transcriptional activity may be tightly regulated by the dynamic interplay of ER coregulators. Fujita \textit{et al.} (20) made an interesting observation that each of the MTA family members could be essential components of distinct subsets of the Mi-2·NuRD complexes that have unique functional properties. MTA1 repression of MTA3 expression could presumably be a mechanism by which the cell type-specific transcription is controlled. We hypothesize that the levels of MTA1 in the cell would determine which subset of the Mi-2·NuRD complex would be active to carry out its specialized function. Because MTA1 has been shown to be associated with more metastasis and invasiveness in tumors, regulation of MTA3 gene expression by MTA1, both under basal conditions as well as in the presence of estrogen, assumes importance. In this context, MTA1 overexpression, leading to the down-regulation of MTA3 and E-cadherin expression, would give the tumors survival and metastatic advantage over other cells. In our model cell lines, the overexpression of MTA1 resulted in non-responsiveness of these cells to estrogen in terms of induction of MTA3 gene by ER. It could be speculated that this could be one of the mechanisms by which tumors eventually stop responding to estrogen and consequently, anti-estrogen therapy. Furthermore, it is also possible that MTA1 may also repress gene expression of both ER and its coactivators in these situations. If it does so, then MTA1 could be targeted for therapeutic intervention to sensitize the tumor cells to anti-estrogen therapies, which could have tremendous clinical impact.

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Fig. 1. Identification of the ER interaction site on the MTA3 regulatory elements.

A, schematic representation of the MTA3 gene around the three possible ERE half-site-α recruitment sites. B, association of ER with the MTA3 chromatin. E$_2$ signaling promotes interaction of ER with one of the possible ERE half-sites in MTA3 chromatin. MCF-7 cells grown in a phenol red-free medium supplemented with 3% charcoal dextran-stripped fetal bovine serum were treated with or without estrogen (10$^{-9}$ M) for 60 min. Chromatin lysates were immunoprecipitated with antibodies against ER, and samples were processed as described under “Experimental Procedures.” The lower panel shows the PCR analysis of the input DNA of the MTA3 chromatin. The upper panel demonstrates the PCR analysis of the MTA3 promoter fragments for possible association with ER ($n$ = 3). C, MCF-7 cells were maintained in medium supplemented with 3% charcoal dextran-stripped fetal bovine serum before treating either with estrogen (10$^{-9}$ M), ICI-182780, both estrogen and ICI, cyclohexamide (cyclohex) (10 μg/ml) or cyclohexamide plus estrogen. The top panel demonstrates the PCR analysis of the MTA3 promoter fragments for possible association with ER. The middle panel shows the PCR analysis of the input DNA of the MTA3 chromatin. The bottom panel shows a Western blot analysis for ER under the same conditions ($n$ = 3). *, band of interest.
Fig. 2. Estrogen-mediated induction of MTA3 promoter activity.

A, induced luciferase activity with 735- and 1078-base-pair fragments of the MTA3 promoter \((n = 3)\) in MCF-7 cells. Cells were maintained in 3% \(N,N'\)-dicyclohexylcarbodiimide serum in phenol-red free medium for 48 h before transfection of the luciferase constructs, luciferase activity was assayed at 48 h post-transfection. Values are normalized to \(\beta\)-galactosidase activity \((n = 3)\).

B, induced luciferase activity with 735 and 1078 base pair of the MTA3 promoter \((n = 3)\) in HeLa cells.

C and D, estrogen \((10^{-9} \text{M})\)-mediated and 4-hydroxyl tamoxifen \((10^{-8} \text{M})\)-mediated regulation of MTA3 promoter activity in MCF-7 cells and in HeLa cells, respectively \((n = 3)\). All treatments with ligands were for 16 h. E, schematic diagram of the MTA3 promoter \((\text{top bar})\) was deleted and mutated. To find out which ERE half-site is responsible for the estrogen induction, a point mutation of the proximal ERE half-site at \(-1241\) is done (TGACC–TGCTC) \((\text{middle bar})\), and the second ERE half-site is deleted at \(-1450\) by PCR \((\text{bottom bar})\). F, the 1078-base-pair wild type MTA3 promoter as well as the deletion and point mutation constructs are transfected in MCF-7 cells and treated with estrogen as before. The point mutation at the proximal ERE half-site abolishes the estrogen induction completely, whereas the deletion of the middle ERE half-site has little effect on estrogen stimulation.
Fig. 3. MTA1-mediated interference of association of ER with the MTA3 chromatin.
Cells were maintained in 3% charcoal dextran-stripped fetal bovine serum before treating with estrogen ($10^{-9}$ M) in the following experiments. A, MTA1 inhibits ER recruitment on the MTA3 chromatin. The lower panel shows input DNA for the ChIP assay. MCF-7 cells overexpressing pcDNA or T7-MTA1 were taken for the above experiment. B, short variant of MTA1 inhibits association of ER with the MTA3 chromatin. The lower panel shows the input DNA for the ChIP assay. ChIP assay was performed in the ZR-75R cells overexpressing vector alone, or T7-MTA1s using the anti-ER antibody. C, reverse transcriptase-PCR analysis of the MTA3 mRNA level in MCF-7 cells stably expressing either control vector or T7-MTA1. D, MTA3 transcription activity in the presence of either control vector or T7-MTA1 with or without estrogen ($10^{-9}$ M, 16 h).
Fig. 4. Expression of MTA3 and its downstream effectors in MTA1 overexpressing cells by confocal microscopy.
Confocal analysis of E-cadherin (top panel), MTA3, and Snail in MCF-7 cells expressing pcDNA (middle panel) and MTA1 (bottom panel). In the top panel, E-cadherin was stained red and in the bottom panel, MTA3 was stained green, and Snail was red. The nuclei were visualized using Topro3 (blue). The cells were all cultured in 10% serum containing medium.
Fig. 5. Direct association of MTA1 with the MTA3 chromatin and consequence of MTA1 and ER silencing on molecules downstream of MTA3.

A, ChIP analysis of direct association of MTA1 with the MTA3 chromatin in the presence or absence of estrogen. ChIP analysis was performed in MCF-7 cells. B, ChIP analysis of recruitment of HDAC1 and HDAC2 on the MTA3 chromatin in MCF-7 cells in the presence or absence of estrogen. C, Western analysis of molecules downstream of MTA3 as a result of knocking down MTA1 by siRNA in MCF-7 cells. The same blot was stripped and analyzed for expression of Snail, E-cadherin, PR-A, and PR-B as well as vinculin as a loading control. D, reverse transcriptase-PCR analysis of the MTA3 mRNA level in MCF-7 cells as a result of MTA1 silencing with or without estrogen. *, band of interest.
Fig. 6. Effect of MTA1 knockdown on MTA3 gene regulation

A, ChIP analysis of the MTA3 chromatin for ER recruitment in MCF-7 cells with or without MTA1 silencing in the presence or absence of estrogen (10^{-9} M). B, ChIP analysis of the MTA3 chromatin for HDAC1 and HDAC2 recruitment in the presence or absence of estrogen in MCF-7 cells having normal or silenced MTA1 expression. C, effect of MTA1 silencing on MTA3 luciferase activity in MCF-7 cells in the presence or absence of estrogen (10^{-9} M). D, immunoblotting of samples having normal ER-α expression and silenced ER expression, which is reprobed for PR (PR-A and PR-B) and vinculin as a loading control. E, ER was knocked down in MCF-7 cells, and ChIP analysis was performed for T7-MTA1 recruitment on the MTA3 chromatin in the presence or absence of estrogen.
Fig. 7. Coregulator recruitment and its effect on MTA3 promoter activity

A, detection of MTA3 protein in MCF-7 cell line. B, increased expression of MTA3 protein in MCF-7 cells in response to different concentrations of estrogen after 24 h of treatment. MTA3 protein levels were quantified using ImageQuant and normalized to vinculin levels in the cells. C, association of PELP1 with MTA3 promoter in a time-dependent and estrogen-sensitive manner in MCF-7 cells stably expressing T7-PELP1. For immunoprecipitation, anti-T7 antibody was used, which is targeted against the T7-PELP1. D, MTA1 inhibits the association of AIB1 with the MTA3 chromatin. MCF-7 cells either overexpressing the vector alone or T7-MTA1 were analyzed by ChIP assay for possible recruitment of AIB1 with or without estrogen signaling. Cross-linked cells were lysed and taken for immunoprecipitation with anti-AIB1 antibody. E, MTA3 luciferase activity was measured in the presence or absence of different coactivators, such as AIB1 and PELP1, and the corepressor MTA1 with or without estrogen treatment.
Table I
Primer and siRNA sequences used in the study

| Method                        | Primer/Target Sequence                      |
|-------------------------------|---------------------------------------------|
| MTA3 promoter cloning         | MTA3-pro1988F: tgtcagagctcttggtgggatctctggta |
|                               | MTA3-pro3066R: gacctgcagctcttgggccaggaa     |
|                               | MTA3-pro2331F: ttatttgagctctttgcctcagctatgca |
|                               | MTA3-pro3066R: gagcctcgaggctcttgggccaggaa   |
| MTA3 promoter deletion/mutation| MTA3pro2073F: tgacacagagctcagaatttgacacac   |
|                               | MTA3pr-mut-F2: aagagagaacagacatagactca      |
|                               | MTA3pr-mut-R2: tgtacctatgctctgtttcctcgt     |
| MTA3 reverse transcriptase-PCR F | MTA3-prmF: accctcgtgttagaagtcacgtgt         |
|                               | MTA3-prmR: gcagcataattaatagcaacaaacgg       |
| MTA3 reverse transcriptase-PCR R | MTA3-prmR: gctggaacccatgcggcgcaacatgttcgcag |
| MTA3 cDNA cloning             | MTA3-start: caacatctcaggttaaagttaaatagccatct |
| MTA3-ChIP primers            | MTA3-ChIPF1: ggaaatagagagaagggacctaacgc     |
|                               | MTA3-ChIPR1: tgacccagacagaggttacaa          |
|                               | MTA3-ChIPF2: catagcgaatttctcttcgaa          |
|                               | MTA3-ChIPR2: tcaagtccccattttataagcag        |
|                               | MTA3-ChIPR3: acacagctgtgctgtcagct          |
|                               | MTA3-ChIPF4: tgggtcatgggctttggt            |
|                               | MTA3-ChIPR4: tgggtctgtggtgaagtt            |
| MTA1 siRNA                   | Mta1siRNA-1: aaccctgtcagctctgcataaa        |
|                               | Mta1siRNA-2: aagacccctgtgctgagataaa        |
|                               | Mta1siRNA-3: aagatccctcccctgtgaaagtt        |
|                               | Mta1siRNA-4: aagacccctgcggcatccaa          |
| Control siRNA                | Nonspecific pooled duplex control catalog number SD-001206–13-80 |