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MTA3, a Mi-2/NuRD Complex Subunit, Regulates an Invasive Growth Pathway in Breast Cancer

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Summary

Estrogen receptor is a key regulator of proliferation and differentiation in mammary epithelia and represents a crucial prognostic indicator and therapeutic target in breast cancer. Mechanistically, estrogen receptor induces changes in gene expression through direct gene activation and also through the biological functions of target loci. Here, we identify the product of human *MTA3* as an estrogen-dependent component of the Mi-2/NuRD transcriptional corepressor in breast epithelial cells and demonstrate that *MTA3* constitutes a key component of an estrogen-dependent pathway regulating growth and differentiation. The absence of estrogen receptor or of MTA3 leads to aberrant expression of the transcriptional repressor Snail, a master regulator of epithelial to mesenchymal transitions. Aberrant *Snail* expression results in loss of expression of the cell adhesion molecule E-cadherin, an event associated with changes in epithelial architecture and invasive growth. These results establish a mechanistic link between estrogen receptor status and invasive growth of breast cancers.

Introduction

Estrogen receptors (ER) are members of the superfamily of nuclear hormone receptors (Mangelsdorf et al., 1998; Tsai and O'Malley, 1994) whose activity is required for the normal function of the female reproductive system. Two isoforms of estrogen receptor (ERα and ERβ) have been described. They function as ligand-dependent transcriptional activators (McDonnell and Norris, 2002). The biological functions downstream of ER result from altered expression of direct transcriptional targets as well as secondary effects mediated by biological activities of direct targets. In the mammary gland, estrogen receptors regulate normal epithelial cell development and differentiation through their well-documented effects on transcription (Nilsson et al., 2001; Couse and Korach, 1999). Estradiol acts as a potent mitogen for many breast cancer cell lines (Prall et al., 1998), and approximately 70% of breast carcinomas are ER positive and estrogen dependent (Masood, 1992). Mechanistically, estrogen enhances growth by stimulating the G1 to S transition (Prall et al., 1998). These mitogenic effects can be blocked by estrogen antagonists (McDonnell and Norris, 2002). In fact, selective estrogen receptor modulators (SERMs) represent principal chemotherapeutic agents for the treatment and prevention of breast cancers (Sommer and Fuqua, 2001).

Ample evidence links alterations in the homotypic cell adhesion molecule E-cadherin with the advent of invasive growth in epithelial tumors. E-cadherin function is required for the production of adherens junctions (Adams and Nelson, 1998). Both functional disruption of E-cadherin using monoclonal antibodies (Imhof et al., 1983) and loss of E-cadherin expression (Behrens et al., 1989) can disrupt these junctional complexes and elicit phenotypic changes including the acquisition of invasive growth (Thiery, 2002). While E-cadherin is produced at normal levels in many well-differentiated tumors, it is frequently lost during disease progression (Hirohashi, 1998). Downregulation of E-cadherin in tumors is most commonly attributed to epigenetic mechanisms, although occasionally the gene is mutated, leading to a nonfunctional protein (Thiery, 2002). The transcription factors Snail and Slug have been implicated as direct transcriptional repressors of E-cadherin (Batlle et al., 2000; Cano et al., 2000; Hajra et al., 2002; Thiery, 2002). Moreover, increased Snail expression has been correlated with loss of differentiation and metastasis in breast tumors (Blanco et al., 2002), suggesting that regulation of Snail may be critical in the maintenance of normal epithelial architecture.

The Mi-2/NuRD complex represents a major macromolecular form of histone deacetylase in vertebrates that was independently isolated in several different laboratories (Tong et al., 1998; Wade et al., 1998; Xue et al., 1998; Zhang et al., 1998; Humphrey et al., 2001). This complex couples a histone deacetylase and nucleosome-stimulated ATPase with several subunits of unknown function (Wade et al., 1999; Xue et al., 1998; Zhang et al., 1999). Genetic evidence (Kehle et al., 1998; Ch'ng and Kenyon, 1999; Solari et al., 1999) implicates the complex in transcriptional repression following recruitment by direct interaction with site-specific DNA binding proteins. A consistent feature is a 70–80 kDa polypeptide encoded by either *MTA1* or *MTA2* (Xue et al., 1998; Wade et al., 1999; Zhang et al., 1999; Humphrey et al., 2001). MTA1 overexpression is associated with metastatic growth of cell lines in vitro and with invasive growth of tumors (Toh et al., 1997). However, the precise function of this subunit is currently unknown.

In this work, we investigated the *MTA1* gene family in the context of breast cancer. A member of this family, *MTA3*, was found to be a component of the Mi-2/NuRD complex. Expression of MTA3 was tightly linked with estrogen action. MTA3 repressed transcription in a histone deacetylase-dependent manner, consistent with function as a component of the Mi-2/NuRD complex. We identified the transcription factor *Snail* as a direct regulatory target of *MTA3* action. Experimental manipulation of *MTA3* strongly impacted *Snail* and *E-cadherin* levels. These data indicate direct regulation of *Snail* transcription by *MTA3* and identify a regulatory pathway

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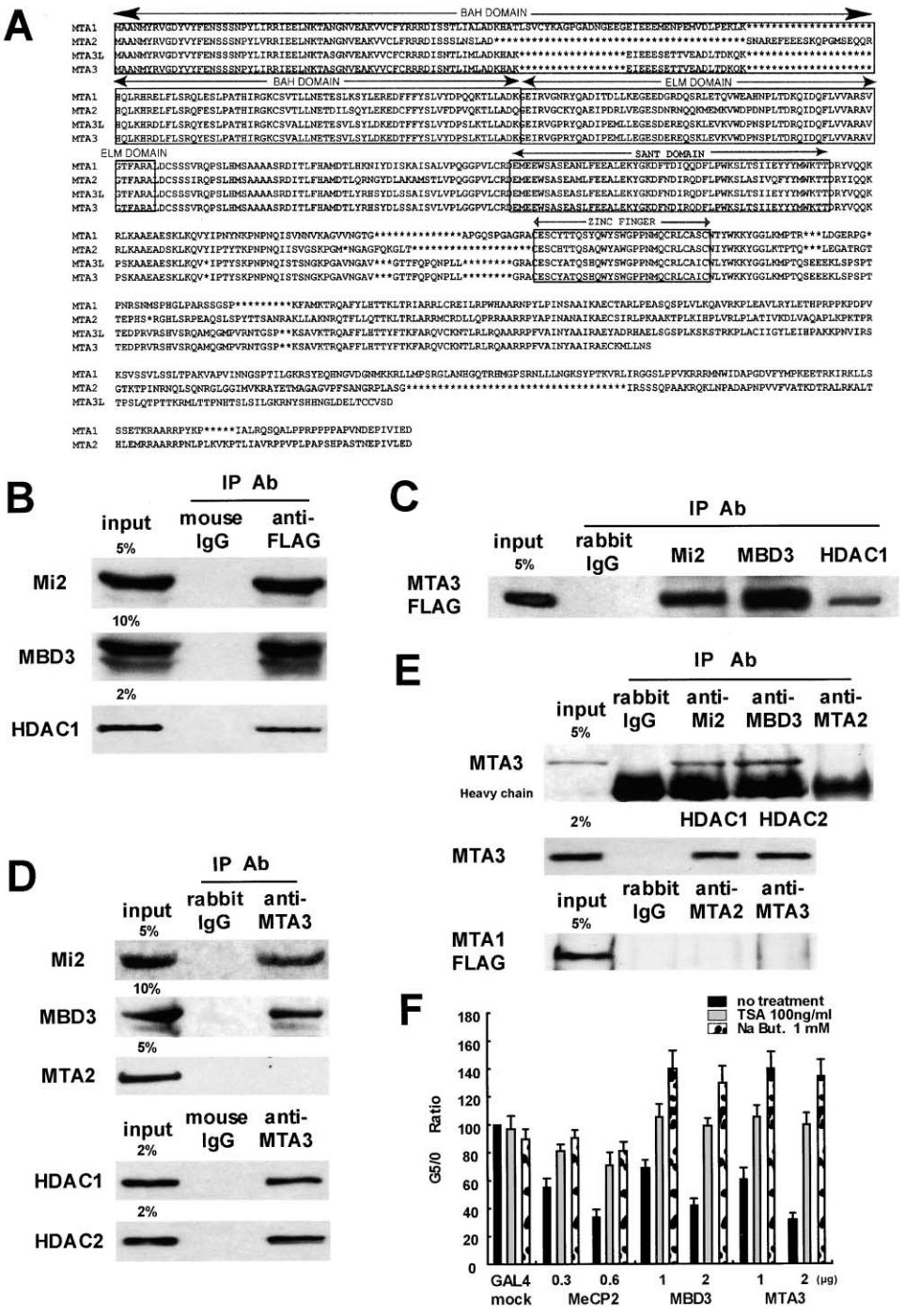

Figure 1. MTA3 Is a Mi-2/NuRD Subunit

(A) Human MTA1, MTA2, and MTA3 were aligned. Conserved sequence motifs (BAH domain, ELM domain, SANT domain and GATA zinc finger) are indicated. Gaps introduced to optimize the alignment are denoted by an asterisk.

(B) MTA3 was expressed in HEK 293T cells with a FLAG tag. FLAG-MTA3 was immunoprecipitated from nuclear extracts with FLAG M2 antibody. Immunoblots were analyzed with the indicated antibodies.

(C) MTA3 was expressed in HEK 293T cells with a FLAG epitope tag. Immunoprecipitations were performed from nuclear extracts using the indicated antibodies. Immunoblots were probed with FLAG antibody.

(D) Endogenous MTA3 was immunoprecipitated from MCF7 nuclear extracts and analyzed by immunoblot. The indicated percentage of input nuclear extract is shown. Immunoblots were probed with antibodies against Mi-2, MBD3, HDAC1, and HDAC2.

(E) Immunoprecipitation of Mi-2/NuRD complex subunits was performed from MCF7 nuclear extracts and analyzed by immunoblot with MTA3 antibodies. The IgG heavy chain is indicated where appropriate. The bottom depicts immunoprecipitations performed on nuclear extracts from HEK 293T cells transfected with FLAG-tagged MTA1. Immunoprecipitations were performed using MTA2 and MTA3 antibodies and probed with FLAG antibody.

downstream of estrogen receptor that contributes to the maintenance of normal epithelial architecture.

Results

MTA3 Is a Component of the Mi-2/NuRD Complex

The Mi-2/NuRD complex has been described in multiple experimental systems. All forms purified to date contain an approximately 80 kDa subunit encoded by *MTA1* or *MTA2* (Tong et al., 1998; Wade et al., 1999; Xue et al., 1998; Zhang et al., 1999; Humphrey et al., 2001). Database searches of genomic and EST sequences revealed a third closely related gene, *MTA3* (LocusLink ID 57504). To initiate characterization of *MTA3* in humans, we cloned a cDNA by RT-PCR. As predicted, there are two RNA species transcribed from the *MTA3* locus. One contains a 1548 bp open reading frame encoding a protein of 515 amino acids (aa), which we named MTA3, and the other includes a 1785 bp open reading frame encoding a longer protein of 594 aa, which we now term MTA3L (Figure 1A). The two mRNAs differ in their 3′ terminal exons and are predicted to produce two highly similar proteins with unique carboxyl termini. The MTA3 protein shares about 80% similarity to human MTA1 and MTA2 proteins, containing several conserved sequence motifs commonly found in chromatin-associated proteins.

To determine whether MTA3 is a bona fide Mi2/NuRD complex subunit like MTA2 and MTA1, we performed biochemical analysis. Endogenous Mi-2, MBD3, and HDAC1 were detected in MTA3 immune complexes whether we precipitated exogenously expressed protein with an epitope tag (Figure 1B) or endogenous MTA3 (Figure 1D). The converse experiment, precipitation of endogenous Mi-2, MBD3, and HDAC1, coprecipitated exogenous (Figure 1C) and endogenous MTA3 (Figure 1E). No association between MTA1 and MTA2 or MTA3 was detected in any case (Figures 1D and 1E). We concluded that MTA3 is a bona fide component of the Mi-2/NuRD complex. Our failure to detect MTA1 or MTA2 in association with MTA3 strongly suggests that multiple, unique forms of the Mi-2/NuRD complex exist that differ in their MTA family subunit.

The Mi-2/NuRD complex was described as a histone deacetylase complex and transcriptional corepressor. We used a GAL4 tethering experiment to investigate the functional relationship between MTA3 and histone deacetylation in transcriptional silencing. We examined the effect of an MTA3-GAL4 DNA binding domain fusion on transcription of a luciferase reporter containing five GAL4 binding elements (G5) just upstream of the constitutively active *SNRPN* promoter (Figure 1F). GAL4 DBD fusions with the known Mi-2/NuRD complex subunit MBD3 and with the transcriptional repression domain (TRD) of MeCP2 served as controls. Levels of transcriptional repression driven by MTA3 were dose dependent and quantitatively similar to repression driven by the MeCP2 TRD and by MBD3. Consistent with previous reports (Jones et al., 1998; Nan et al., 1998), transcriptional repression by the TRD domain of MeCP2 was partially reversed by both TSA and sodium butyrate. In contrast, repression by MTA3 and by MBD3 was completely reversed by both inhibitors. We conclude that MTA3 is a component of the Mi2/NuRD complex and that this complex likely functions as a negative regulator of gene expression.

MTA3 Expression Is Dependent on Estrogen Action

MTA1 was originally isolated by differential display comparing breast cancer cell lines with different growth properties (Pencil et al., 1993; Toh et al., 1994). To determine expression patterns of *MTA3*, Northern and immunoblot analyses were performed. An *MTA3* cDNA probe identified a single abundant transcript of approximately 2.2 kb (Figure 2A). This transcript was differentially expressed and was abundant only in the ER-positive cell lines, MCF7 and T47D (Figure 2A). We infer that this transcript represents MTA3 (not MTA3L) based on size. Control probes for MTA2 hybridized to an abundant transcript of the appropriate size in all cell lines. Immunoblot analysis of several breast and endometrial cell lines also identified a single species of approximately 60 kDa, closely matching the predicted size of MTA3 (Figure 2B). Like the transcript, MTA3 protein was abundant only in cells expressing estrogen receptor. In this report, we focus on the function of the readily detectable isoform, MTA3.

The correlation of MTA3 with estrogen receptor suggested that ER might regulate MTA3 expression. Consistent with this notion, steroid depletion led to a decline in the expression of MTA3, but not β-actin (Figure 2C). As a further demonstration of specificity, we grew the endometrial cell line Ishikawa in the presence of tamoxifen, a specific estrogen receptor modulator. Over a period of 7 days, a gradual decline in MTA3 protein levels was observed, while actin levels were unaffected (Figure 2C). Next, we overexpressed ER in two ER-positive cell lines, MCF7 and HeLa. ER overexpression led to commensurate increases in MTA3 expression levels in both cell lines (Figure 2D). Finally, we exogenously expressed ERα in Hs578t cells and observed de novo expression of MTA3 (Figure 2E). These data firmly demonstrate the dependence of MTA3 expression on estrogen action in cancer cell lines.

MTA3 Selectively Represses *Snail*

The analyses of MTA3 expression suggested that the MTA3 complex participates in the genetic program initiated by ligand bound ER. We hypothesized that changes in epithelial architecture manifested in many ER-negative breast cancer cell lines might result from a loss of

(F) GAL4 DNA binding domain fusions to MBD3, MTA3, and the MeCP2 TRD were cotransfected with a luciferase reporter construct. Data are reported as the ratio of activity from GAL4 site containing to GAL4 site-deficient templates (G5/G0 ratio). The ratio obtained on cotransfection of the GAL4 DNA binding domain alone was arbitrarily assigned a value of 100. Two different amounts of each effector molecule were transfected, amounts of DNA used are indicated in the Figure for each GAL4 DBD fusion. The cells were treated with HDAC inhibitors as described in Experimental Procedures.

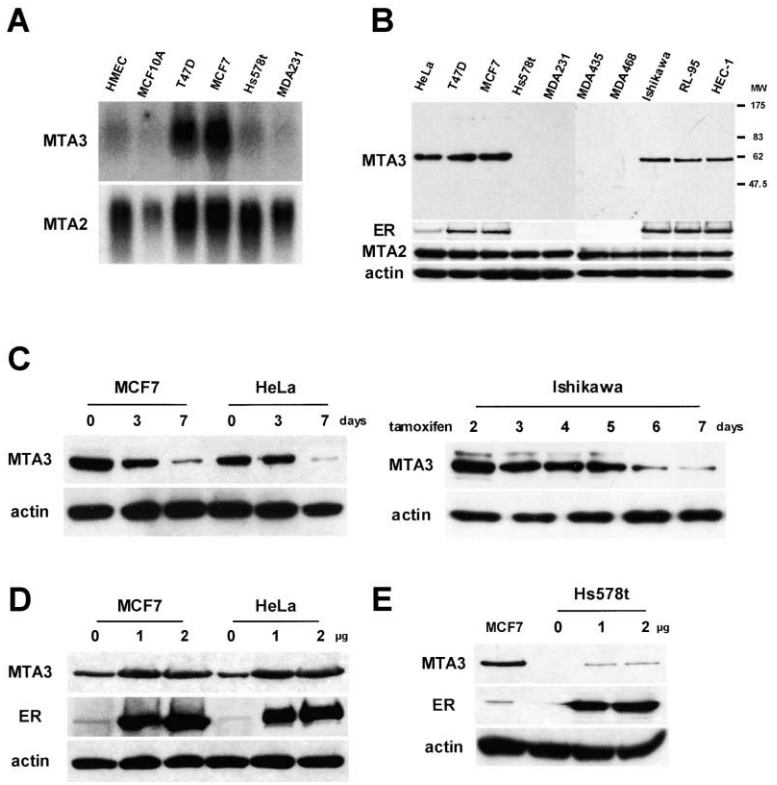

Figure 2. MTA3 Biosynthesis Requires Ligand Bound Estrogen Receptor

(A) Northern blot of total RNA from the indicated cell lines was hybridized with probes for MTA2 and MTA3.

(B) Immunoblot of whole cell lysates from the indicated cell lines probed with antibodies for MTA3, estrogen receptor α, MTA2, and β-actin.

(C) MCF7 and HeLa cells were grown for the indicated number of days in media stripped of steroids. Immunoblots were probed for MTA3 and β-actin. Ishikawa cells were grown in normal growth media supplemented with 1 μM tamoxifen for the indicated number of days. Immunoblots were probed for MTA3 and β-actin.

(D) MCF7 and HeLa cells were transfected with ERα cDNA. Whole-cell lysates were analyzed for MTA3, ER, and β-actin. The growth media for these experiments (DMEM with 10% FBS) contains sufficient steroids to provide normal ER function.

(E) Hs578t cells were transfected with an ERα cDNA, using the amounts indicated. Whole-cell lysates were analyzed for MTA3, ER, and β-actin.

MTA3 function. Recent reports have documented roles for the transcription factors *Snail* and *Slug* in repression of the cell adhesion molecule *E-cadherin* (Batlle et al., 2000; Cano et al., 2000; Hajra et al., 2002). As *E-cadherin* is crucial to maintenance of normal epithelial cell contact (Hajra and Fearon, 2002), we reasoned that *Snail* and/or *Slug* might represent regulatory targets of MTA3. Therefore, *Snail* and *Slug* promoter-luciferase fusions were constructed and assayed. Cotransfection of either ERα or MTA3 repressed transcription from *Snail*, but not from *Slug*, in a dose-dependent manner (Figures 3A and 3B). In contrast, MTA1 had little effect. As controls for promoter specificity, we analyzed the *p16* and *cyclin D1* promoters. None of the protein effector molecules inhibited *p16* or *cyclin D1* (Figure 3C and 3D). If MTA3 functions as a component of the Mi-2/NuRD complex, repression should be sensitive to inhibitors of histone deacetylase. Therefore, we asked whether the repressive activities of MTA3 and ERα were sensitive to HDAC inhibitors (Figure 3E). Repression by both MTA3 and ER was completely reversed. In aggregate, these data strongly suggest that MTA3 represses *Snail* in an HDAC-dependent manner but has no effect on *Slug*.

MTA3 Complex Localizes on the *Snail* Promoter

To determine whether MTA3 physically associates with the endogenous *Snail* promoter, we performed a chromatin immunoprecipitation assay (ChIP). We compared the ability of MTA3 antibodies to precipitate the *Snail* promoter in cell lines expressing MTA3 at activated (MCF7) and basal levels (Hs578t). Cells were fixed with formaldehyde with or without an initial protein-protein crosslinking step using dimethyl 3,3′-dithiobispropionimidate-2HCl (DTBP). As previously described for the histone deacetylase *RPD3* (Kurdistani et al., 2002), coprecipitation of both MTA3 and MBD3 with the *Snail* promoter in the ER-positive MCF7 cell line was strongly enhanced by protein-protein crosslinking with DTBP. This observation suggests that MTA3 and MBD3, a known component of the Mi-2/NuRD complex, associate with the *Snail* promoter through interaction with unidentified DNA binding proteins (Figure 4A). We further characterized this interaction by performing a primer walk across a genomic region encompassing the *Snail* promoter. MTA3 and MBD3 were strongly associated with genomic DNA immediately upstream of the *Snail* transcription start site (Figure 4B). As expected from the reporter assays, we did not detect an interaction between MTA3 and the *Slug* promoter.

In the ER-negative Hs578t cell line, neither MTA3 nor MBD3 immunoprecipitates contained *Snail* promoter DNA (Figure 4C). The MTA3 and MBD3 antisera failed to precipitate negative controls, the G6PD promoter and the far upstream region of *Snail*, under any circumstances. Consistent with our inability to coprecipitate MTA2 with MTA3, we did not observe evidence for association of MTA2 with the *Snail* promoter (Figures 4A–4C). The negative control rabbit IgG immune complexes also did not contain *Snail* DNA. To determine whether experimental manipulation of MTA3 would result in de novo association with *Snail*, we transfected Hs578t with ER. MTA3 immune complexes clearly contained *Snail* promoter DNA (Figure 4D). Neither control rabbit IgG nor anti-ER antibodies precipitated the *Snail* promoter.

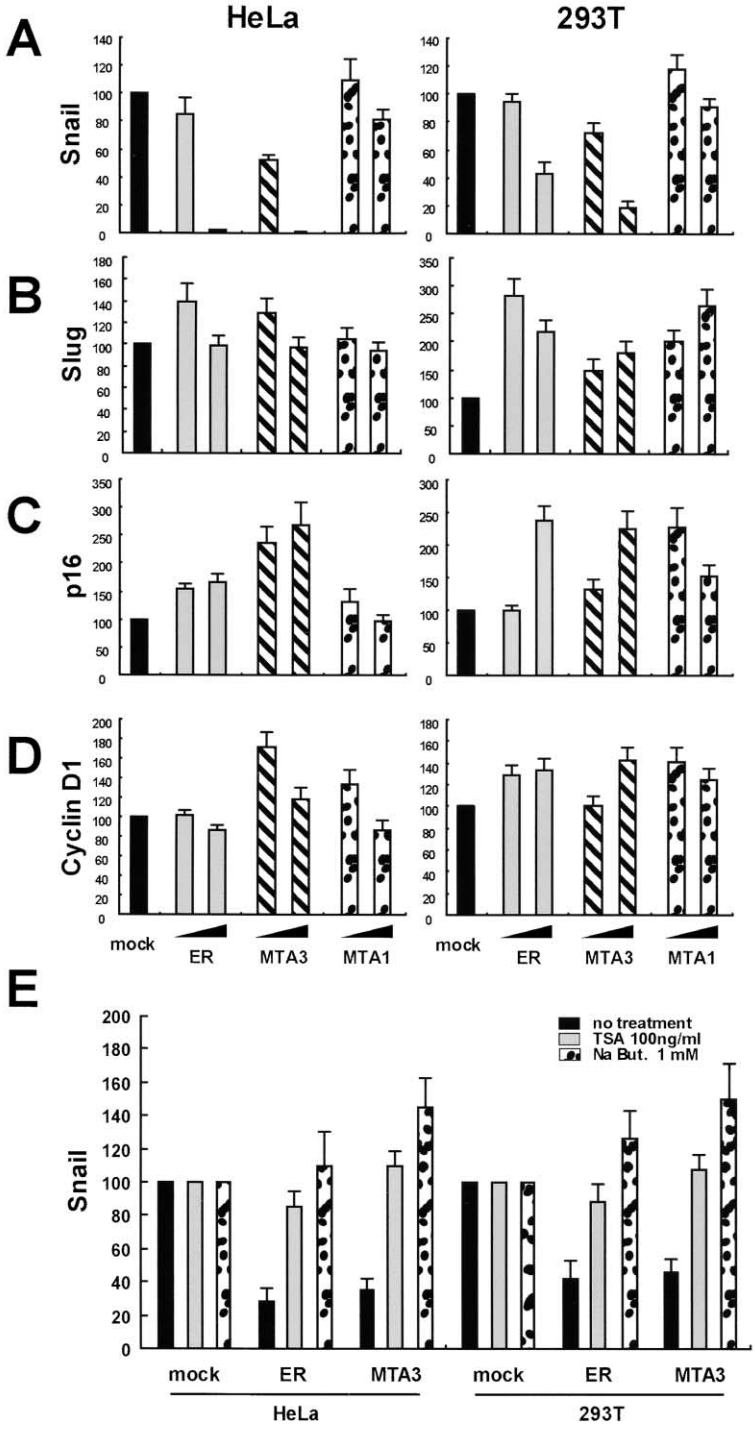

Figure 3. MTA3-Dependent Transcriptional Repression of Snail

(A–D) HeLa and HEK 293T cells were transfected with the indicated luciferase reporter and the effector molecules ERα, MTA3, and MTA1. Luciferase values (normalized) for mock effector transfections were arbitrarily assigned a value of 100.

(E) Transfections were performed with the Snail-luciferase reporter as in (A). The indicated histone deacetylase inhibitors were added and luciferase activity was determined as above.

These data demonstrate that MTA3 forms a complex containing other components of Mi-2/NuRD on the *Snail* promoter in an ER-dependent fashion.

MTA3 Expression Correlates with Snail Repression and with E-Cadherin Transcription

The ability to manipulate MTA3 through experimental alterations in estradiol constitutes a valuable tool. The ER-positive T47D cell line was grown in media depleted of steroids for 7 days. Subsequently, estradiol was added, and cells were collected at various time points for RT-PCR. While depletion of steroids led to a dramatic decline in MTA3 transcript levels, the addition of estradiol restored expression to control levels within 2 hr (Figure 4F, black bars). In contrast to *MTA3*, *Snail* transcript levels were increased in the steroid-depleted media. However, the addition of estradiol reverted steady-state levels of *Snail* back to control levels (Figure 4F).

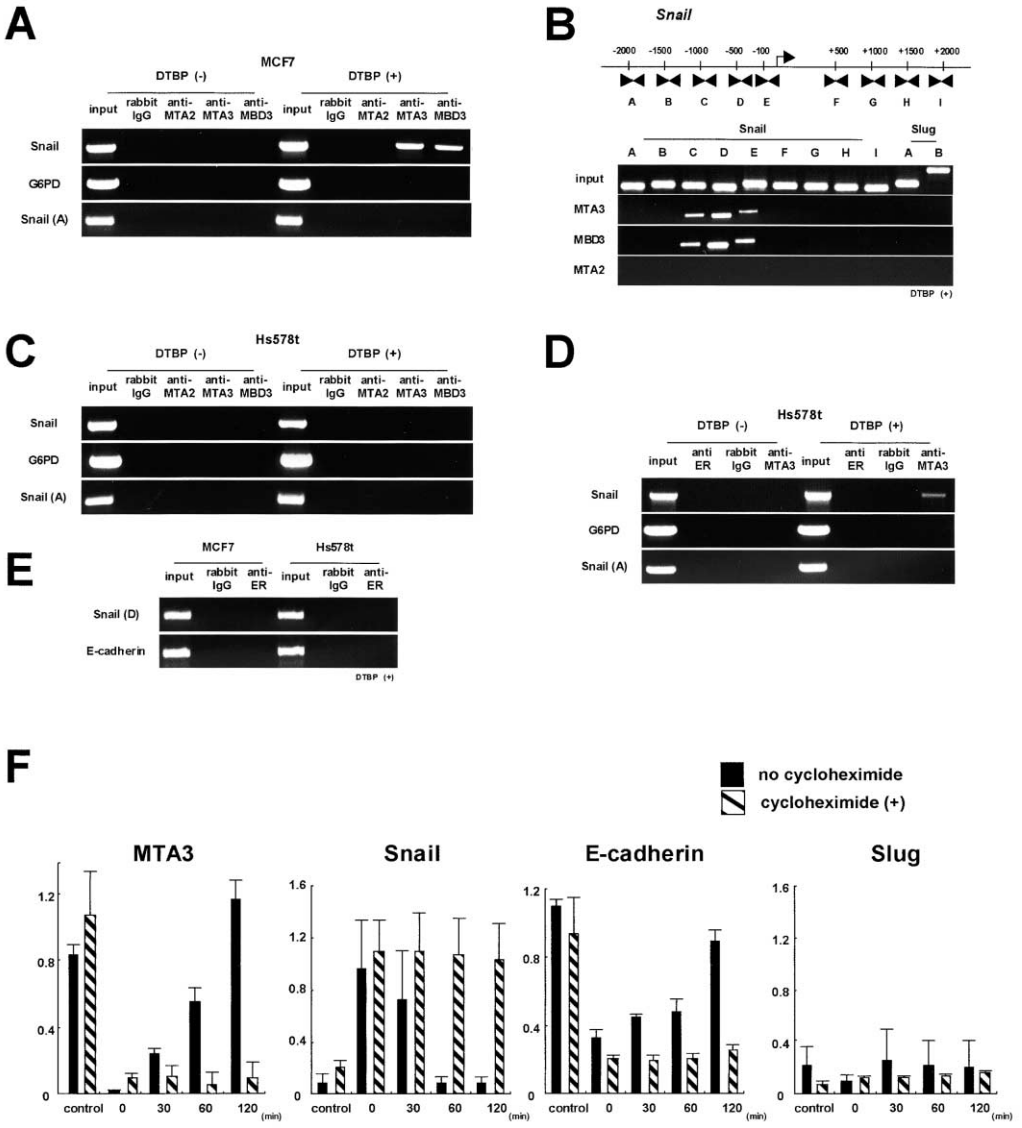

Figure 4. MTA3 Is Present on the Repressed Snail Promoter

(A) Chromatin immunoprecipitations (ChIP) were performed on MCF7 chromatin using the indicated antisera. Crosslinking with DTBP preceded formaldehyde fixation in the indicated lanes. PCR detection of G6PD, the Snail proximal promoter (−990 to −3), and Snail far upstream (Snail [A], approximately −2000) sequences are indicated.

(B) A primer walk was performed across the Snail promoter with DNA precipitated by the indicated antibodies. The diagram indicates the location of the primer sets. All chromatin was crosslinked with DTBP prior to formaldehyde.

(C) ChIP assay was performed on Hs578t chromatin using the indicated antisera.

(D) ChIP assay was performed on chromatin from Hs578t cells transfected with ERα. PCR products from Snail and G6PD are indicated.

(E) ChIP assay was performed on chromatin from MCF7 and Hs578t cells using control rabbit IgG or estrogen receptor antibodies. Detection of Snail promoter (primer set D) and E-cadherin promoter sequences are depicted. All chromatin was crosslinked with DTBP then formaldehyde.

(F) T47D cells were grown in the absence of steroids for 7 days. At time 0, estradiol was added and cells were collected at the indicated time points. RT-PCR was performed with real time detection for the indicated mRNAs as described in Experimental Procedures. RNA levels were determined without (black bars) or with (hatched bars) at 1 hr pretreatment with cycloheximide (25 ng/ml) prior to addition of estradiol at time 0.

Consistent with the identification of *Snail* as a negative regulator of *E-cadherin*, levels of *E-cadherin* mRNA closely paralleled those of *MTA3* (Figure 4F). This strong correlation between the appearance of *MTA3* and the decline in *Snail* transcript levels is consistent with a direct role for *MTA3* in regulation of *Snail*. Steady-state levels of *Slug* were unaltered in this experiment, demonstrating that *Slug* expression is independent of estrogen action.

The very rapid reactivation of E-cadherin in these experiments suggested that ER might contribute to its transcription. To determine whether estrogen receptor directly regulated E-cadherin transcription, we again grew T47D in the absence of endogenous steroids and pretreated cells with cycloheximide to block new protein synthesis prior to the addition of estradiol. RNA was prepared and analyzed by RT-PCR (Figure 4F, hatched bars). To our surprise, MTA3 mRNA levels failed to re-

spond to estrogen addition, indicating that estrogen receptor does not directly activate MTA3. As expected, Snail RNA levels were unaffected by addition of estradiol in the presence of cycloheximide. E-cadherin mRNA also failed to respond to estrogen addition, indicating that ER does not directly activate its transcription.

MTA3 Regulates the Expression of E-Cadherin

These results suggested that *MTA3*, *Snail*, and *E-cadherin* are components of a pathway downstream of ER. Therefore, we tested whether *MTA3* was both necessary and sufficient for regulation of *E-cadherin*. MTA3 was exogenously expressed in Hs578t (low level ER, MTA3, and E-cadherin). RT-PCR analysis demonstrated that *Snail* mRNA levels declined in response to expression of MTA3. *E-cadherin* transcript was detectable solely when MTA3 was overexpressed and not in the mock transfection control (Figure 5A). This result strongly argues that MTA3 expression is sufficient for estrogen-dependent transcriptional regulation of *E-cadherin* through alterations in *Snail* levels. To address the requirement for *Snail* and for *Slug* in this system, we utilized RNA interference. We designed siRNAs specific for *Snail* and for *Slug* and demonstrated that transfection with these siRNAs resulted in decreases in levels of the appropriate mRNA (Figure 5B). Analysis of *E-cadherin* transcript levels in this experiment revealed that depletion of Snail led to increases in *E-cadherin* mRNA. Likewise, depletion of Slug led to increased *E-cadherin* transcription (Figure 5C). We concluded that MTA3 regulates E-cadherin through alterations in Snail levels. Furthermore, Slug also regulates E-cadherin transcription in an ER- and MTA3-independent fashion.

To address the necessity for *MTA3* in estrogen dependent expression of E-cadherin, we depleted endogenous MTA3 in HeLa and RL-95 cells (Figure 5D). As controls for RNAi, we used green fluorescent protein (GFP) (Elbashir et al., 2001). MTA3 expression was not affected by GFP siRNA, and MTA3 siRNA did not influence the expression of either MTA2 or β-actin (Figure 5D). However, MTA3 siRNA led to dramatic declines in both MTA3 protein and in *E-cadherin* transcript levels compared with controls (buffer or GFP). *Snail* transcript levels were significantly increased by depletion of MTA3 while *Slug* was unaffected. We concluded that MTA3 is both necessary and sufficient for estrogen-dependent regulation of *E-cadherin* transcription and that this regulation is mediated through alterations in Snail levels.

Estrogen Receptor, MTA3, and E-Cadherin Gene Expression Are Correlated in Breast Cancer Patients

Immunohistochemical staining was performed on clinical samples of breast carcinoma to ascertain whether findings in our tissue culture model would be corroborated in situ. Photomicrographs from two selected cases are presented in Figure 6. Prominent nuclear MTA3 immunoreactivity (Figure 6A) was observed in carcinoma cells from a case that was positive for estrogen (Figure 6C) and progesterone receptor (data not shown). Staining of nonepithelial cells is notably absent. E-cadherin staining of carcinoma cells from the same tumor sample demonstrated strong staining at the cellular periphery (Figure 6E). A separate specimen that was negative for ER (Figure 6D) and PR (data not shown) failed to demonstrate MTA3 immunoreactivity (Figure 6B) and also lacked strong E-cadherin staining (Figure 6F). However, this case did stain with the anti-MTA2 antibody, which demonstrated strong staining in all nuclei, regardless of cell type, in every case examined (data not shown). In addition, we performed immunostaining of MTA3 and ER in a larger sample of clinical specimens (total 21 cases; see Supplemental Tables S1 and S2 online at http://www.cell.com/cgi/content/full/113/2/207/DC1). In these cases, MTA3 expression was strongly correlated with ER status (Pearson correlation coefficient of 0.69 with $p = 0.0013$).

To determine whether our observations regarding MTA3 and ER can be extended in clinical samples to Snail and E-cadherin, we analyzed a publicly available microarray gene expression data set from a study of 115 women (van't Veer et al., 2002). Analyses were performed on the entire dataset of all patients (Figure 7A), as well as four subsets (Figures 7B–7E). Two of the subsets, ER positive and ER negative as determined by immunohistochemical staining, were provided in the public datasets. The final two patient subsets were separated based on the gene expression data for MTA3 and divided into MTA3-positive and MTA3-negative subsets, respectively. The correlation between ER and MTA3 expression was statistically significant for the entire patient dataset and for three of the four patient subsets analyzed (Figure 7F). The finding that ER and MTA3 were not correlated in the ER-positive subset probably reflects the involvement of intermediary factors downstream of ER that directly mediate activation of MTA3 transcription, as demonstrated in our cycloheximide experiment (Figure 4F). In addition, ER and E-cadherin expression were positively correlated in all datasets examined, and this was statistically significant for both the ER-negative and MTA3-negative patient subsets (Figures 7C, 7E, and 7F). A significant negative correlation was observed between ER expression and Snail expression in the ER-negative patient subset (Figures 7C and 7F). Finally, a statistically significant positive correlation was observed between MTA3 and E-cadherin expression for the MTA3 positive patient subset (Figure 7F).

Discussion

Loss of normal tissue architecture is a hallmark event in disease progression in epithelial cancers. Primary tumors generate cells that invade locally or migrate from the initial site, and invasive growth and metastasis is estimated to be the root cause of 90% of human cancer mortality (Sporn, 1996). Cellular changes leading to the acquisition of invasive growth represent one of the acquired capabilities that define malignant growth (Hanahan and Weinberg, 2000). Alterations in cell surface adhesion molecules that modulate interactions of epithelial cells with their tissue microenvironment are likely prerequisites for invasive growth. Cell adhesion molecules, like E-cadherin, constitute an important class of molecules that anchor epithelial cells. In addition to physical attachment, cell adhesion molecules transmit regulatory information through cytoplasmic adaptors (Hajra and

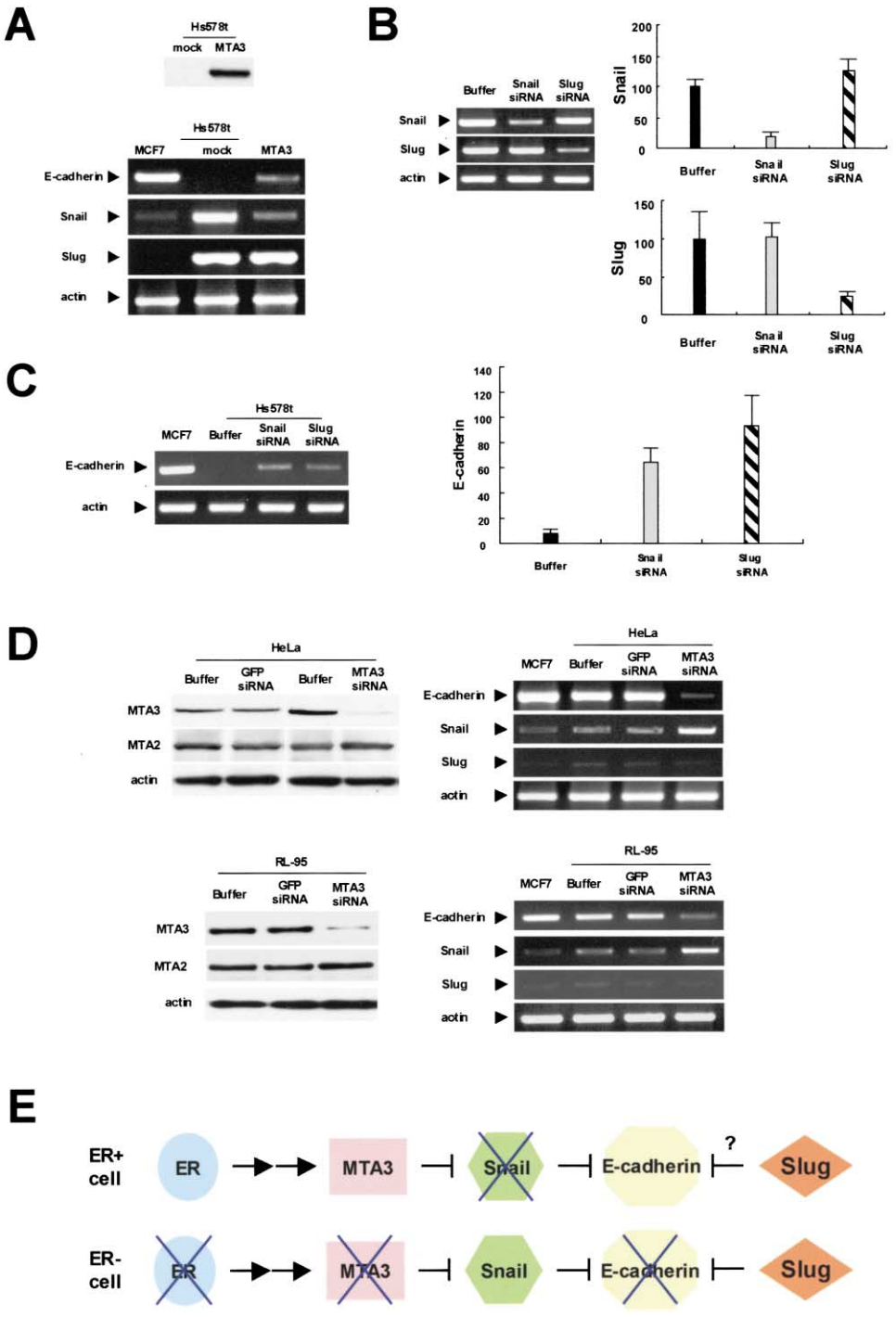

Figure 5. MTA3 Is Necessary and Sufficient for Estrogen-Dependent Regulation of E-Cadherin Transcription

(A) Hs578t cells were transfected with MTA3 cDNA. The top shows an immunoblot probed with MTA3 antiserum. The bottom depicts RT-PCR products from the E-cadherin, Snail, Slug, and β-actin loci.

(B) RNAi was performed in Hs578t using siRNA for Snail and Slug (see Experimental Procedures). The right depicts RT-PCR products of Snail, Slug, and β-actin following transfection with the indicated siRNAs. The graphs at the right summarize quantitative RT-PCR of the same mRNAs.

(C) cDNAs from Hs578t transfected with Snail and Slug siRNAs were analyzed by RT-PCR for E-cadherin and β-actin. The ethidium-stained gel is depicted alongside quantitation by real time RT-PCR.

(D) RNAi was performed in HeLa and RL-95 cells using the MTA3 siRNA (see Experimental Procedures). Following transfection, whole-cell lysates were analyzed by immunoblot (see left). The right depicts RT-PCR products from the E-cadherin, Snail, Slug, and β-actin loci.

(E) The model depicts the pathway leading from activation of estrogen receptor to transcription of E-cadherin. Details of the model are discussed in the text.

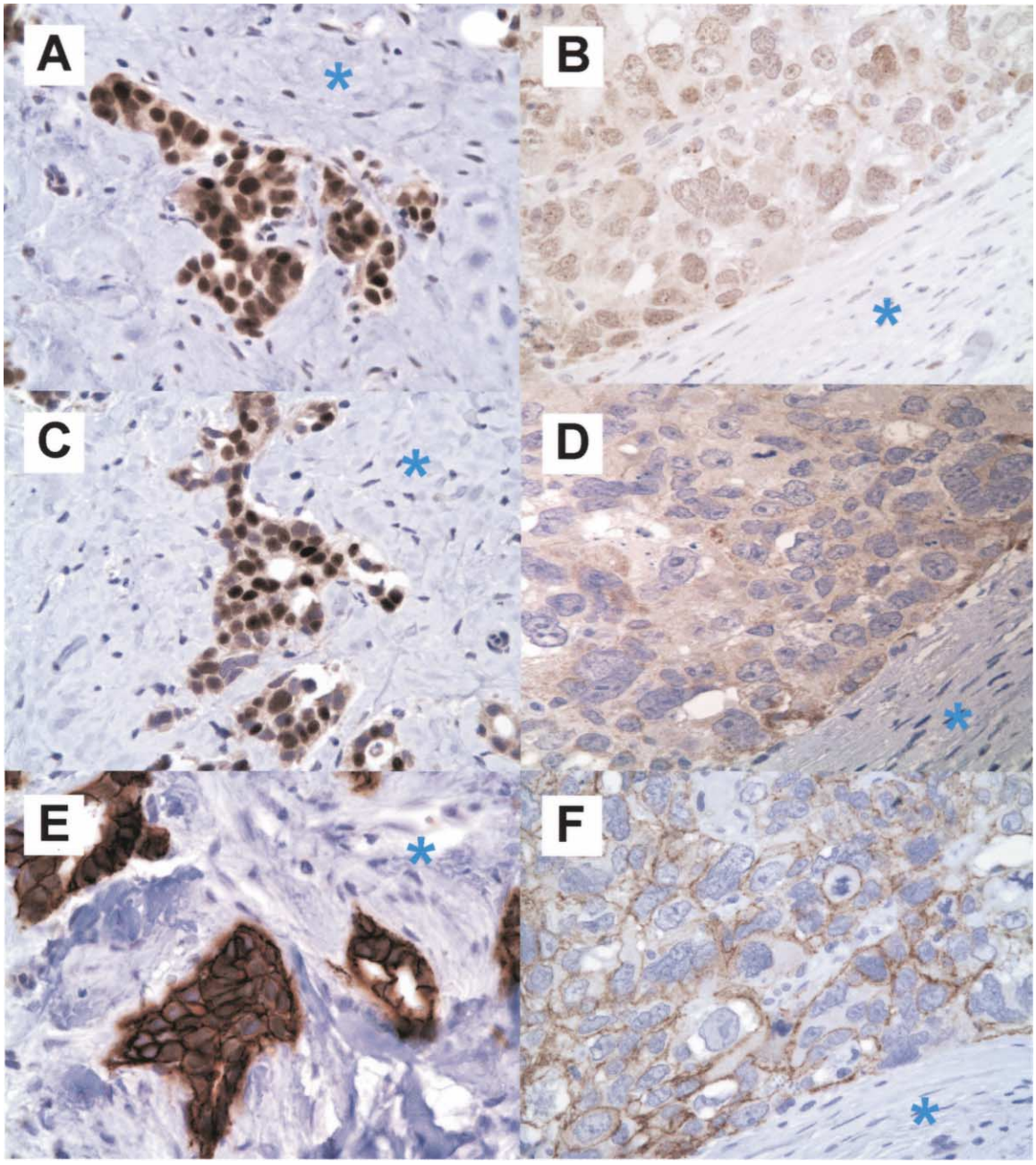

Figure 6. Immunoreactivity for MTA3 in Clinical Breast Carcinoma Specimens Is Concordant with Estrogen and Progesterone Receptor Immunoreactivity

(A) MTA3 was analyzed in a breast carcinoma sample. MTA3 detection is dark brown, nuclei were counterstained with hematoxylin (light blue). Magnifications is 400× for all images. Asterisks indicate nonepithelial cells.

(B) A second breast carcinoma demonstrating background staining of MTA3.

(C) A serial section of the carcinoma specimen from (A) was analyzed for ERα.

(D) A serial section of the carcinoma specimen from (B) was analyzed for ERα.

(E) E-cadherin staining in the carcinoma specimen used in (A) and (C).

(F) E-cadherin staining in the carcinoma specimen used in (B) and (D).

Fearon, 2002). Thus, loss of cell adhesion molecules has multiple ramifications on cell physiology. Changes in E-cadherin abundance or function have been widely observed in many different tumor types and are predicted to contribute to the acquisition of invasive growth (Hanahan and Weinberg, 2000; Hajra and Fearon, 2002). Our results now document one pathway regulating transcription of E-cadherin. Loss of function of this pathway occurs in a clinically important subset of breast cancers, ER-negative carcinomas that have a generally poor clinical prognosis (Lapidus et al., 1998).

The dependence of MTA3 levels on ER predicts a mech-

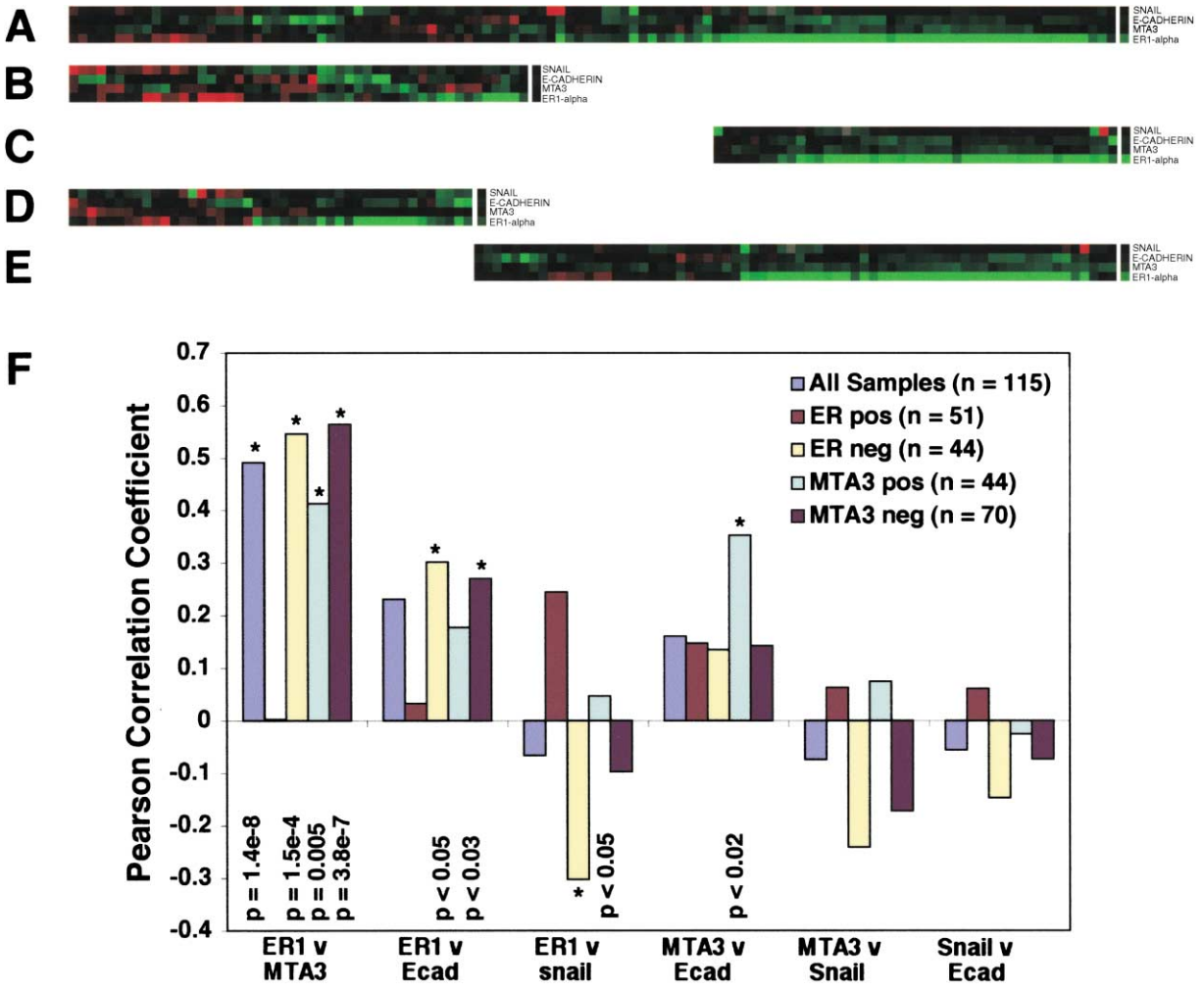

Figure 7. Microarray Expression Analysis of Breast Cancer Patients Shows Correlations between Estrogen Receptor, MTA3, and E-Cadherin

(A) Hierarchical clustering of gene expression data for ER, MTA3, E-cadherin, and Snail from 115 breast cancer patients. Primary data analyzed was from van't Veer et al. (2002).

(B) Hierarchical clustering of the same gene set using the subset of 51 patients that were designated ER positive by immunohistochemistry.

(C) Expression analysis using the data from the subset of 44 patients that were designated ER negative by immunohistochemistry.

(D) Expression analysis of the subset of 44 patients with an MTA3 expression ratio >0.

(E) Expression analysis of the subset of 70 patients with an MTA3 expression ratio <0.

(F) Graphical representation of the Pearson correlation coefficients of the gene expression patterns shown in (A)–(E). The theoretical maximum for Pearson correlation coefficients of perfect positive correlation is $+1$, and perfect negative correlation is -1. A value of zero represents no correlation. Statistically significant correlations are marked with an asterisk, and computed p values represent the probability that the null hypothesis of zero correlation is true.

anism by which receptor status can impact E-cadherin biosynthesis (Figure 5E). ER indirectly activates transcription of MTA3 in response to estrogen signaling. MTA3, in turn, forms a transcriptional corepressor complex containing histone deacetylase and ATP-dependent chromatin remodeling functions. This complex is dedicated to gene repression, and one direct target is *Snail*. Thus, ER action indirectly results in transcriptional repression at *Snail*. Ultimately, one biological readout of estrogen signaling through ER is manifested in the expression of E-cadherin and the maintenance of normal epithelial architecture. In this manner, ER contributes to the maintenance of differentiation in breast epithelia. This pathway utilizes chromatin modifications as a basis for gene regulation. During female development and adult life, such a mechanism endows the system with the ability to respond to biological signals through reversible repression of Snail or E-cadherin. Such a strategy imparts considerable regulatory potential to a seemingly simple event—activation of a ligand-inducible transcription factor. Activation of ER leads not only to transcriptional activation, but it also leads to locus specific transcriptional repression through biosynthesis of MTA3. The ability to modulate transcription in both a positive and negative fashion contributes both to the complexity and to the plasticity of the regulatory circuit.

In clinical samples, we observed that loss of ER leads to loss of MTA3, expression of Snail, and loss of E-cadherin. Further, tumors expressing MTA3 also express E-cadherin. These data support the data derived from our cell-

culture model and strongly suggest that ER is essential for MTA3 expression in mammary epithelium. However, it is also clear from the clinical data that E-cadherin transcriptional regulation is complex. We did not observe a statistically significant correlation between ER and MTA3 expression in the microarray data subset of ER-positive tumors. This lack of correlation potentially reflects the involvement of additional factors in the pathway between ER and MTA3. The cycloheximide experiments performed in cultured cells further support the existence of such a factor(s). We initially considered the progesterone receptor as a prime candidate for direct regulation of MTA3 transcription, based on the documented roles of PR in differentiation of mammary epithelia (Couse and Korach, 1999). However, PR antagonists failed to alter MTA3 RNA or protein levels (data not shown), eliminating PR involvement in MTA3 expression. From these data, we conclude that input to the E-cadherin promoter comes from many different regulatory circuits, including the pathway described here.

We propose that the MTA protein family constitutes an essential structural component of the Mi-2/NuRD complex. Based on our finding that MTA3 is physically associated with several components of the complex, but not with MTA1 or MTA2, we predict that each MTA family member serves as a molecular marker for a distinct subset of a heterogeneous family of complexes. We observed that MTA3, but not MTA2, is associated with the Snail promoter in ER-positive cells. Further, MTA3 supported repression of the Snail promoter in transfection assays where MTA1 did not. These data strongly support the notion that individual MTA family members can impart unique functional properties to the complex. For instance, MTA1 is reported to repress estrogen-dependent transcription through a direct interaction with ER (Mazumdar et al., 2001). We propose that MTA2 is a constitutively expressed Mi-2/NuRD subunit and that MTA1 and MTA3 serve specialized functions in cell type-specific transcription. In the mammalian SWI/SNF complex, subunit composition differs with cell type (Wang et al., 1996), and specific subunits are known to impart functional specificity (Armstrong et al., 1998; Nie et al., 2000). Alternative subunits have the capacity to impart unique properties to corepressors and coactivators, contributing to cell type-specific patterns of transcription.

Estrogens have long been known to have mitogenic functions in breast cancer cell lines and in breast tumors (Gruber et al., 2002). Selective estrogen receptor modulatory compounds (SERMs), which bind directly to ER, can block the growth stimulatory function of estrogens (McDonnell et al., 2002). It is now clear that the receptor adopts physically unique confirmations when bound to distinct ligands, providing unique surfaces for interaction of the receptor with coactivators and corepressors (McDonnell and Norris, 2002). The regulatory circuit described in this work has significant implications for SERM therapy. Any compound that interferes with the ability of ER to activate transcription of MTA3 would be predicted to lead to decreased expression of E-cadherin in breast epithelia and predisposition to invasive growth.

Approximately 30% of breast carcinomas lack ER expression. Presumably, these breast cancers become estrogen independent through genetic alterations that bypass the requirement for ER-dependent stimulation of cell proliferation. Examples include amplification of cyclin D1, c-*myc*, and c-ErbB2 (Lapidus et al., 1998). These genetic alterations produce a cellular state in which ER function is unnecessary for continued growth and proliferation. In this paradigm, loss of ER, MTA3, and E-cadherin expression should provide a selective growth advantage in the tumor microenvironment by increasing the probability of invasion and metastasis. Thus, the pathway described in this work provides a molecular mechanism by which loss of ER expression promotes tumor progression.

Experimental Procedures

Cloning of Human MTA3

A tblastn search of the Genbank database using the amino acid sequence of mouse MTA3 (AF288138) revealed several human EST clones, including KIAA1266 (XM 038567). The full-length cDNAs for *MTA3* were PCR-amplified from HeLa and MCF7 cells. Please see Supplemental Tables S1 and S2 for PCR primer sequences.

Cell Culture and Transfection

Cells were cultured in DMEM (Cellgro) with 10% (v/v) fetal bovine serum (Gibco). Steroids were depleted from serum with dextran-charcoal (Reddel and Sutherland, 1984). For estrogen depletion, cells were grown in phenol red-free RPMI with 10% dextran-charcoal-stripped serum and 10 μg per ml insulin (Invitrogen). Cells were transfected with Lipofectamine (Invitrogen). Transfected cells were treated with 100 ng/ml trichostatin A and 1 mM sodium n-butyrate (Sigma) for 12 hr where indicated. Luciferase activities were determined as described (Fujita et al., 2000). Values are the means and standard deviations of the results from three independent experiments.

Plasmids

Human *MTA3* and human *MTA1* were cloned into pcDNA3 (pcDNA3-MTA3) (Invitrogen). The promoter regions of human *p16*, *Snail*, *Slug*, *Cyclin D1*, and *SNRPN* were amplified from human genomic DNA and cloned into pGL2-Basic (Promega). Primer sequences are available in Supplemental Tables S1 and S2. The *GAL4* cDNA (amino acids 1–147) was amplified from pBIND (Promega) and ligated into pcDNA3. The resulting plasmid was used to make GAL4 DNA binding domain fusions with MTA3, MBD3, and the MeCP2 TRD (amino acids 207–310). The fragment containing five GAL4 binding sites from pG5*luc* (Promega) was inserted into the *SNRPN*-promoter-containing pGL2-Basic vector.

Antibodies and Western Blotting

MTA3 antibodies were generated in rabbits using GST-fused MTA3 (amino acids 1–147). For affinity purification, MTA3 (1–147) was coupled to Affi-Gel 15 activated matrix (BioRad). Antibodies were raised against MTA2, Mi2, and MBD3 in rabbits. Other antibodies utilized were anti-HDAC1, anti-HDAC2, and anti-ERα (Santa Cruz), anti-β-actin (Chemicon), and anti-FLAG (M2) (Sigma).

Immunoprecipitation

Cells were lysed with hypotonic buffer: 0.5% Triton X-100, 10 mM Tris (pH 7.5), 10 mM NaCl, 3 mM MgCl2, 0.2 mM PMSF, and 1 mM dithiothreitol (DTT). After centrifugation at 4000 rpm for 2 min, nuclear pellets were extracted with 10 mM Tris (pH 7.5), 500 mM NaCl, 3 mM MgCl2, 10% glycerol, 1% CHAPS, 0.2 mM PMSF, and 1 mM DTT. After centrifugation, the supernatants were dialyzed versus 10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM MgCl2, 20% glycerol, 0.2 mM PMSF, and 1 mM DTT. Aliquots of nuclear extract (200–400 μg) were incubated with specific antibodies or with control IgG (Santa Cruz) for 1 hr at 4°C followed by incubation for 1 hr with 25 μl of protein G/A agarose (Oncogene).

RNA Extraction, Northern Blotting, cDNA Synthesis, and RT-PCR

Total RNA was extracted by a modified guanidium thiocyanate-phenol-chloroform extraction method and analyzed by Northern blotting. The membranes were probed with a 1.5 kb fragment of *MTA3*, a 2 kb fragment of *MTA2*, and a 0.5 kb *GAPDH* fragment labeled with [α-^{32}P]dCTP by random priming.

cDNA was synthesized with M-MLV reverse transcriptase (Life Technologies) using random hexamers. The cDNA was amplified with primers for *Slug*, *Snail*, *E-cadherin*, *MTA3*, or β-*actin* (please see Supplemental Tables S1 and S2 for primer sequences). RT-PCR with real-time quantitation was carried out using the iCycler system (BioRad) as follows: 3 μg total RNA were reverse transcribed using random hexamers. PCR amplification reactions included SYBR green (Sigma) at a 1:10,000 dilution. Positive control reactions were performed to determine the linear range of detection and establish a standard curve for each transcript. Unknowns were amplified in triplicate and cDNA was diluted so that threshold values fell within the linear range of detection. Transcripts were then quantified from the corresponding standard curve. β-actin served as an internal control.

Chemical Crosslinking and Chromatin Immunoprecipitation Assay (ChIP)

Cells (5×10^5) were treated for 30 min on ice with or without 5 mM dimethyl 3,3′-dithiobispropionimidate-2HCl (DTBP) (Pierce) in PBS, rinsed (100 mM Tris-HCl [pH 8.0], 150 mM NaCl) and crosslinked (PBS) with 1% formaldehyde. Crude cell lysates were sonicated to generate 300 to 3000 bp DNA fragments. Immunoprecipitation was performed with specific and control antibodies (Upstate). Promoter regions were detected by PCR amplification with specific primers (see Supplemental Tables S1 and S2). G6PD amplification followed the conditions of Coffee et al. (1999).

siRNA Knockdown Experiments

siRNA duplexes were designed in the open reading frames of *MTA3*, *Snail*, and *Slug* and BLASTed against the human genome to ensure target specificity. RNAs were obtained in annealed form (Dharmacon). siRNAs were transfected using Oligofectamine following the manufacturer's instructions (Invitrogen). Target sequences were: MTA3 (5′-AACCAAAUAGCCCACUUACGG-3′), GFP (5′-GGCTACGTCCAGGAGCGCACC-3′), Snail (5′-GCGAGCUGCAGGACUCUAA-3′), Slug (5′-GCUACCCAAUGGCCUCUCU-3′).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were obtained from Grady Memorial Hospital and Emory University Division of Surgical Pathology. Appropriate institutional approval for human tissue studies was obtained. Sections were deparaffinized and rehydrated followed by antigen unmasking in 10 mM citrate buffer (pH 6.0) (MTA2, PR, ERα, E-cadherin) or 0.1 M Tris (pH 10.0) (MTA3) for 10 min at 15 psi and 120°C. After blocking (PBS, 2% milk) sections were stained for 1 hr. Antibody dilutions were: MTA3 (1:1200), MTA2 (1:2000), PR (1:50), E-cadherin (1:50), and ERα (1:20). ERα and PR positivity criterion was $>$10% of carcinoma cell immunoreactive. Detection with the LSAB2 System (DAKO) for immunoperoxidase staining followed the manufacturer's recommendations. Sections were counterstained with hematoxylin before mounting for microscopy.

Microarray Data Analysis

Publicly available gene expression data from 115 patients on 24481 genes (van't Veer et al., 2002) was downloaded and analyzed using Microsoft Excel, Spotfire Decisionsite 7.0, Cluster, and Treeview software packages. Pearson correlation coefficients were computed between all possible comparisons of ER (estrogen receptor 1 α, NM_00012), MTA3 (KIAA1266, AB033092), Snail (NM_00598), and E-cadherin (NM_00436), and statistical significance was assessed based on a two-tailed, unpaired T test using the R statistical programming language. Two-dimensional hierarchical clustering, using average Euclidian distance linkages, was performed using Cluster and visualized with Treeview.

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