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14. ABSTRACT
The overall goal of this research is to determine the role of LMO4 in breast cancer progression. New insight into these concepts will likely provide new targets and strategies for therapeutic intervention. We have identified estrogen receptor alpha (ERα) and its corepressors MTA1 and MTA1s as novel binding partners of LMO4 and have shown that LMO4 is essential to maintain ER transcriptional repression in breast cancer cells. This work was reported in a recent *Cancer Research* article. Since LMO4 is up regulated in human breast cancers, repression of ERα transactivation functions by LMO4 might contribute to the process of breast cancer progression by allowing the development of ERa-negative phenotypes and hormone resistance, leading to increased aggressiveness of breast cancer cells.

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Introduction

LIM domain transcriptional regulators are critical mediators of pattern formation, organogenesis and cell differentiation. The LIM-only proteins (LMO) consist nearly entirely of two LIM domains and utilize these cysteine-rich, zinc-coordinating regions to help dictate patterns of gene expression and cell fate through mediating protein-protein interactions with DNA binding proteins and transcriptional coregulators. In addition to their developmental roles, LMO proteins may also be critical mediators of cancer development. LMO4, the most divergent LMO protein, was originally cloned from a breast cancer cDNA library and is overexpressed in more than 50% of invasive breast cancers. While investigating the function of a new cytoplasmic protein, MTA1s (metastasis-associated protein 1 (MTA1) short form) in breast cancer, we have found that MTA1s physically interacts with LMO4. Cytoplasmic localization of LMO4 has been noted in late stage human breast cancers. Since MTA1s has been shown to contribute to the cytoplasmic localization of estrogen receptor alpha (ER), enhancement of nongenomic ER signaling, and the development of hormone-resistant breast cancer, we tested the hypothesis that LMO4 is a new ER coregulator that facilitates MTA1s-mediated ER cytoplasmic localization and nongenomic signaling.The overall goal of this research is to determine the role of LMO4 in breast cancer progression and gain novel insight into the molecular mechanisms of altered ER localization and tamoxifen resistance in human breast cancer. New insight into these concepts will likely provide new targets and strategies for therapeutic intervention.

Body

To address our hypothesis that LMO4 contributes to breast cancer progression through the cytoplasmic sequestration of ER, we have developed paired tamoxifen-sensitive and tamoxifen-resistant, ER expressing breast cancer cells that over express LMO4, MTA1s, or both proteins. Routine biochemical, molecular biology and confocal microscopy techniques have been employed in these studies. Results indicate that LMO4 and ER physically interact in vitro and in vivo. Deletion mapping determined that the first 164 amino acids of MTA1s are required for this interaction, while both LIM domains of LMO4 are required for optimal protein-protein interaction. Using transient transfection of an estrogen response element (ERE)-luciferase reporter system, we determined that LMO4 is a potent suppressor of ER-mediated transcriptional activity. Likewise, treatment of ERE-luciferase transfected cells with siRNA directed against LMO4 resulted in a more than four fold increase in both basal and estrogen-induced reporter activity. Although both proteins showed significant cytoplasmic localization under different conditions using immunofluorescent labeling and confocal microscopy, most LMO4-ER colocalization appeared to be in the cell nucleus. Thus LMO4 appears to be an important regulator of ER function. This regulation may encompass both nuclear and cytoplasmic ER.

The LMO4 interaction domain of MTA1s covered the first 164 amino acids of the protein,a region that is identical in both the full legth (MTA1) and the alternatively spliced (MTA1s) forms of the protein. Also, MTA1 is a known ER interacting coregulator

localized in the nucleus and most of the observed LMO4-ER interaction was in the nucleus. These data led us to hypothesize that MTA1 might also bind LMO4 in the nuclear compartment and these two proteins might coordinately block ER-regulated transcription. Interestingly, LMO4 exhibited binding with both ERα and MTA1, and existed as a complex with ER, MTA1 and histone deacetylases (HDACs), implying that LMO4 was a component of the MTA1 corepressor complex. Consistent with this notion, LMO4 over expression repressed ERα transactivation functions in an HDAC-dependent manner. Accordingly, silencing of endogenous LMO4 expression with specific small inhibitory RNA resulted in significantly increased recruitment of ER to target gene chromatin, stimulation of ERα transactivation activity (including an estrogen response element-luciferase reporter) and enhanced expression of ERα-regulated genes (including the pS2 gene) and recruitment of ER to target chromatin.

Key Research Accomplishments

The following new information relevant to the biology of breast cancer has emerged as a result of the work performed under this contract:

1) We have identified estrogen receptor alpha (ERα) and its corepressors MTA1 and MTA1s as novel binding partners of LMO4.
2) We have mapped the protein-protein interaction domains between these molecules.
3) We have demonstrated that LMO4 is a component of the MTA1 nuclear corepressor complex in vivo and represses ER transcriptional activity in an HDAC-dependent manner.
4) We have shown that LMO4 is essential to maintain ER transcriptional repression in breast cancer cells.
5) LMO4 may also function in some circumstances in the cytoplasmic sequestration of ER through interaction with MTA1s, but these investigations are ongoing.

Reportable Outcomes

LMO4 is a potent repressor of ER transactivation functions. This repression involves binding both ER and MTA1, and ER coregulator that is part of the NuRD corepressor complex. LMO4 may contribute to the development of hormone resistance in breast cancer progression through one or more mechanisms. This work was reported in a recent *Cancer Research* article. Please see Appendix 1.

Conclusions

Our findings suggest that LMO4 is an integral part of the molecular machinery involved in the negative regulation of ERα transactivation function in breast cells. Since LMO4 is up regulated in human breast cancers, repression of ERα transactivation functions by LMO4 might contribute to the process of breast cancer progression by allowing the development of ERα-negative phenotypes, leading to increased aggressiveness of

breast cancer cells. Thus LMO4 may be an important component of oncogenic events in breast epithelial cells.

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Appendices

Appendix 1.

Singh RR. Barnes CJ Talukder AH. Fuqua SAW. and Kumar R. Negative regulation of estrogen receptor alpha transactivation functions by LIM domain only 4 protein. Cancer Res. 65(22):10594-601, 2005.

Negative Regulation of Estrogen Receptor α Transactivation Functions by LIM Domain Only 4 Protein

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Abstract

LIM domain only 4 (LMO4), a member of the LIM-only family of transcriptional coregulatory proteins, consists of two LIM protein-protein interaction domains that enable it to function as a linker protein in multiprotein complexes. Here, we have identified estrogen receptor α (ERα) and its corepressor, metastasis tumor antigen 1 (MTA1), as two novel binding partners of LMO4. Interestingly, LMO4 exhibited binding with both ERα and MTA1 and existed as a complex with ERα, MTA1, and histone deacetylases (HDAC), implying that LMO4 was a component of the MTA1 corepressor complex. Consistent with this notion, LMO4 overexpression repressed ERα transactivation functions in an HDAC-dependent manner. Accordingly, silencing of endogenous LMO4 expression resulted in a significant increased recruitment of ERα to target gene chromatin, stimulation of ERα transactivation activity, and enhanced expression of ERα-regulated genes. These findings suggested that LMO4 was an integral part of the molecular machinery involved in the negative regulation of ERα transactivation function in breast cells. Because LMO4 is up-regulated in human breast cancers, repression of ERα transactivation functions by LMO4 might contribute to the process of breast cancer progression by allowing the development of ERα-negative phenotypes, leading to increased aggressiveness of breast cancer cells. (Cancer Res 2005; 65(22): 10594-601)

Introduction

The LIM-only subclass of LIM proteins is a family of nuclear transcription coregulators that are characterized by the exclusive presence of two tandem LIM domains and no other functional domains. Each LIM domain has two cysteine-rich zinc finger motifs that are involved in protein-protein interactions but have no direct DNA-binding properties (reviewed in refs. 1, 2). These proteins regulate gene transcription by functioning as "linker" or "scaffolding" proteins by virtue of their LIM domains and are involved in the formation of multiprotein complexes of DNA-binding factors and transcriptional regulatory proteins. Four members of the LIM-only family [LIM domain only 1 (LMO1) to LMO4] have been identified to date. These proteins have been shown to play important roles in cell fate determination, tissue patterning, and organ development. As might be expected, their deregulated expression has been implicated in oncogenesis. *LMO1* and *LMO2* genes were discovered as oncogenes and are deregulated in acute T-cell lymphocytic leukemia (3–5). LMO2 is an obligate regulator of hematopoiesis and angiogenesis (6, 7) and blocks the terminal differentiation of hematopoetic cells when overexpressed (8). LMO3 was discovered on the basis of sequence homology and nothing much was known regarding its biological and pathologic significance. Recently, it was found that LMO3 interacts with neural transcription factor HEN2 and functions as an oncogene in nueroblastoma, where the expression level of both *LMO3* and *HEN2* genes was high and associated with poor prognosis (9).

LMO4 was the latest addition to this family and isolated as an interacting protein of Ldb1/NLI/CLIM and also identified in an expression screen with autologous serum of breast cancer patients (10–13). It has 165 amino acid residues and shares only ~50% amino acid sequence homology with the LIM domain regions of LMO1, LMO2, and LMO3; thus, it is considered the most distant relative of the family (11). It has a very broad spectrum of expression in human tissue (13). In mice, targeted disruption of LMO4 led to defects in neural tube closure, sphenoid bone formation, and altered anterior-posterior patterning (14, 15), revealing its importance in cell patterning and embryogenesis. Its expression is developmentally regulated in the mammary gland and overexpression blocks the differentiation of mammary epithelial cells (16). LMO4 is overexpressed in 50% of primary breast tumors (16), in squamous cell carcinomas of the oral cavity (17), and in primary prostate cancer (18), implicating it as an oncogene. It has been identified as a binding partner and a participant in multiprotein complexes with several transcriptional regulatory proteins, such as HEN1, deformed epidermal autoregulatory factor 1 (DEAF1), and BRCA1 (10–16). HEN1 (also known as NSCL1/NHLH1) is a basic helix-loop-helix protein. It functions as a transcriptional activator important in hematopoiesis and is specifically expressed in the developing nervous system (19). LMO2 and LMO4 were found to be binding partners of HEN1 by yeast two-hybrid analysis, but LMO4 and not LMO2 was found to be a repressor of its transcriptional activating functions (20). LMO4 was found to physically interact with CtBP-interacting protein and the breast and ovarian tumor suppressor protein BRCA1 (21). This study showed that LMO4 represses BRCA1-mediated transcriptional activation in yeast and mammalian cells but the mechanism of repression was not established (21). LMO4 also interacts with the coregulatory proteins Clim-2/ldb-1/NL1 and DEAF1 in the same complex. DEAF1 is a DNA-binding protein that interacts with regulatory sequences and modulates transcriptional outcome (12). Providing additional implication of the role of LMO4 in breast carcinogenesis, it was shown in a recent study that overexpression of LMO4 in mice under the control of the mouse mammary tumor virus induced mammary hyperplasia and mammary intraepithelial neoplasia in two transgenic strains (22).

Note: C.J. Barnes and A.H. Talukder contributed equally to this work.

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Estrogen receptors (ER) are ligand-dependent transcription factors that control a variety of essential physiologic and developmental processes in humans. The nuclear receptors primarily regulate the initiation of transcription by directly binding to specific DNA sequences in the regulatory region of target genes called hormone response elements and recruiting diverse ancillary factors characterized as coregulators along with the basal transcriptional machinery (23). Ligand binding results in the dismissal of histone deacetylase (HDAC)–containing corepressor complexes and the concomitant recruitment of coactivator complexes. One of such corepressors of ERα is the metastasis tumor antigen 1 (MTA1), a component of nuclear remodeling complex (24). It functions by recruiting HDACs, which deacetylate histones and subsequently facilitate the compaction of chromatin and transcriptional repression.

In the present study, we have identified LMO4 as a potent repressor of transcriptional activity of ERα. We have also identified ERα and its corepressor protein, MTA1, as LMO4 binding partners and established that a multiprotein complex of LMO4, ERα, MTA1, and HDACs existed *in vivo*. LMO4 was found to be an important component of the MTA1 corepressor complex and to negatively regulate the expression of the endogenous ERα target genes in a physiologic setting. The potential implications of these regulatory interactions and a role for LMO4 in modulating ERα functions in breast cancer cells are presented.

Materials and Methods

Cell culture and reagents. Human breast cancer cells were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS). For estrogen treatment experiments, cells were grown in dextran-charcoal–stripped medium containing 5% charcoal-stripped FBS. Antibodies against the T7 tag were from Bethyl Laboratories (Montgomery, TX); anti-ERα was from Chemicon Inc. (Pittsburgh, PA); and anti-MTA1, HDAC1, and HDAC2 were from Santa Cruz (Santa Cruz, CA). Antimouse and antirabbit horseradish peroxidase or alkaline phosphatase–conjugated antibodies were from Amersham Biosciences (Piscataway, NJ).

Glutathione *S*-transferase pull-down assay. *In vitro* transcription and translation of MTA1s, MTA1, LMO4, and ERα was done using a T7-TNT kit (Promega Biosciences, San Luis Obispo, CA), where 1 μg cDNA in pcDNA 3.1 vector was translated in the presence of [^{35}S]methionine in a reaction volume of 50 μL. The reaction mixture was diluted to 1 mL with NP40 lysis buffer (25 mmol/L Tris, 50 mmol/L NaCl, and 1% NP40). An equal aliquot was used for each glutathione *S*-transferase (GST) pull-down assay. Translation and product size were verified by subjecting 2 μL of the reaction mixture to SDS-PAGE and autoradiography. The GST pull-down assays were done by incubating equal amounts of GST, GST-tagged full-length proteins, and GST-tagged deletion constructs immobilized on glutathione Sepharose beads (Amersham Biosciences) with *in vitro* translated ^{35}S-labeled protein to which the binding was being tested. Bound proteins were isolated by incubating the mixture for 3 hours at 4°C, washing five times with NP40 lysis buffer, eluting the proteins with 2× SDS buffer, and separating them by SDS-PAGE. The bound proteins were then visualized by autoradiography.

Immunoprecipitation and immunoblotting. Cell extracts for immunoprecipitation were prepared by washing cells thrice with PBS. Cells were then lysed using a minimum volume of high-salt lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 500 mmol/L NaCl, 100 mmol/L NaF, 200 mmol/L $NaVO_5$, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Life Technologies, Gaithersburg, MD)] for 15 minutes at −80°C and 15 minutes on ice to freeze and thaw the cells to aid lysis. Lysates were centrifuged in an Eppendorf centrifuge at 4°C for 15 minutes. Lysates were diluted with 1 mL lysis buffer without added NaCl and immunoprecipitation was done for 3 hours at 4°C using 1 μg of antibody per milligram of protein. For immunoblotting, the immunoprecipitated proteins were resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose membrane, and probed with appropriate antibodies.

Immunofluorescence and confocal microscopy studies. We determined the cellular localization of proteins by indirect immunofluorescence as described (24). Briefly, cells grown on glass coverslips were fixed in 4% phosphate-buffered paraformaldehyde for 15 minutes. Cells were permeabilized in methanol at −20°C for 4 minutes. Following permeabilization, cells were incubated with primary antibodies for 2 hours at room temperature, washed thrice in PBS, and then incubated with secondary antibodies conjugated with 546-Alexa (red) or 488-Alexa (green) from Molecular Probes (Eugene, OR). The DNA dye Topro-3 (Molecular Probes) was used for nuclear localization (blue). Confocal scanning analysis was done using an Olympus FV300 laser scanning confocal microscope in accordance with established methods using sequential laser excitation to minimize the possibility of fluorescence emission bleed through. Each image is a three-dimensional reconstructed stack of serial Z sections at the same cellular level and magnification. Colocalization of two proteins is shown yellow for red and green fluorescence.

Transfection and promoter assays. Cells were maintained in DMEM/F-12 (1:1) supplemented with 10% FCS. For reporter assays, the required plasmids were transiently transfected using FUGENE6 kit from Roche Biochemicals (Indianapolis, IN) as per instructions of the manufacturer. Cells were cotransfected with β-galactosidase and luciferase assay was done using Luciferase assay kit (Promega).

RNA interference transfection and reverse transcription-PCR analysis. RNA interference (RNAi) transfections were done using Oligofect-AMINE (Invitrogen) according to the protocol of the manufacturer. RNAi against LMO4 was purchased from Qiagen. A pool of four individual RNAi was used and the sequences have been provided below. Forty-eight hours were allowed to elapse after transfection to allow efficient silencing of the gene. Reverse transcription-PCR (RT-PCR) was done using Access RT-PCR kit (Promega) using specific primers shown below:

LMO4 RNAi 1: 5′-ACGUCCUGUUACACCAAAAUU-3′.
LMO4 RNAi 2: 5′-CAUGAUCCUUUGCAGAAAUUU-3′.
LMO4 RNAi: 5′-CGCAAGGCAAUGUGUGUAUCAUU-3′.
LMO4 RNAi 4: 5′-CUACAUCCAUGGCSGUUUAUU-3′.
LMO4: 5′-GGACCGCTTTCTGCTCTATG-3′ and
5′-ACGAGTTCACTCGCAGGAAT-3′.
pS2: 5′-ATACCATCGACGTCCCTCCA-3′ and
5′-AAGCGTGTCTGAGGTGTCCG-3′.
Total progesterone receptor (PR): 5′-CAAATGAAAGCCAAGCCCTA-3′
and 5-TGCCTCTCGCCTAGTTGATT-3′.
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH):
5′-CCATCTTCCAGGAGCGAGATC-3′ and
5′-CGTTCAGCTCAGGGATGACC-3′.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assay was done in MCF-7 cells following the procedure as described elsewhere (24). Briefly, LMO4 expression in MCF-7 cells was silenced using RNAi against LMO4. Cells were later cultured in dextran-charcoal–stripped medium for 24 hours, treated with estrogen (10^{-9} mol/L) for 1 hour, and cross-linked with 1% formaldehyde. Cells were lysed by sonication and chromatin immunoprecipitation was done with an ERα-specific antibody. Immunoprecipitated DNA fragments were analyzed for *pS2* chromatin by amplifying specific region by PCR using *pS2* chromatin–specific primers with the following sequence: 5′-GAATTAGCTTAGGCCTAGACGGAATG-3′ and 5′-AGGATTTGCTGATAGGACAGAG-3′.

Silencing of LIM domain only 4 expression in metastasis tumor antigen 1 stable clones and Northern blotting. MTA1-overexpressing cells were transfected with LMO4-specific RNAi. After 24 hours, cells were maintained for 24 hours in dextran-charcoal–stripped medium and later treated with estrogen (10^{-9} mol/L) for 16 hours. Total RNA from the cells was extracted, resolved on an RNA gel, and blotted onto a nitrocellulose membrane. Levels of specific mRNAs were analyzed by probing the blot with appropriate radiolabeled probes and were measured by autoradiography.

Results

LIM domain only 4 represses estrogen receptor α transactivation activity. To gain insight into the functional role of dysregulated LMO4 in breast cancer, we decided to test the effect of LMO4 on ERα transactivation functions. First, we examined the effect of LMO4 overexpression on transcription from an estrogen response element (ERE)-luciferase reporter plasmid in two ERα-positive breast cancer cell lines, MCF-7 and ZR-75 (Fig. 1*A* and *B*). LMO4 overexpression in both cell lines led to a distinct repression of ERE transcription activity independent of estrogen stimulation, with a 3-fold repression in MCF-7 cells and a 2-fold repression in ZR-75 cells. To further validate the observed repression of ERα transactivation by LMO4, we examined the effects of increased amounts of LMO4 plasmid on ERE-luciferase transcription in MCF7 cells (Fig. 1*C*). As little as 250 ng of LMO4 was found to be sufficient to exert a potent repression of ER transactivation function in breast cancer cells and the extent of repression increased with increasing amount of LMO4 expression in the cells.

LIM domain only 4 represses estrogen receptor α transactivation in a histone deacetylase–dependent manner. To test the possibility that the repression of ERα functions by LMO4 could be HDAC dependent, we examined the effect of trichostatin A, a specific inhibitor of HDACs, on LMO4-induced repression of ERE transcription in both MCF-7 and ZR-75 cells (Fig. 2*A*). We found that LMO4-mediated repression of ERα transactivation activity could be effectively relieved by inhibiting HDAC activity. These results suggest that LMO4 requires functional HDACs for its noticed corepressor function of ERα activity.

We have previously reported that MTA1, a component of the NURD complex, functioned as a corepressor of ERα transactivation functions by binding and recruiting HDACs to the repressor complex (24). To see if LMO4 may be functioning via this MTA1 corepressor complex, we tested the effect of silencing LMO4 expression using LMO4-specific RNAi on MTA1 repression of ERα transactivation function in MCF-7 cells. As expected, overexpression of MTA1 effectively repressed ER transactivation functions. However, silencing of LMO4 expression resulted in a >3-fold enhancement of the ERE-luciferase activity, demonstrating that the repression of ERα transactivation functions was released on knocking down LMO4 expression (Fig. 2*B*) both with and without MTA1 overexpression. These findings suggest that LMO4 might be an essential component of the MTA1 corepressor complex.

We next tested whether LMO4-mediated repression was dependent on MTA1. Results indicate that the LMO4-induced repression of ERE-luciferase activity was partially relieved by cotransfection of MTA1-specific RNAi (Fig. 2*C*). These assays showed that, functionally, LMO4 and MTA1 corepressor functions were interlinked and that LMO4 could be a part of the MTA1 corepressor complex. These observations suggested an inherent role of the endogenous LMO4 in influencing the status of ERα transactivation function and that LMO4 may also be important in the corepressor activity of MTA1 in breast cancer cells.

LIM domain only 4 binds to metastasis tumor antigen 1. To test whether LMO4 could physically interact with MTA1, *in vitro* binding studies were done using ^{35}S-labeled full-length LMO4 and GST-tagged full-length MTA1 and GST-MTA1 deletion constructs. Results indicated that LMO4 binds with the full-length MTA1 (Fig. 3*A*). Full-length LMO4 bound to both the NH_2-terminal BAH and ELM domains of MTA1 (Fig. 3*A, deletion construct A*) as well as the COOH-terminal region (deletion construct D) of MTA1, encompassing the Src homology 2 (SH2)– and SH3-binding domains (Fig. 3*A*). Binding studies of ^{35}S-labeled MTA1 with GST-tagged full-length LMO4 and its deletion constructs showed that the first LIM domain (LIM1, amino acids 20-89) of LMO4 was sufficient to bind ^{35}S-labeled MTA1 (Fig. 3*B, deletion construct B*). Weak or no binding was observed between the MTA1 and the second LIM domain of LMO4. This bidirectional *in vitro* binding study showed that MTA1 and LMO4 were binding partners and strengthened the possibility that LMO4 could be a part of the MTA1 corepressor complex.

LIM domain only 4 also interacts with estrogen receptor α and histone deacetylase but not estrogen receptor β. Because LMO4 has been shown to be up-regulated in malignant breast cancers that were, in general, functionally ERα negative, and because MTA1 functions as a corepressor of ERα transcriptional functions (24), we next investigated the possibility that LMO4 directly interacted with ERα. Indeed, ^{35}S-labeled ERα interacted with GST-tagged full-length LMO4 *in vitro* (Fig. 4*A*). Binding studies using the individual GST-tagged domains of LMO4 with full-length ^{35}S-labeled ERα showed that the first LIM domain of LMO4 along with additional NH_2-terminal region (Fig 4*A, deletion construct A*,

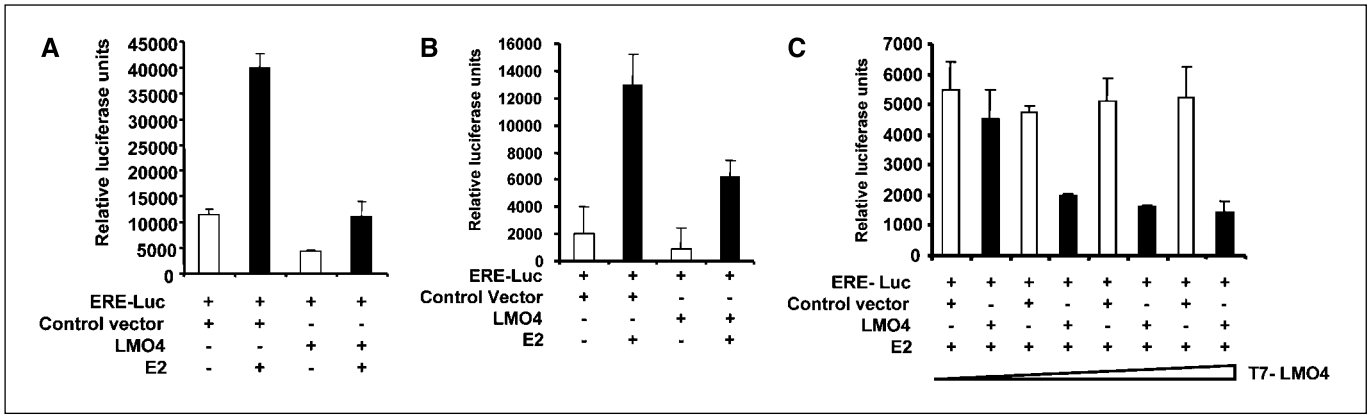

Figure 1. ERE-luciferase (*ERE-Luc*) activity was used as a functional assay to study the effect of LMO4 on ERE transcription in the cells. The expression construct (1 μg) for T7-tagged LMO4 was transiently transfected in MCF7 cells (*A*) and ZR-75 (*B*) breast cancer cells. Repression of estradiol (*E2*, 10^{-9} mol/L)–mediated ERE transcription by 3-fold (MCF-7) and 2-fold (ZR-75) showed the repression of ER transcriptional functions by LMO4. *C,* increasing amounts of T7-LOM4 expression plasmid (250-1,000 ng with increments of 250 ng) were transfected in MCF-7 cells and a dose-dependent repression of ERE-transcription was observed. In all the assays, cells transfected with suitable vector (pcDNA) were used as controls for comparison. *Columns,* average of at least two separate experiments.

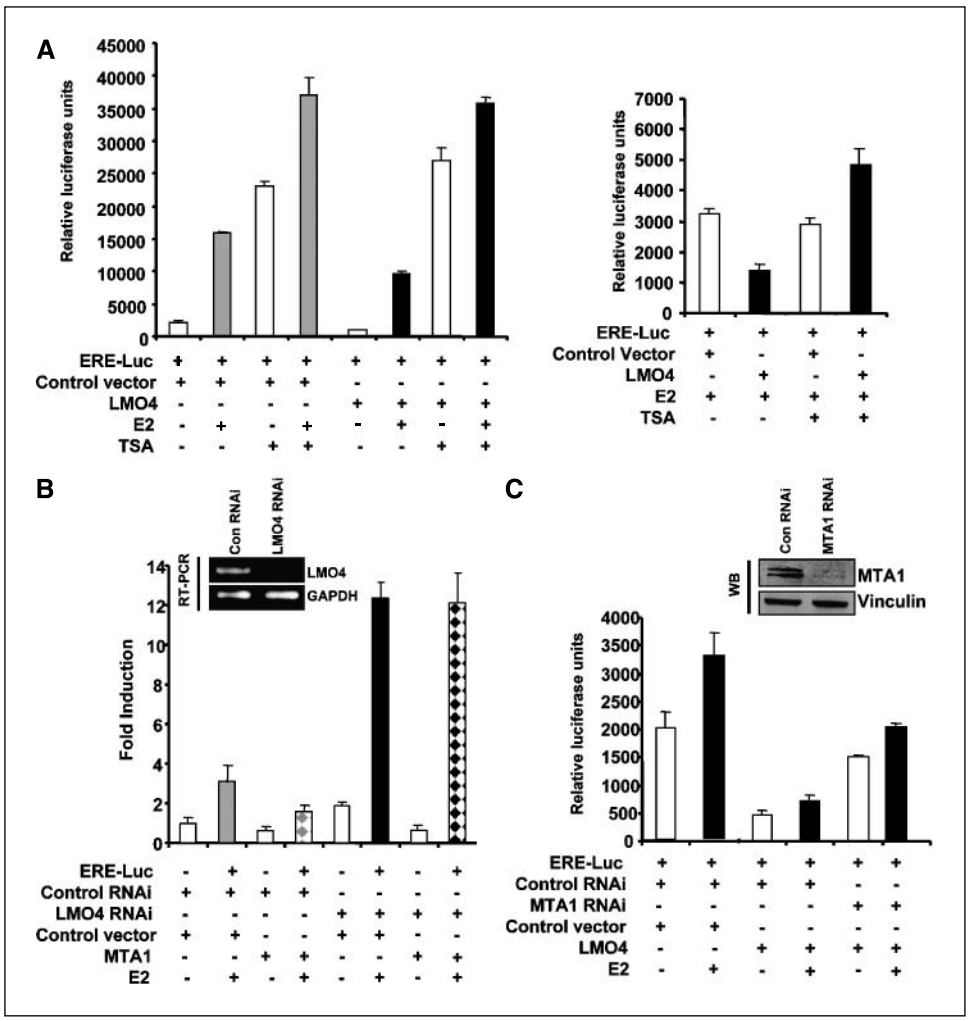

Figure 2. *A,* treatment of cells with the specific HDAC inhibitor trichostatin A (*TSA*; 300 nmol/L per milliliter of culture medium) led to a drastic relieving of the LMO4-induced repression in both MCF-7 (*left*) and ZR-75 (*right*) cell lines as measured by ERE-luciferase assay, exhibiting that the repression was brought about by the involvement of HDACs. *B,* ERE-luciferase activity was enhanced in MCF-7 cells on silencing the LMO4 expression using LMO4-specific RNAi. A 4-fold enhancement of luciferase activity indicated a drastic relieving of repression. A similar relieving of repression when MTA1 was overexpressed showed that LMO4 was essential for MTA1 to function as ERα corepressor. *C,* silencing of MTA1 expression in MCF-7 cells also led to the alleviation of LMO4-induced repression of ERE-luciferase activity, indicating that LMO4 functions as a ER corepressor via MTA1. *Columns,* average of two separate experiments. Evidence for effective silencing of LMO4 and MTA1 expression by their RNAi was tested by RT-PCR and Western blotting analysis and the results are provided here.

amino acids 1-89) had binding affinity to ERα, whereas the second LIM domain had no appreciable binding affinity. Full-length ^{35}S-labeled LMO4 was found to bind with the C domain (DNA-binding domain, amino acids 181-263) and the activation function 2 domain (domain E/AF-2, amino acids 301-552) of ERα with high affinity (Fig. 4*B*). No binding was observed with the activation function-1 domain (Fig. 3*B, deletion constructs A* and *B*, amino acids 1-180). We also tested the *in vitro* binding of LMO4 to HDAC2, which is an important component of the MTA1 corepressor complex (24). GST-tagged HDAC2 clearly showed binding affinity to ^{35}S-labeled LMO4 *in vitro* (Fig. 4*C*). In addition to being a binding partner of ERα, we wanted to know whether LMO4 could also interact with ERβ. To address this question, we have done an *in vitro* binding experiment using ^{35}S-labeled *in vitro* translated ERβ to GST-tagged full-length LMO4. No binding of LMO4 to ERβ was observed, indicating that LMO4 is likely a specific binding partner of ERα. The experiment was repeated twice for confirmation (Fig. 4*D*). Overall, this series of *in vitro* binding studies showed that LMO4 strongly interacts with the three integral components of the ERα-MTA1 corepressor complex, namely, ERα, MTA1, and HDACs.

LIM domain only 4 is a component of metastasis tumor antigen 1 corepressor complex. To confirm the binding of LMO4 with ERα and MTA1 *in vivo*, we did coimmunoprecipitation followed by Western blot analysis. Due to lack of a commercial antibody for LMO4 suitable for immunoprecipitation or Western immunoblotting of endogenous protein, we transfected MCF-7 cells with an expression vector of T7-tagged LMO4. Results indicate that immunoprecipitated T7-tagged LMO4 was present in the same multiprotein complex as ERα and MTA1 (Fig. 5*A*). Because MTA1 functions as a corepressor by recruiting HDACs (24), we next tested whether HDACs were also an integral part of the LMO4 and MTA1 complex. MCF-7 cells were transfected with Myc-tagged LMO4. Cell lysates were immunoprecipitated with anti-Myc antibody and analyzed for the presence of HDACs among the LMO4-associated endogenous proteins. Both HDAC1 and HDAC2 were immunoprecipitated along with LMO4 and MTA1 (Fig. 5*B*), demonstrating that LMO4 was a part of the MTA1 corepressor complex. These findings suggested that LMO4 may repress the ER transactivation function as an integral component of HDAC-containing corepressor complexes.

LIM domain only 4 colocalizes with metastasis tumor antigen 1 and estrogen receptor α in breast cancer cells. To confirm the protein-protein interactions between LMO4, MTA1, and ERα *in situ*, we next examined whether Myc-LMO4 colocalizes with the endogenous MTA1 and ERα in MCF-7 cells. Immunofluorescence studies indicated that LMO4 colocalizes individually with both MTA1 and ERα predominantly in the cell nucleus.

Overlap of red and green fluorescence resulted in the yellow spots (Fig. 5*D* and *E, G* and *H*), representing colocalization. Some LMO4 was also localized to the cytoplasm; however, such localization of LMO4 did not noticeably change in either serum-starved or estrogen-deprived MCF7 cells (data not shown). Together, these experiments confirmed MTA1 and ERα as new binding partners of LMO4.

LIM domain only 4 is a natural inhibitor of endogenous estrogen receptor α functions. To determine whether LMO4 could negatively regulate ERα transactivation functions in a physiologic context, we investigated changes in the expression level of the endogenous ERα target genes with knockdown of LMO4 expression in MCF-7 cells. Treatment of cells with LMO4-specific RNAi enhanced the level of pS2 mRNA by >2-fold when compared with cells treated with control RNAi (Fig. 6*A, second panel*). We also did RT-PCR analysis of another ER-regulated gene (i.e., *PR*) in cells transfected with control RNAi or LMO4-specific RNAi. Results indicate that RNAi-mediated down regulation of LMO4 expression increased total PR expression levels at least 2-fold (Fig. 6*A, third panel*). To further validate these results, we used Northern blot analysis of estrogen-responsive genes in MTA1-overexpressing stable cell lines. As expected from the earlier data, the expression levels in these stable clones were considerably repressed by overexpression of MTA1 when compared with the parental cells (24). Interestingly, knockdown of LMO4 expression led to a marked increase of pS2 mRNA levels (Fig. 6*B*). These data indicated that by decreasing the level of LMO4 expression, MTA1-induced repression of estrogen-responsive genes was relieved. Thus, LMO4 may be a functionally essential component of the MTA1 corepressor complex.

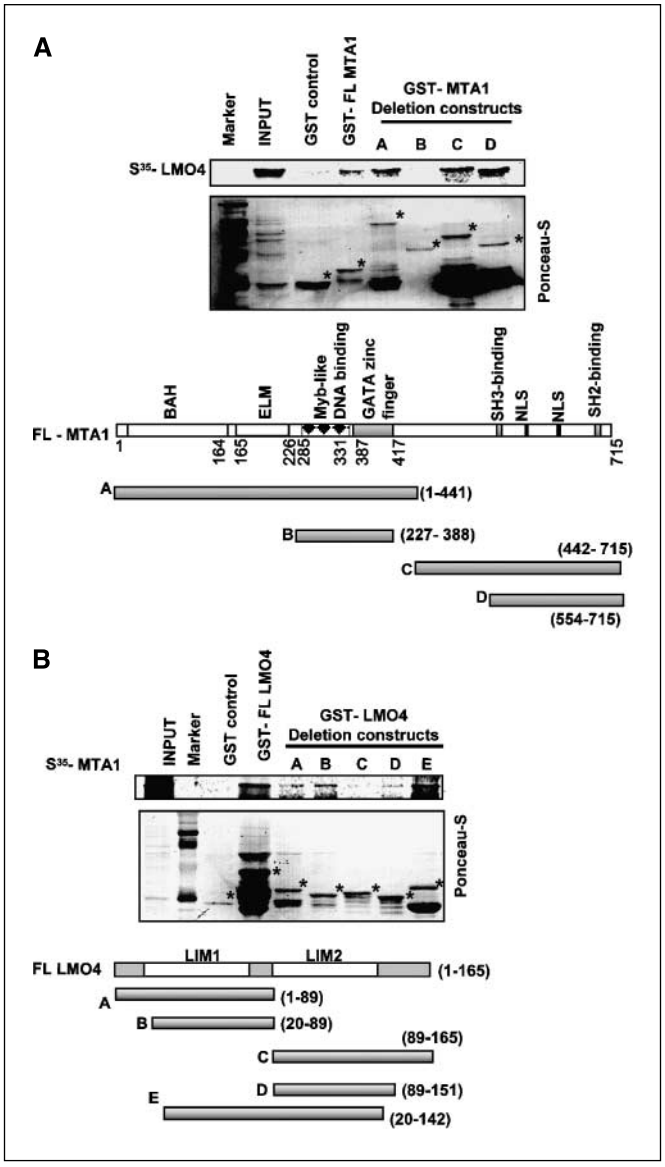

Figure 3. *In vitro* binding studies of LMO4 with MTA1. *A,* binding of GST-tagged FL-MTA1 and its deletion constructs to ^{35}S-labeled LMO4. GST-tagged FL-MTA1 binds with LMO4 (*GST-FL MTA1*). The NH_2-terminal stretch of MTA1, encompassing the BAH and ELM domains, showed strong binding to LMO4 (*deletion construct A*). No binding was seen to deletion construct B. The COOH-terminal constructs showed a strong binding to LMO4 (*deletion constructs C* and *D*), indicating that LMO4 can interact with both NH_2- and COOH-terminal regions of MTA1. *B, in vitro* translated ^{35}S-labeled full-length MTA1 binds to full-length GST-tagged LMO4 (*GST-FL LMO4*). LIM1 domain of LMO4 showed binding to MTA1 (*deletion constructs A* and *B*), whereas the LIM2 domain showed very weak or no binding (*deletion constructs C* and *D*). Schematic representation of MTA1 and LMO4 domains and the deletion constructs used are shown below the respective figures.

Silencing of LIM domain only 4 expression promotes estrogen receptor α recruitment to its target chromatin. To gain further insight into the observed negative regulatory function of LMO4 in relation to ERα-regulated genes, we next investigated whether the levels of the endogenous LMO4 also affected the recruitment of ERα to its target gene chromatin. To test this possibility, we examined the effect of silencing of LMO4 expression by LMO4 RNAi upon the ability of ERα to interact with the *pS2* gene chromatin by chromatin immunoprecipitation assay in MCF-7 cells. On estrogen treatment, the level of recruitment of ERα to the *pS2* gene chromatin was 2-fold higher in cells treated with LMO4 RNAi when compared with cells with control RNAi (Fig. 6*C*), clearly showing that silencing of LMO4 expression increased the recruitment of ER to its target genes. Together, these results clearly showed that LMO4 may be an endogenous regulator of ERα transactivation activity and functions in breast cancer cells, and up-regulation of LMO4 as has been observed in human breast cancer may lead to inhibition of ERα-transcriptional responsiveness.

Discussion

LMO4 is overexpressed in 50% of primary breast tumors and its enhanced expression blocks mammary gland differentiation. At the mechanistic level, very little is known regarding the mode of LMO4 functioning, LMO4 interacting molecular partners, or of the signaling pathways affected by LMO4 through which its effects in the breast cancer cells are manifested. In this context, we sought to investigate the effects of LMO4 upon estrogen signaling and functioning by focusing on key proteins of breast cancer tumorigenesis, such as the ERα and its coregulatory proteins. Preliminary ERE-luciferase assays conducted in MCF-7 and ZR-75 cell lines showed that LMO4 might be functioning as a negative regulator of ERα transactivation functions (Fig. 1). The LMO4-induced repression of ERE-luciferase could be reversed or relieved by trichostatin A, which is a specific inhibitor of HDACs (Fig. 2*A*) in both MCF-7 and ZR-75 cell lines. These data clearly showed that the repression exhibited by LMO4 was HDAC dependent. Studies from our laboratory had identified MTA1 as an ERα corepressor. MTA1 is a part of the NURD complex and functions by recruiting

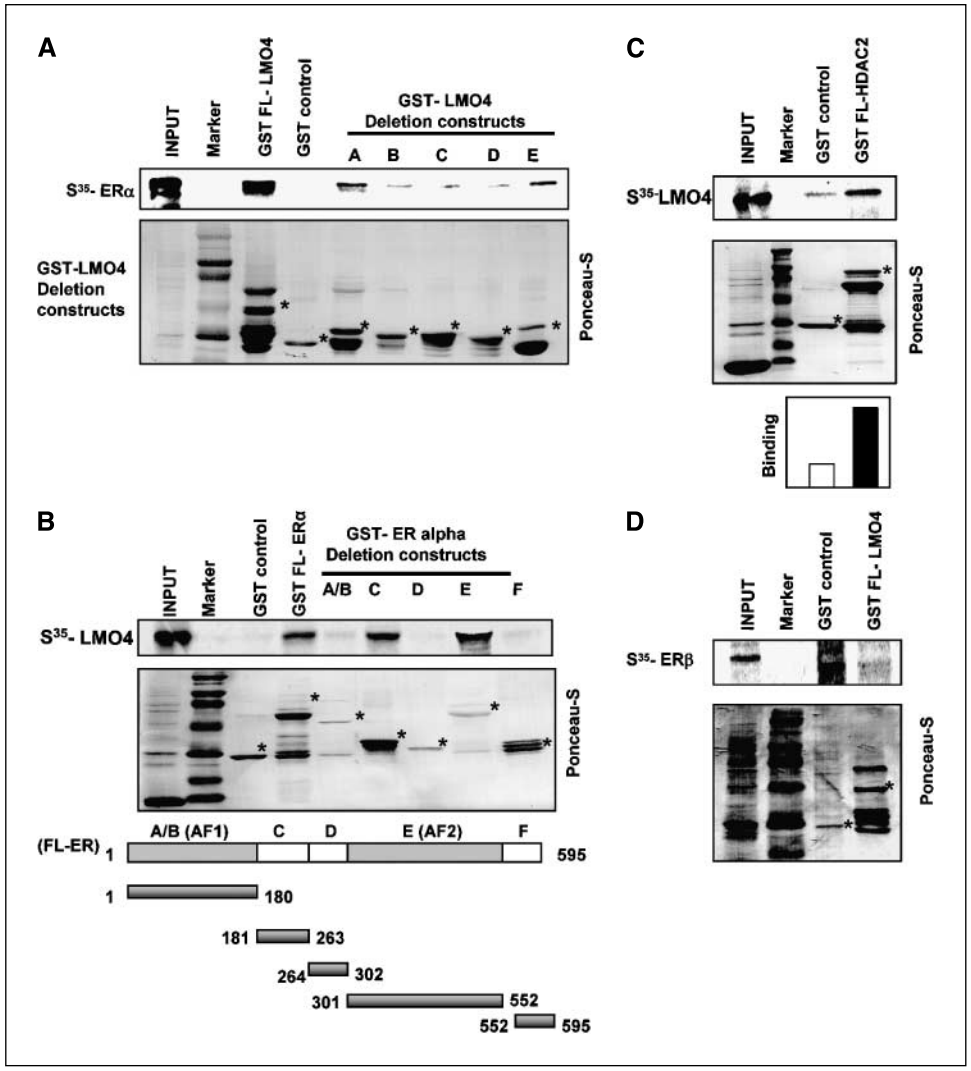

Figure 4. LMO4 binds to ERα and HDAC. *A,* full-length ^{35}S-labeled ERα exhibits binding to GST-tagged full-length LMO4 (*GST FL-LMO4*). LIM domain 1 of LMO4 with additional NH_2-terminal region showed most binding affinity with ERα (*deletion construct A*). *B,* ^{35}S-labeled LMO4 showed strong binding to the DNA-binding domain (*deletion construct C*) and the ligand-binding domain (AF2, *deletion construct E*) of ERα. Different domains of ERα have been shown schematically below. *C,* GST-tagged full-length HDAC2 showed strong binding to ^{35}S-labeled LMO4 (*GST FL-HDAC2*) whereas GST alone (*GST control*) showed very little binding, emphasizing the specificity of LMO4 and HDAC interaction. *D,* ^{35}S-labeled ERβ showed no binding affinity to GST-tagged full-length LMO4, showing that it was a specific binding partner of ERα and not ERβ.

HDACs, which are a class of enzymes involved in deacetylation of hyperacetylated histone tails, leading to compaction of chromatin and transcriptional repression (25).

Because both LMO4 are MTA1 are predominantly nuclear coregulatory proteins, our results prompted us to check whether the repression of ERα by MTA1 and the repression by LMO4 were interrelated. Silencing of LMO4 repression resulted in the relieving of MTA1-induced repression of ERα functions (Fig. 2*B*) and silencing of MTA1 expression partially relieved LMO4-induced ERα repression (Fig. 2*C*). These data showed that the functions of these two proteins were interlinked and raised the possibility of LMO4 physically participating in the MTA1 corepressor complex. A series of different *in vitro* binding studies established that ERα, MTA1, and HDACs are novel binding partners of LMO4. The *in vitro* binding results were confirmed *in vivo* with T7-LMO4 interacting with both ERα and MTA1 (Fig. 5*A*). We also showed that transfected Myc-LMO4 could also be coimmunoprecipitated along with MTA1, HDAC1, and HDAC2 (Fig. 5*B*). Colocalization studies lent further support to the notion that LMO4 was a part of an MTA1 corepressor complex *in vivo*.

LIM domains are exclusively involved in protein-protein interactions. LMO4, with two tandem LIM domains with a capability of individually interacting with ERα, MTA1 and HDACs, may be playing the role of a "linker" or "scaffolding" protein involved in stabilizing the corepressor complex. In the same context, a recent study showed that LMO4 associated with glycoprotein 130 (gp130) subunit, a common receptor subunit for interleukin (IL)-6 type cytokines, and functioned as a part of the gp130 complex. Overexpression of LMO4 enhanced the transcription of IL-6 target genes like *Stat3*, whereas silencing of LMO4 expression by RNAi led to a decrease of transcription of IL-6 target genes, implying the function of LMO4 as a scaffolding protein in the stabilization of gp130 complex (26).

Silencing of LMO4 expression in cells with LMO4 RNAi led to a drastic increase of ERE transcription as measured by ERE-luciferase functional assay (Fig. 2) and this raised the possibility that LMO4 could act as a natural negative regulator of ERα pathway. Indeed, we discovered that down-regulation of LMO4 increased expression of estrogen-responsive genes *pS2* and *PR* and also stimulated the recruitment of ERα to the endogenous *pS2* gene chromatin (Fig. 6). Together, these findings established that LMO4 is a potent endogenous repressor of ERα transactivation function and that the levels of endogenous LMO4 may influence the status of ERα functions in breast cancer cells. In addition, there was also partial relieving of LMO4-induced repression of ERE transcription with MTA1 knockdown (Fig. 2*C*), suggesting the

possibility of potential involvement of additional corepressors in the noted corepressor function of LMO4. This, we believe, would open new avenues of study directed toward recognizing other ER-corepressor complexes of which LMO4 might be an integral part.

MTA1 is expressed virtually in all human cell lines and overexpressed in breast, ovarian, lung, gastric, colorectal, and pancreatic cancers. The level of MTA1 in rapidly growing breast cancer cells was found to be twice that in the normal epithelial cells (27). It functions as a part of HDAC or nucleasome remodeling complexes and acts as a major modulator of transcription. MTA1 was found to be a potent repressor of ERE transcription and overexpression of MTA1 in breast cancer cells enhanced the ability of cells to invade and grow in an anchorage-independent manner, implicating its role in metastatic potential of cells. Heregulin also promoted the interaction of MTA1 with ER (24). In addition, MTA1 expression could also be induced by the growth factor Heregulin, a ligand for HER3 and HER4, which is also frequently deregulated in human epithelial cancers (24). Incidentally, it has also been reported that the expression of LMO4 is also significantly up-regulated by Heregulin treatment (28). Indeed, breast cancer cell lines that highly express MTA1, such as MDA-MB-231 (29), BT474, and T47D (28), also express high levels of LMO4 (28). In addition, down-regulation of LMO4 expression in MDA-MB-231 cells, an invasive breast cancer cell line, resulted in 3- to 4-fold decrease in cell motility and a 2-fold decrease in cell invasion. Overexpression of LMO4 in MCF-10A, which is a normal breast epithelial cell line, resulted in a 3-fold increase in cell migration and 2-fold increase in cell invasion (22). Overexpression of MTA1 in MCF-7 breast cancer cell line also had an identical effect of increased cell invasiveness and anchorage-independent growth (24). Up-regulation of both *MTA1* and *LMO4* genes by a common signal (i.e., Heregulin) enhanced expression of these proteins in common breast cancer cell lines. Similar phenotypic changes resulting from overexpression all strongly support the notion of functional synergy between MTA1 and LMO4.

To summarize, in the present study, we have identified ERα and MTA1 as two novel binding partners of LMO4 in a physiologically relevant context and that LMO4 functions as an integral part of the MTA1 corepressor complex. LMO4 effectively repressed ERα transactivation functions in an HDAC-dependent manner. Down-regulation of LMO4 expression resulted in a significant enhancement of ERα functions. Because LMO4 is overexpressed in 50% of breast tumors and not much is known regarding the mechanistic role played by it at the molecular level as an oncogenic protein,

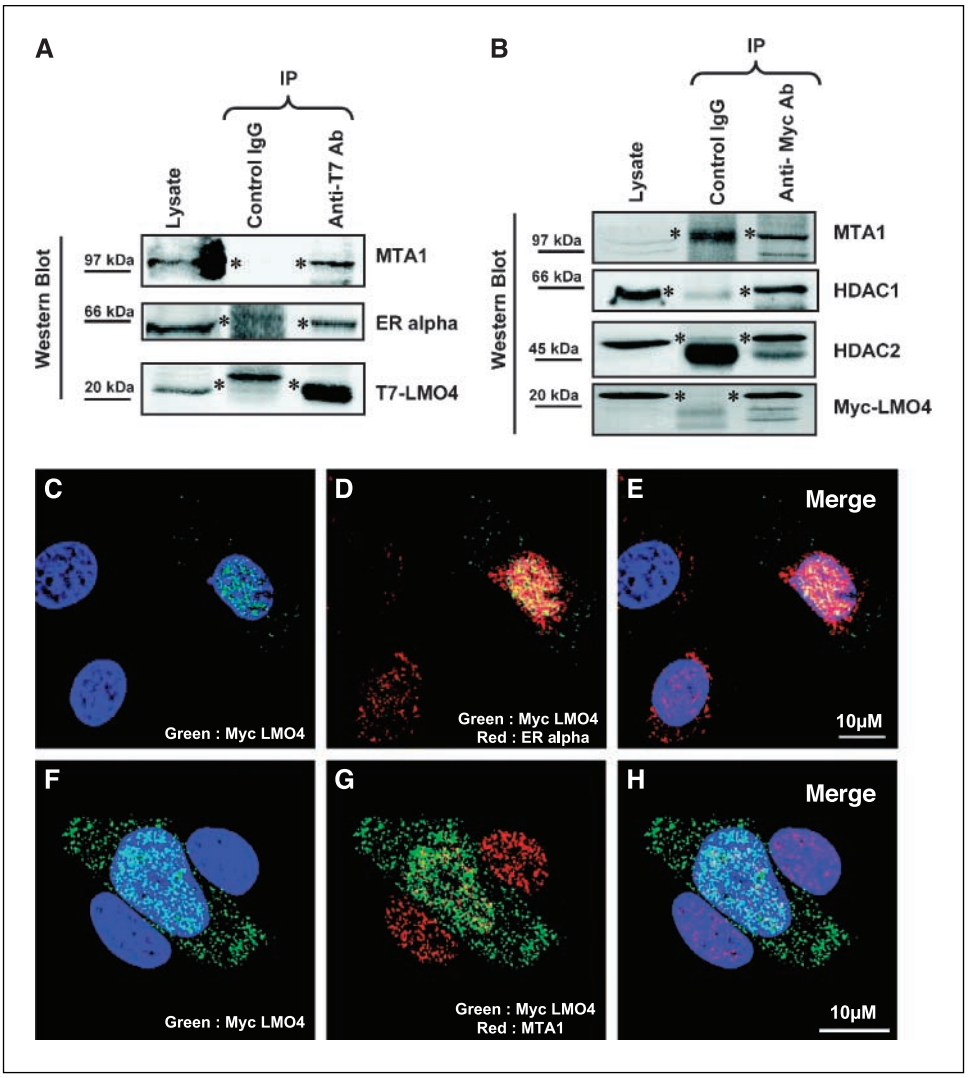

Figure 5. LMO4 forms a part of MTA1 corepressor complex *in vivo* and colocalizes with MTA1 and ERα in the nucleus. *A,* MCF-7 cells were transfected with expression constructs encoding T7-tagged LMO4. After 48 hours, cell lysate was prepared and the T7-tagged LMO4 was immunoprecipitated (*IP*) with T7-specific antibody. Immunoblotting was done with anti-T7 antibody (*Anti-T7 Ab*) and clear bands in Lysate and Anti-T7 Ab lanes (*third panel*) showed the expression of T7-LMO4 in the cells and the successful immunoprecipitation of it with anti-T7 antibody, respectively. Immunoblotting with ERα- and MTA1-specific antibodies recognized ERα (*second panel, Lysate* and *Anti-T7 Ab*) and MTA1 (*first panel, Lysate* and *Anti-T7 Ab*) among the proteins coimmunoprecipitated along with LMO4, exhibiting that LMO4 binds ER and MTA1 *in vivo*. Absence of these bands in the Control IgG lane (immunoprecipitation with control IgG) proved the specificity of immunoprecipitation and Western blot analysis. *B,* immunoprecipitation of Myc-tagged LMO4 expressed in MCF-7 cells and subsequent Western blotting showed that HDAC2 (*third panel*), HDAC1 (*second panel*), and MTA1 (*first panel*) were present among the coimmunoprecipitated proteins and proved that LMO4 was a part of MTA1 corepressor complex. *C,* MCF7 cells were transiently transfected with Myc-LMO4 and 48 hours later were processed as described in Materials and Methods for immunofluorescent localization of Myc-LMO4 (*green*), ERα (*red*), and DNA (*blue*); Cells were examined by confocal microscopy for localization of Myc-LMO4 (*green fluorescence*; in *C* and *F*) and colocalization of Myc-LMO4 with ERα and MTA1 (*yellow fluorescence*). Distinct yellow spots in the nucleus showed the colocalization of Myc-tagged LMO4 with endogenous ERα (*D* and *E*) and with endogenous MTA1 (*G* and *H*), proving them to be LMO4 binding partners *in vivo*.

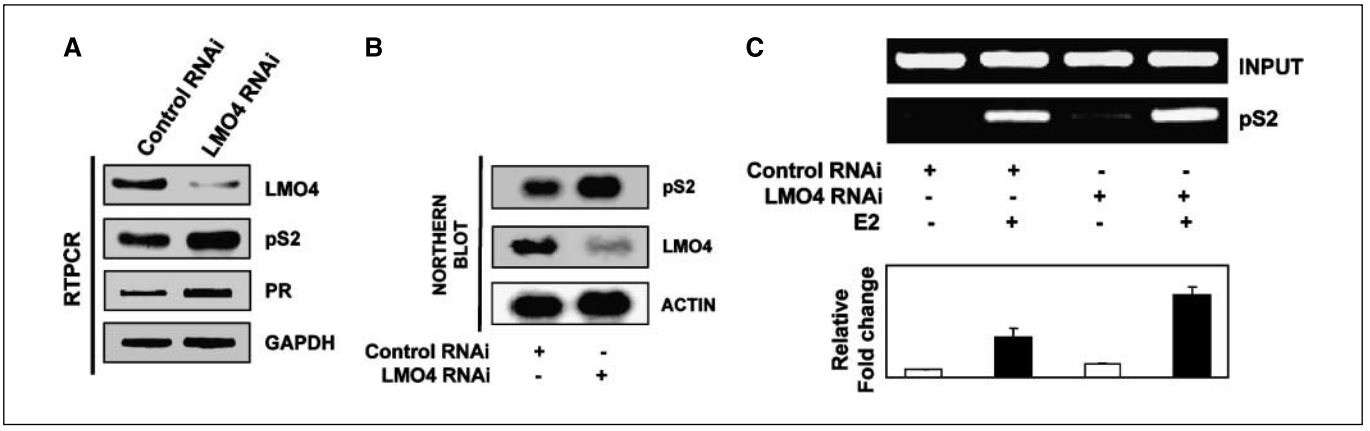

Figure 6. Knockdown of LMO4 expression leads to increased expression of ER target genes and enhanced recruitment of ER to the target chromatin. *A,* expression of estradiol-responsive genes *pS2* and *PR* were tested by RT-PCR analysis on silencing LMO4 expression in MCF-7 cells. A substantial increase of the expression of both *pS2* and *PR* was evident in cells with silenced LMO4 expression (*second* and *third panels, right lane*) compared with the control cells, indicating elevated levels of ERE transcription. A decreased level of LMO4 expression (*first panel, right lane*) shows the effective knockdown of LMO4 by the LMO4-RNAi. GAPDH control for both the RNA samples is shown below (*fourth panel*). *B,* LMO4 expression was silenced in MTA1-overexpressing stable clones and Northern blotting for pS2 mRNA clearly showed elevated levels of pS2 mRNA (*first panel*). Effective silencing of LMO4 expression is shown in the middle panel by probing for LMO4 mRNA and actin control for the RNA samples is shown in the bottom panels for comparison. *C,* MCF-7 cells were transfected with LMO4 RNAi, chromatin immunoprecipitation analysis was done with ERα-specific antibody, and the recruitment of ER to one of its target gene *pS2* was studied. Decreased LMO4 expression resulted in a 2-fold increased recruitment of ER to *pS2* gene chromatin on estrogen stimulation compared with cells with intact LMO4 expression. Quantification of the recruitment of ER to *pS2* chromatin is shown below for comparison. Quantification was done using the program ImageQuant version 5.1.

our present findings raise the possibility that LMO4 up-regulation might contribute to the process of breast cancer progression by repressing ERα transactivation functions and, consequently, allowing the development of ERα-negative phenotypes, leading to increased aggressiveness and invasiveness of breast cancer cells.

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