Role of metadherin in estrogen-regulated gene expression

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Abstract. The disruption of estrogen signaling is widely associated with the development of breast, endometrial and ovarian cancers. As a multifunctional mediator of carcinogenesis, metadherin (MTDH)/astrocyte elevated gene-1 (AEG-1) overexpression has been associated with numerous types of cancer, with reported roles in tumor initiation, proliferation, invasion, metastasis and chemoresistance. At the molecular level, MTDH has been shown to interact with proteins that drive tumorigenesis, including nuclear factor-κB (NF-κB), promyelocytic leukaemia zinc finger (PLZF), BRCA2- and CDKN1A (p21Cip1/Waf-1/mda-6)-interacting protein α (BCCIPα) and staphylococcal nuclease and tudor domain containing 1 (SND1). Through the analysis of the Cancer Genome Atlas (TCGA) datasets for estrogen receptor (ER)-positive endometrial and breast cancers, we found that over 25% of all gene expression correlated with MTDH. Using Affymetrix microarrays, we characterized the differences in gene expression between estrogen-treated parental and MTDH-deficient endometrial and breast cancer cells. We also explored a possible interaction between MTDH and ER by immunoprecipitation, and found that MTDH and ER associated in both breast and endometrial cancer cells in response to estrogen. Reciprocal co-immunoprecipitation analysis demonstrated that acute estrogen stimulation promoted the interaction of MTDH with ER in the nucleus. These data, to the best of our knowledge, provide the first evidence that MTDH and ERα interact in the nucleus with estrogen treatment to regulate gene expression.

Introduction

Metadherin (MTDH), also known as astrocyte elevated gene-1 (AEG-1) or LYRIC, is a protein encoded by the *MTDH* gene. MTDH is frequently overexpressed in cancers of the breast, prostate, ovary and endometrium, and is associated with poor clinical outcomes (1,2). MTDH influences several oncogenic signaling pathways and transcription factors, such as the Ras, Myc, PI3K/AKT, nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK) and Wnt pathways (3,4), suggesting that MTDH plays an important role in disease development and progression.

MTDH is highly basic and contains a transmembrane domain and multiple nuclear localization signals, with reported localizations in the cell membrane, cytoplasm, nucleus, nucleolus and endoplasmic reticulum. Not surprisingly, distinct interaction partners have been reported for the various locales, allowing MTDH to contribute to a diverse range of intracellular events (4). For example, MTDH has been shown to interact with several proteins to drive tumorigenesis, including NF-κB, promyelocytic leukaemia zinc finger (PLZF), BRCA2- and CDKN1A (p21Cip1/Waf-1/mda-6)-interacting protein α (BCCIPα) and staphylococcal nuclease and tudor domain containing 1 (SND1) (5-8). MTDH also interacts with the steroid hormone receptors RXRs (RXRα, β, γ) to negatively regulate RXR function and RXR-mediated transcriptional regulation (9). Recently, some reports have suggested that MTDH overexpression inversely correlates with the estrogen receptor (ER) status, and MTDH may induce resistance to tamoxifen in ERα-positive breast cancer cells (10-12).

In this study, we examined the potential association between MTDH and ER. Estrogens control multiple cellular functions via estrogen receptors (ER-α and ER-β), modular proteins that belong to the steroid/nuclear receptor family of intracellular homeostatic regulators. Ligand binding in the cytosol results in conformation changes that promote receptor translocation into the nucleus, thereby promoting gene expression or repression through interactions with chromatin and transcriptional co-regulatory complexes. In this study, we found that the expression of a number of genes correlated with MTDH expression in ER positive cancers from the Cancer Genome Atlas (TCGA) dataset, and we also found a transient interaction of MTDH with ERα following estrogen treatment.

Materials and methods

The Cancer Genome Atlas (TCGA) data analysis. Gene expression data from the RNA sequencing (RNA-seq) experiments were downloaded from the TCGA (https://portal.gdc.cancer.gov/) data portal for breast and endometrial cancer. For

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RNA-seq only level 3 data was available. Data at level 3 are already preprocessed, normalized and log2-transformed (for more details see https://cancergenome.nih.gov/abouttcga/about-data/datalevelstypes). In addition, metadata and clinical data were extracted and downloaded to identify clinical parameters, such as endometrial histological types (serous vs. endometrioid), and molecular characteristics, such as ER-positive vs. ER-negative breast cancer. Table I shows the patients included in the analysis.

To abstract unique gene expression data we used the Biometric Research Branch (BRB) ArrayTools (version 2.13.2 for X64 systems) software suite. BRB-ArrayTools was developed by Dr Richard Simon and colleagues, and is an integrated package for visualization and statistical analysis utilizing Excel (Microsoft, Redmond, WA, USA) as the front end, with tools developed in the R statistical system. MTDH expression was correlated with the expression of all other genes in breast and endometrial RNA-seq experiments using rank-based Spearman correlation to allow for non-linear relationships between several gene expression values. To control the false discovery rate (FDR) we used the q-value for statistical significance (qFDR) and Bonferroni correction for multiple comparisons. A significant correlation was considered a correlation with a p-value $<10^{-6}$ after controlling for multiple comparisons.

Cell culture and generation of MTDH-deficient cells. The human endometrial cancer cell line, ECC1 (American Type Culture Collection; ATCC, Manassas, VA, USA), was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, MCF-7 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% FBS and 1% penicillin/streptomycin (all from Gibco-BRL, Carlsbad, CA, USA); all cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. Genomic deletions of MTDH were created in the ECC1 cells using 3 pairs of chimeric single guide RNAs (sgRNAs) as previously described (13). Briefly, sgRNA oligos were synthesized and cloned into the lentiCRISPR transfer plasmid for virus production. Viral vectors were produced in 293T cells (ATCC), followed by infection of ECC1 cells as described. MTDH-deficient ECC1 cells were selected with 5 μg/ml puromycin after 48 h of transduction. MTDH-deficient ECC1 cells were grown at low density for 2 weeks to select clones. MTDH expression was determined by western blot analysis to identify clones with no or low MTDH protein expression.

Microarray assay and data analysis. ECC1 and MTDH-deficient ECC1 cells were incubated in RPMI-1640 supplemented with 5% charcoal stripped FBS for 24 h then treated with 10 nM estrogen (Sigma-Aldrich, St. Louis, MO, USA) or treated with ethanol as a control for 24 h. Total RNA was isolated using the Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA). RNA sample preparation for hybridization and the subsequent hybridization to the Illumina beadchips were performed at the University of Iowa IIHG Genomics Division using the manufacturer's recommended protocols. Briefly, 100 ng total RNA were converted to amplified biotin-aRNA using the Epicentre TargetAmp-Nano Labeling kit for Illumina Expression BeadChip (Illumina, Inc., San Diego, CA, USA). The amplified

Biotin-aRNA product was purified through a Qiagen RNeasy MinElute Cleanup column (Qiagen) according to modifications from Epicentre (Madison, WI, USA). Subsequently, 750 ng of this product were mixed with Illumina hybridization buffer, placed onto Illumina-Human H12 v4 BeadChips (part no. BD-103-0204), and incubated at 58°C for 17 h, with rocking, in an Illumina hybridization oven. Following hybridization, the arrays were washed, blocked and then stained with streptavidin-Cy3 (Amersham/GE Healthcare, Piscataway, NJ, USA) according to the Illumina Whole-Genome Gene Expression Direct Hybridization Assay protocol. Beadchips were scanned with the Illumina iScan system (ID #N0534) and data were collected using the GenomeStudio software v2011.1. Microarray data have been deposited at GEO (www.ncbi.nlm. nih.gov/geo) under the accession no. GSE78182. The microarray data were analyzed for data summarization, normalization and quality control by using BRB ArrayTools (version 2.13.2 for X64 systems) software suite. To select the differentially expressed genes, we used threshold values of ≥2 and ≤-2-fold-change and a Benjamini-Hochberg corrected p-value of 0.001. The data were log2-transformed and median centred by genes using the Adjust Data function of Cluster 3.0 software, and then further analyzed with hierarchical clustering with average linkage. The microarray experiment was performed using biological triplicates.

Immunoprecipitation (IP) and western blot analysis. The ECC1 cells were incubated in RPMI-1640 and the MCF-7 cells were incubated in DMEM supplemented with 5% charcoal stripped FBS for 24 h then treated with 10 nM estrogen for 0, 1, 2 and 4 h. For IP, the cells were lysed in 400 μl of NP-40 buffer and 5% of the lysates were saved as input. BCA protein assay was used to determine the protein concentrations of each lysate. A total of 1 mg of the protein lysates were incubated with 1 μg primary antibodies followed by incubation with protein A/G agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), which were then washed 4 times with NP-40 buffer. Immuno-complexes were boiled in 2X Laemmli sample buffer for 5 min before they were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes and immunoblotted with MTDH antibody (cat. no. 40-6500; Invitrogen) or ERα antibody (cat. no. sc-543; Santa Cruz Biotechnology, Inc.). The procedures for cytoplasmic and nuclear extraction were carried out according the manufacture'rs instructions (Active Motif, Carlsbad, CA, USA), and the fractions were then immunoprecipitated with ERα antibody, followed by immunoblotting with ERα or MTDH antibodies.

Immunostaining. Breast cancer and endometrial cancer specimens obtained from the Tissue Procurement Core were subjected to immunohistochemical staining for either ER or MTDH as previously described (13).

RT-qPCR. Four significant genes from microarray data were randomly selected and validated by RT-qPCR analysis in the ECC1 cells. Total RNA was isolated using the Qiagen RNeasy mini kit (Qiagen), and reverse transcription with SuperScript® III First-Strand Synthesis system (Invitrogen). SYBR-Green-based real-time PCR (SYBR-Green I; ABI, Warrington, UK) was used to quantitatively detect the expres-

Table I. Number of genes correlated with MTDH expression ($p<10^{-6}$).

	Endometrial cancer (18,023 genes)		Breast cancer (17,660 genes)	
	ER-Pos (endometrioid) (N=142)	ER-Neg (serous) (N=38)	ER-Pos (N=705)	ER-Neg (N=207)
Significant r^2 with MTDH	4479	88	4,451	332
% Correlation	24.9%	0.5%	25.2%	1.9%

MTDH, metadherin; ER, estrogen receptor; Pos, positive; Neg, negative.

sion of Phosphatidic Acid Phosphatase Type 2B (PPAP2B), Plexin Domain Containing 2 (PLXDC2), Transmembrane And Tetratricopeptide Repeat Containing 1 (TMTC1), Protease, Serine 23 (PRSS23) and 18S RNA. The fold variation in the RNA samples was calculated using the $2^{-\Delta\Delta ct}$ method following normalization with 18S. The primer sequences were as follows: PPAP2B forward, 5'-TGA GAG CAT CAA GTA CCC ACT-3' and reverse, 5'-ACG TAG GGG TTC TGA ATC GTC-3'; PLXDC2 forward, 5'-TTC TCA AGG CGG TAG ACA CGA-3' and reverse, 5'-CGA TCT GAG TGT TAT TGT CCT GC-3'; TMTC1 forward, 5'-ACG GTG TCT CCC TTC TTC TTG-3' and reverse, 5'-ATT GCT CGA CTT GTC TTG CTT-3'; PRSS23 forward, 5'-CAG TGT CAT AAG GGA ACT CCA C-3' and reverse, 5'-CCT GAG TCT CGG TGT TGG G-3'; 18S forward, 5'-AAC TTT CGA TGG TAG TCG CCG-3' and reverse, 5'-CCT TGG ATG TGG TAG CCG TTT-3'.

Online tools. Gene ontology (GO) analyses were based on the online tool PANTHER (http://pantherdb.org/).

Results

The expression of a number of genes correlates with MTDH expression in ER-positive endometrial cancer and breast cancer. Using the Cancer Genome Atlas (TCGA) dataset, with a cut-off set at p-value <10^{-6} between endometrial cancer and serous endometrial cancer, or between ER-positive and ER-negative breast cancer, we found that the expression of 24.9% of the genes correlated with MTDH expression in endometrial cancer, and the expression of 25.2% of the genes correlated with MTDH expression in ER-positive breast cancer (Table I). By contrast, only approximately 0.5 or 1.9% of the genes correlated with MTDH expression in serous endometrial cancer and ER-negative breast cancer, respectively. These results indicate that MTDH may be associated with ER or estrogen-regulated gene expression.

Microarray analysis of estrogen-treated ECC1 cells and MTDH-deficient ECC1 cells. To further examine the association between MTDH and ER, genomic deletions of MTDH were created in ECC1 cells (MTDH-deficient ECC1 cells). The expression levels of MTDH and ER were detected by western blot analysis in these cells (Fig. 1A), and the differences in gene expression between estrogen-treated parental ECC1 cells and MTDH-deficient ECC1 cells were then investigated using microarray assay. By using threshold values of p<0.001 and

fold-change ≥2 or ≤-2, the ECC1 cells had 46 and the MTDH-deficient ECC1 cells had 42 significantly altered genes induced by estrogen treatment (Fig. 1B). Of note, 27 genes were induced by estrogen in both parental the ECC1 and MTDH-deficient ECC1 cells (Table II). These genes are estrogen induced and not related with MTDH status, and thus these genes may be associated with the function of estrogen receptors independent of MTDH. There were 19 estrogen-regulated genes that were dependent on MTDH expression (Table III). Molecular network analysis did not find that these differentially expressed genes had direct interactions with MTDH (data not shown). We also found 15 genes significantly regulated by estrogen treatment in the MTDH-deficient ECC1 cells (Table IV).

We randomly selected 2 upregulated and 2 downregulated genes and validated the expression changes in the ECC1 cells by RT-qPCR. The fold-change of these genes was similar with the microarray data (Fig. 1C). The differential gene expression induced by estrogen in the ECC1 and MTDH-deficient ECC1 cells was represented graphically in a heatmap (Fig. 1D and F).

Inputting 46 differentially expressed genes induced by estrogen in the ECC1 cells into the online tool PANTHER (http://pantherdb.org/) identified 47 terms belonging to molecular functions (Fig. 1E) and 90 terms belonging to biological processes (data not shown). Inputting 42 differentially expressed genes induced by estrogen in the MTDH-deficient ECC1 cells into the online tool PANTHER identified 46 terms belonging to molecular functions (Fig. 1G) and 97 terms belonging to biological processes (data not shown). The most representative molecular functions were binding and catalytic. On the other hand, the most significant biological processes involving the ER interacting proteins identified are represented by metabolic processes and cellular process (data not shown).

ER and MTDH interact in the nucleus. In order to define the involvement of MTDH in ER or estrogen-regulated gene expression, the ECC1 endometrial cancer cells were treated with 10 nM estrogen for 0, 1, 2 and 4 h, and the interaction of MTDH and ERα was queried using IP assays. The IP results revealed that MTDH and ERα can 'pull down' each other following estrogen treatment for 2 h (Fig. 2A). We acquired similar results in the MCF-7 cells (Fig. 2B).

We then wished to determine where MTDH and ERα interact in the cell. Cytoplasmic and nuclear fractions of ECC1 cells treated with 10 nM estrogen were immunoprecipitated with anti-ERα antibody, followed by immunoblotting with ERα or MTDH antibody. The results indicated that the interaction of
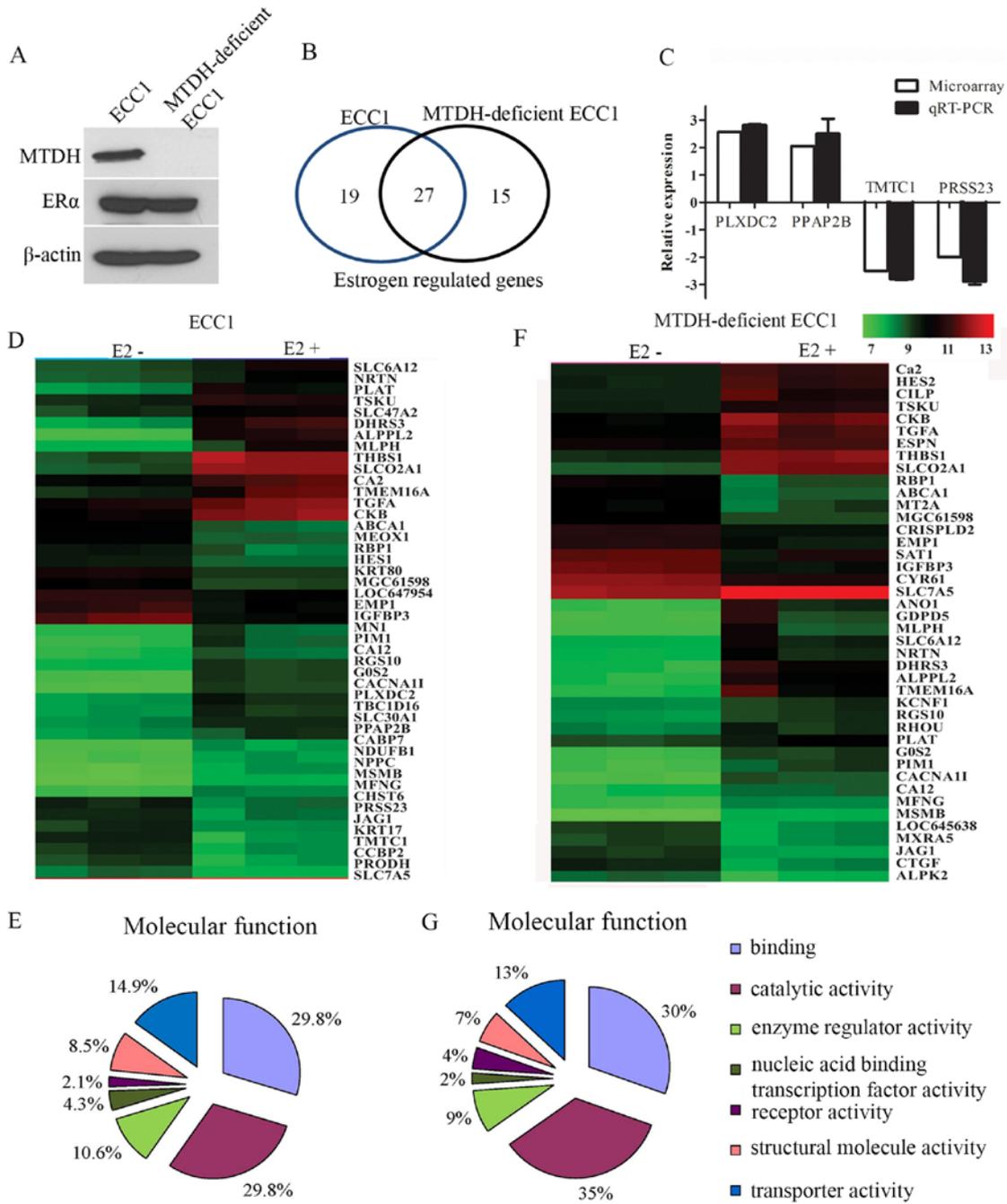
Figure 1. Microarray data analysis of estrogen-treated control and metadherin (MTDH)-deficient ECC1 cells. (A) The expression of MTDH and estrogen receptor α (ERα) in ECC1 cells and MTDH-deficient ECC1 cells. (B) Venn diagram of differentially expressed genes induced by estrogen between ECC1 and MTDH-deficient ECC1 cells. (C) Two upregulated genes and two downregulated genes were amplified from estrogen-treated ECC1 cells and control ECC1 cells, and the fold-change compared with microarray data. (D and F) Heatmap of estrogen induced differently expressed genes in ECC1 cells (D) or in MTDH-deficient ECC1 cells (F). (E and G) GO analysis of estrogen-induced differently expressed genes in ECC1 cells (E) or MTDH-deficient ECC1 cells associated with molecular functions (G).

MTDH and ERα occurs in the nucleus (Fig. 2C). Furthermore, we detected the co-localization of MTDH and ER by immunostaining in a subset of cells in ER-positive breast cancer and endometrial cancer tissues (Fig. 3).

Discussion

Cancer progression is a multifactorial process that includes alterations in proliferation, cell death, apoptosis, genetic instability, and changes in the expression and activity of tumor suppressor genes, oncogenes, growth factors and cell adhesion molecules (14-16). The overexpression of MTDH is frequently observed in most human cancers, and several studies have suggested that MTDH may play a pivotal role in the pathogenesis, invasion, metastasis, angiogenesis, progression and regulation of apoptosis. Estrogen receptors are overexpressed in approximately 70% of breast and endometrium tumors. Similar to MTDH, estrogen signaling through ER promotes cell proliferation and survival and inhibits apoptosis, thereby promoting tumorigenesis. In this study, we found that the

Table II. MTDH-independent gene expression regulated by estrogen in ECC1 cells (MTDHWT) and MTDH-deficient ECC1 (MTDH KO) cells (p<0.001 and fold-change ≥2 or ≤-2).

UniqueID	Name	Map Location	Fold-change (treat/no-treat)	
			MTDH WT	MTDH KO
ALPPL2	Alkaline phosphatase, placental-like 2 (ALPPL2)	2q37.1c	12.06	7.50
THBS1	Thrombospondin 1 (THBS1)	15q14d	9.50	6.45
SLCO2A1	Solute carrier organic anion transporter family, member 2A1 (SLCO2A1)	3q22.1e	9.45	8.33
DHRS3	Dehydrogenase/reductase (SDR family) member 3 (DHRS3)	1p36.22a-p36.21d	7.92	9.38
MLPH	Melanophilin (MLPH), transcript variant 2	2q37.3b	5.19	4.60
CACNA1I	Calcium channel, voltage-dependent, T type, alpha 1I subunit (CACNA1I), transcript variant 1	22q13.1d	4.18	3.81
G0S2	G0/G1 switch 2 (G0S2)	1q32.2b	4.04	3.81
PLAT	Plasminogen activator, tissue (PLAT), transcript variant 1	8p11.21a	3.66	2.02
TMEM16A	Transmembrane protein 16A (TMEM16A)	11q13.3b-q13.3c	3.60	8.52
CKB	Creatine kinase, brain (CKB)	14q32.33a	3.41	3.68
PIM1	Pim-1 oncogene (PIM1)	6p21.2c	3.01	3.59
CA2	Carbonic anhydrase II (CA2)	8q21.2b	2.82	3.29
RGS10	Regulator of G-protein signaling 10 (RGS10), transcript variant 1	10q26.11d	2.80	2.16
TSKU	Tsukushin (TSKU), mRNA.	11q13.5c	2.64	2.42
TGFA	Transforming growth factor alpha (TGFA), transcript variant 2	2p13.3c	2.52	2.86
SLC6A12	Solute carrier family 6 (neurotransmitter transporter, betaine/GABA), member 12 (SLC6A12)	12p13.33d	2.41	4.11
CA12	Carbonic anhydrase XII (CA12), transcript variant 1	15q22.2b	2.30	2.28
MFNG	MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase (MFNG)	22q13.1a	2.15	2.10
SLC7A5	Solute carrier family 7 (cationic amino acid transporter, y⁺ system), member 5 (SLC7A5)	16q24.2b	2.07	2.22
MSMB	Microseminoprotein beta (MSMB), transcript variant PSP57	10q11.23b	2.03	2.08
NRTN	Neurturin (NRTN)	19p13.3b	2.03	3.89
JAG1	Jagged 1 (Alagille syndrome) (JAG1)	20p12.2a	-2.18	-2.41
RBP1	Retinol binding protein 1, cellular (RBP1)	3q23a	-2.19	-2.98
MGC61598	PREDICTED: *Homo sapiens* similar to ankyrin-repeat protein Nrarp (MGC61598)	9q34.3f	-2.22	-2.16
EMP1	Epithelial membrane protein 1 (EMP1)	12p13.1b	-2.23	-2.29
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1 (ABCA1)	9q31.1d	-2.83	-2.81
IGFBP3	Insulin-like growth factor binding protein 3 (IGFBP3), transcript variant 2	7p13b	-3.32	-3.82

MTDH, metadherin; treat/no treat, treatment or not with estrogen.

expression of many genes correlated with the expression of MTDH in ER-positive cancers using TCGA data, and we defined an interaction between MTDH and ER by co-IP. Our data suggest that the interaction is dependent on estrogen and is a short-term process.

ERs are involved in pathological processes, including breast cancer, endometrial cancer, osteoporosis, hypertension and coronary artery diseases (17-19). One of the main risk factors for endometrial cancer is high levels of estrogen (20). In this study, we used the human endometrial cancer cell line, ECC1, and MTDH-deficient ECC1 cells, treated with or without estrogen, and assessed the gene expression profiles by microarray assay. Our results demonstrated that most estrogen-induced genes were common in the ECC1 cells and MTDH-deficient ECC1 cells, indicating that approximately 60% of the genes induced by estrogen were unrelated with the MTDH status, and that MTDH may only participate in parts of ER function.

Previous studies have indicated that MTDH interacts with and activates the transcription factor, NF-κB, to regulate gene expression (3,8,21). In this study, we found that MTDH transiently interacted with ER in the nucleus of cancer cells, the interaction is dependent on estrogen and it is a short-term

Table III. List of differently expressed MTDH-dependent genes regulated by estrogen in ECC1 cells (p<0.001 and fold-change ≥2 or ≤-2).

UniqueID	Fold-change (treat/no-treat)	Name	MapLocation
PLXDC2	2.57	Plexin domain containing 2 (PLXDC2)	10p12.32a-p12.31c
MN1	2.54	Meningioma (disrupted in balanced translocation) 1 (MN1)	22q12.1b
NPPC	2.36	Natriuretic peptide precursor C (NPPC)	2q37.1b
TBC1D16	2.27	TBC1 domain family, member 16 (TBC1D16), mRNA.	17q25.3d
SLC30A1	2.21	Solute carrier family 30 (zinc transporter), member 1 (SLC30A1)	1q32.3a
SLC47A2	2.17	Solute carrier family 47, member 2 (SLC47A2), transcript variant 1	17p11.2d
CABP7	2.06	Calcium binding protein 7 (CABP7)	22q12.2a
PPAP2B	2.05	Phosphatidic acid phosphatase type 2B (PPAP2B), transcript variant 2	1p32.2c
NDUFB1	2.03	NADH dehydrogenase (ubiquinone) 1β subcomplex, 1, 7 kDa (NDUFB1)	14q32.12b
LOC647954	-2.00	PREDICTED: *Homo sapiens* misc_RNA (LOC647954)	5q23.2b
PRODH	-2.00	Proline dehydrogenase (oxidase) 1 (PRODH)	22q11.21b
CHST6	-2.00	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6 (CHST6)	16q23.1a
HES1	-2.01	Hairy and enhancer of split 1, (*Drosophila*) (HES1)	3q29c
PRSS23	-2.01	Protease, serine, 23 (PRSS23)	11q14.2a
MEOX1	-2.34	Mesenchyme homeobox 1 (MEOX1), transcript variant 3	17q21.31b
CCBP2	-2.44	Chemokine binding protein 2 (CCBP2)	3p22.1a
TMTC1	-2.49	Transmembrane and tetratricopeptide repeat containing 1 (TMTC1)	12p11.22a
KRT80	-2.58	Keratin 80 (KRT80), transcript variant 1	12q13.13d
KRT17	-3.02	Keratin 17 (KRT17)	17q21.2b

MTDH, metadherin; treat/no treat, treatment or not with estrogen.

Table IV. MTDH negatively regulated gene expression regulated by estrogen in MTDH-deficient ECC1 cells (p<0.001 and fold-change ≥2 or ≤-2).

UniqueID	Fold-change (treat/no-treat)	Name	MapLocation
ANO1	6.46	Anoctamin 1, calcium activated chloride channel (ANO1), transcript variant 1	11q13.3b-q13.3c
GDPD5	5.85	Glycerophosphodiester phosphodiesterase domain containing 5 (GDPD5)	11q13.4c-q13.5a
HES2	3.24	Hairy and enhancer of split 2 (*Drosophila*) (HES2)	1p36.31a
CILP	2.90	Cartilage intermediate layer protein, nucleotide pyrophosphohydrolase (CILP)	15q22.31b
RHOU	2.07	Ras homolog gene family, member U (RHOU)	1q42.13c
KCNF1	2.02	Potassium voltage-gated channel, subfamily F, member 1 (KCNF1)	2p25.1c
ESPN	2.01	Espin (ESPN)	1p36.31a
MXRA5	-2.00	Matrix-remodelling associated 5 (MXRA5)	Xp22.33b
CRISPLD2	-2.07	Cysteine-rich secretory protein LCCL domain containing 2 (CRISPLD2)	16q24.1a
LOC645638	-2.08	PREDICTED: *Homo sapiens* misc_RNA (LOC645638), miscRNA	17q23.1a
MT2A	-2.21	Metallothionein 2A (MT2A)	16q13b
ALPK2	-2.22	Alpha-kinase 2 (ALPK2)	18q21.31b-q21.32a
SAT1	-2.44	Spermidine/spermine N1-acetyltransferase 1 (SAT1)	Xp22.11a
CTGF	-2.45	Connective tissue growth factor (CTGF)	6q23.2b
CYR61	-2.50	Cysteine-rich, angiogenic inducer, 61 (CYR61)	1p22.3e

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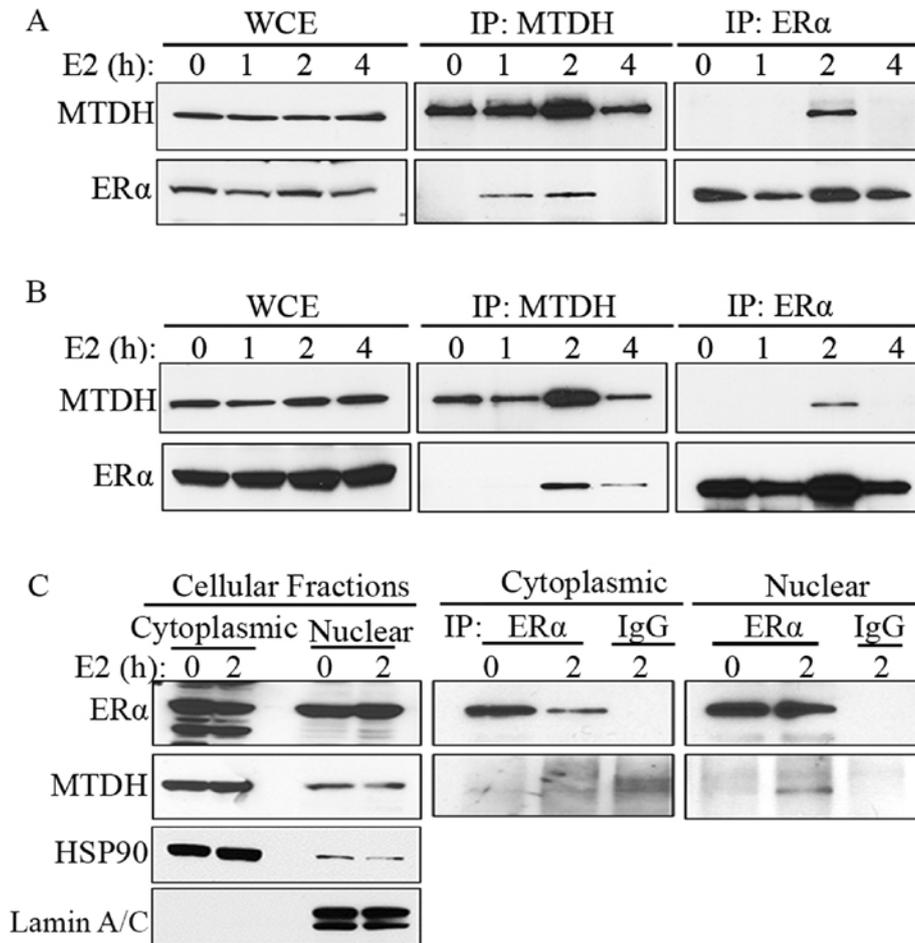
Figure 2. The interaction of estrogen receptor (ER) and metadherin (MTDH) in endometrial cancer and breast cancer cell lines. (A) Reciprocal co-IP analysis of the interaction between MTDH and ER in ECC1 cells. Cell lysates of ECC1 cells treated with 10 nM estrogen were immunoprecipitated with anti-MTDH or anti-ERα antibody. (B) Reciprocal co-IP analysis of the interaction between MTDH and ER in MCF-7 cells. Cell lysates of MCF-7 cells treated with 10 nM estrogen were immunoprecipitated with anti-MTDH or anti-ER antibody. (C) Reciprocal co-IP analysis of the fractions in ECC1 cells. Cytoplasmic and nuclear fractions of ECC1 cells treated with 10 nM estrogen were immunoprecipitated with anti-ERα antibody.
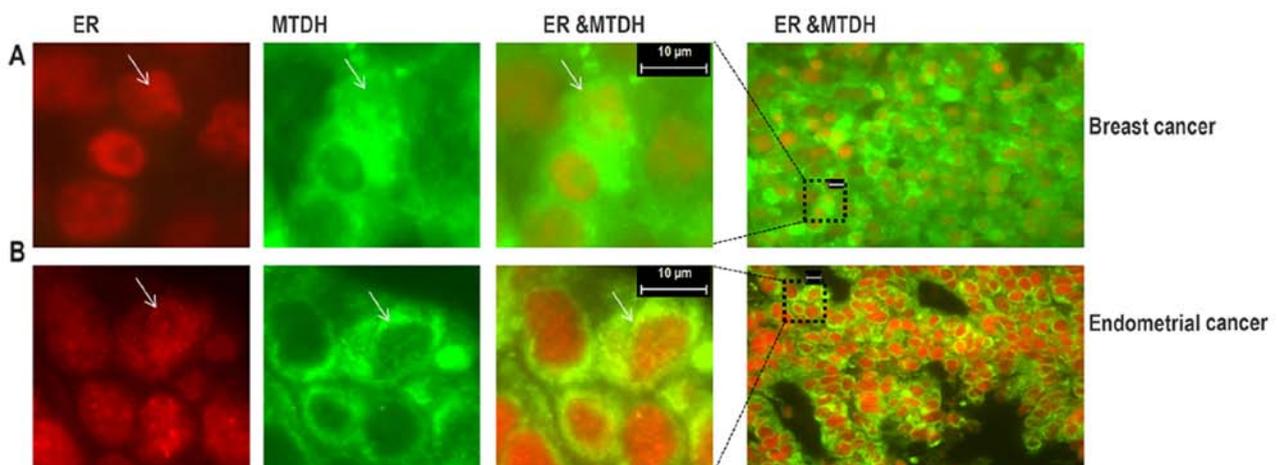
Figure 3. Co-localization of estrogen receptor (ER) and metadherin (MTDH) in breast cancer (A) and endometrial cancer (B) tissues. Co-localization was analyzed by dual immunostaining with anti-MTDH and anti-ER antibodies. Cells with co-localized MTDH and ER in the nucleus are indicated by arrows.

process. Based on this time course, the biological function of this transient interaction may relate to the transcriptional activity of ERα. Indeed, the long-standing dogma in the hormone receptor field is that ligand binding and receptor-mediated transcriptional activation are highly transient events. Microarray data indicated that MTDH had some effect on estrogen-regulated genes, and they jointly control the expression of a subset of ER-regulated genes. MTDH thus may

play an important role early in ER-mediated transcriptional regulation. Further studies on understanding the interaction of MTDH with ER and the related genes may provide new insight into the function of MTDH and ER in tumors.

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