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Xenoestrogen-Induced Epigenetic Repression of *microRNA-9-3* in Breast Epithelial Cells

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Abstract

Early exposure to xenoestrogens may predispose to breast cancer risk later in adult life. It is likely that long-lived, self-regenerating epithelial progenitor cells are more susceptible to these exposure injuries over time and transmit the injured memory through epigenetic mechanisms to their differentiated progeny. Here, we used progenitor-containing mammospheres as an *in vitro* exposure model to study this epigenetic effect. Expression profiling identified that, relative to control cells, 9.1% of microRNAs (82 of 898 loci) were altered in epithelial progeny derived from mammospheres exposed to a synthetic estrogen, diethylstilbestrol. Repressive chromatin marks, trimethyl Lys27 of histone H3 (H3K27me3) and dimethyl Lys9 of histone H3 (H3K9me2), were found at a down-regulated locus, *miR-9-3*, in epithelial cells preexposed to diethylstilbestrol. This was accompanied by recruitment of DNA methyltransferase 1 that caused an aberrant increase in DNA methylation of its promoter CpG island in mammosphere-derived epithelial cells on diethylstilbestrol preexposure. Functional analyses suggest that *miR-9-3* plays a role in the p53-related apoptotic pathway. Epigenetic silencing of this gene, therefore, reduces this cellular function and promotes the proliferation of breast cancer cells. Promoter hypermethylation of this microRNA may be a hallmark for early breast cancer development, and restoration of its expression by epigenetic and microRNA-based therapies is another viable option for future treatment of this disease. [Cancer Res 2009;69(14):5936–45]

Introduction

Prolonged exposure to xenoestrogens, or environmental estrogens, may be linked to breast cancer risk (1, 2). These environmental chemicals can mimic the actions of estrogens that negatively influence the differentiation of breast epithelial cells and promote the development of breast neoplasms (1–4). The growth of ~60% of breast tumors depends on estrogen stimulation. This action is mediated via nuclear hormone receptors (genomic functions) that activate the transcription of target genes for cellular proliferation (4–6). Alternatively, estrogens stimulate the phosphorylation of membrane-bound proteins that subsequently activate signaling of mitogen-activated kinase proteins (nongenomic functions) for regulating downstream gene transcription (4).

As of today, environmental screening has identified many xenoestrogens that potentially support abnormal development of breast epithelial cells (1, 2). Among these chemicals are plasticizers (e.g., bisphenol A), disinfectants (e.g., *o*-nonylphenol), pesticides (e.g., permethrin and heptachlor), flame retardants (e.g., polybrominated diphenyl ether), and heavy metals (e.g., cadmium and arsenite; refs. 7–9). Exposure to these chemicals is probably a major causal factor in the increased incidence of breast cancer. For example, diethylstilbestrol, a synthetic estrogen, was prescribed to prevent miscarriages between the 1940s and the 1960s (10). Whereas diethylstilbestrol was subsequently shown to have no efficacy in preventing early delivery, these mothers had a 20% to 25% increased risk of breast cancer (11). Moreover, daughters prenatally exposed to diethylstilbestrol were found to have a 2.5-fold risk of developing breast cancer after age 40 years (12).

Animal and epidemiologic studies suggest an imprinting phenomenon in which early developmental exposure to xenoestrogens potentiates a carcinogenic process later in adult life (1, 3, 13, 14). Increasing evidence indicates that epigenetic mechanisms play an important role in transmitting this molecular information from parental cells to the progeny (3, 15). This process involves remodeling of DNA structure without altering the nucleotide sequence itself (16). Frequent epigenetic changes are DNA methylation and post-translational modifications of histone tails observed in the promoter CpG islands of silent genes in many types of cancer (17).

Although studies of xenoestrogen-induced epigenetic modifications primarily rely on animal models (13, 18), the challenge encountered is the lack of an *in vitro* system that can be used to validate these exposure effects on human cells. We have recently established a breast progenitor model for epigenetic studies of xenoestrogen exposure (19). These progenitors are slow-dividing, immature cells that are capable of self-renewal and, in response to signaling, differentiate into different types of breast epithelial cells (20). Because of their long lifespan, breast progenitors are more susceptible to environmental injuries and may transmit the memory of these injuries through epigenetic mechanisms to differentiating progeny. In our previous study, DNA methylation profiling of epithelial progeny derived from progenitors exposed to 17β-estradiol detected hypermethylated loci in 0.5% of protein-coding genes (19). Here, we extended this exposure study to noncoding microRNA genes and found that epigenetic alteration of microRNAs was also induced in epithelial cells having a prior exposure to diethylstilbestrol and other xenoestrogens. Promoter hypermethylation of one candidate gene, *miR-9-3*, was found in

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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breast cancer cell lines and primary tumors. Functional analysis suggested that the p53-ARF apoptotic pathway is partially attenuated by this epigenetic silencing in breast cancer cells. This *in vitro* model, therefore, provides a new paradigm to test potential xenoestrogens in epithelial cells and is useful to uncover novel tumor suppressors important for regulating early breast cancer development.

Materials and Methods

Tissue samples and cell culture. Breast samples were collected by the tissue procurement service in accordance with the protocols approved by the Institutional Review Board of the Ohio State University. For isolation of breast progenitors, normal tissue sections were obtained from individuals undergoing reduction mammoplasties mainly due to macromastia. These tissues were dissociated mechanically and enzymatically, and single cells were isolated and grown into mammospheres (2,000-10,000 per mammosphere) in ultralow attachment plates (Corning) in serum-free mammary epithelial growth medium (Cambrex) as described (21). These mammospheres could be repopulated in the suspension culture up to six cell passages (21). Mammospheres were then exposed to 70 nmol/L diethylstilbestrol (Sigma) or DMSO (control) in phenol red-free medium for 3 weeks (medium changed twice weekly). After the exposure, mammosphere-containing progenitors were placed on a collagen substratum (BD Biosciences) in phenol red-free medium for 2 to 3 weeks. Under this culture condition, progenitors were differentiated into breast epithelial cells as described previously (19). Breast cancer cell lines MCF-7, MDA-MB-134, MDA-MB-231, MDA-MB-435S, T47D, and SKBR3 and the immortalized epithelial MCF-10A cell line were obtained from the American Type Culture Collection for *in vitro* studies.

Immunofluorescence staining. Approximately 2,000 mammosphere-derived epithelial cells (MDEC)-seeded collagen I-coated coverslips were fixed with methanol/acetone for 10 min. After blocking with 2.5% bovine serum albumin (Sigma) for 1 h, they were incubated with anti-estrogen receptor (ER)-α antibody (Santa Cruz Biotechnology) overnight at 4°C. The corresponding secondary FITC-conjugated antibody was applied followed by 4′,6-diamidino-2-phenylindole staining (Molecular Probes) for the nuclei. Photographs were captured by Zeiss fluorescence microscopy (Zeiss). The percentages of ER-α subcellular localization were calculated in 10 different optical fields (~10 cells per field) by two independent researchers.

microRNA microarray analysis. Total RNA of three independent MDEC samples was isolated with Trizol (Invitrogen) according to the manufacturer's instructions. RNA (5 μg/sample) was biotin-labeled during reverse transcription using random hexamers. Hybridization was carried out with a microRNA microarray panel (Ohio State University Comprehensive Cancer Center, version 2.0), which contains probes for 898 microRNAs of *Homo sapiens* (including both precursor and mature forms) spotted in replicate with annotated active sites. Hybridization signals were detected with the streptavidin-Alexa 647 conjugate. Processed slides were scanned using a Perkin-Elmer ScanArray XL5K scanner with the laser set to 635 nm, at fixed PMT setting, and scan resolution of 10 nm.

Microarray images were analyzed by using GENEPIX PRO. Average values of the replicates spots of each microRNA were normalized and analyzed in BRB-Array Tools version 3.2.3. After excluding negative values with hybridization intensity below background, normalization was done by using the median normalization method and normalized to median array as reference. We identified genes that were differentially expressed among groups using Student's t test or F tests, and genes are considered statistically significant if their parametric $P < 0.05$. To increase stringency, we considered only microRNA probes that had at least 1.3-fold differences in intensities out of the replicates in the array.

Quantitative reverse transcription-PCR. Total RNA (2 μg) was reverse transcribed to cDNA with oligo(dT) (SuperScript III; Invitrogen) for coding genes and microRNA-specific primers (ThermoScript; Invitrogen) for microRNAs. Quantitative reverse transcription-PCR (qRT-PCR) was done by using SYBR Green dye chemistry (Applied Biosystems) on a 7500 Real-time PCR System apparatus (Applied Biosystems). Coding gene expression was measured by the ΔΔCt method using *β-actin* as the internal control. microRNA expression was calculated by absolute quantitation of standard curves of cloned PCR products. The curves were amplified from universal human reference RNA (Stratagene) with primers for each microRNA. Results of microRNA expression are presented as the relative copy number of the given microRNA to *U6* reference control for each sample.

Quantitative chromatin immunoprecipitation-PCR. Cells were fixed with 1% formaldehyde at room temperature for 10 min. The resultant DNA-protein complexes were sonicated followed by immunoprecipitation using Dynabeads protein G (Invitrogen) coated with control IgG antibody or with respect antibodies for H3K27me3 (Upstate), H3K9me2 (Abcam), and DNA methyltransferase 1 (Imgenex). Pull-down DNAs were used for chromatin immunoprecipitation-PCR assays. PCR primers targeting 3.5 kb upstream to 1.0 kb downstream regions of the predicted *miR-9-3* transcriptional start site (TSS) were used to amplify DNA samples using the SYBR Green-based detection method (Applied Biosystems). Quantitative values measured by a standard curve (50-0.08 ng; 5-fold dilution; $R^2 > 0.99$) of input DNA amplified with the same primer set. Results are presented as the mean ± SE of two independent experiments.

Quantitative methylation analysis using MassARRAY. Genomic DNA (1 μg) was treated with sodium bisulfite using the EZ DNA Methylation kit (ZYMO Research). Bisulfite-treated DNA was amplified with specific primers for the *miR-9-3* CpG island. The MassARRAY platform was used to perform quantitative methylation analysis (Sequenom). This system uses matrix-assisted laser desorption ionization time-of-flight mass spectrometry in combination with RNA base cleavage (MassCLEAVE). Details of quantitative analysis of methylation status have been described elsewhere (22). The average value of the CpG units for each patient tumor was calculated. Patients were separated based on ER status and the difference between groups was assessed by the Mann-Whitney rank-sum test using SigmaPlot 11.

Inhibitor treatments. MCF-7 cells were treated with 1 μmol/L 5-aza-2′-deoxycytidine for 5 days in MEM containing 10% fetal bovine serum and 6 ng/μL insulin. During the final 72 h of the 5-aza-2′-deoxycytidine treatment, cells were hormone deprived by culturing in phenol red-free MEM supplemented with 4% charcoal-dextran-treated fetal bovine serum. During the final 24 h of hormone deprivation, cells were treated with 1 μmol/L trichostatin A and/or an ER antagonist, ICI182780, or DMSO followed by treatment with DMSO (control) or 70 nmol/L diethylstilbestrol for 6 h. Total RNA was collected for qRT-PCR analysis.

Transfection studies. MCF-7 cells (10^6) were seeded in a 10 cm dish and transfected the next day with two predesigned matured mimics of *miR-9-3* (Dharmacon), *miR-9* (5′-uc∩uuugguuaucuagcug∩uauga) and *miR-9** (5′-ua∩aagcuagauaaccgaa∩agu), using the Fugene HD (Roche). After 48 h, transfected cells were used for gene expression analysis and apoptosis assay. Scrambled oligonucleotide (Dharmacon) was used as a negative control.

Expression microarray analysis. Total RNA was extracted from transfectants and control cells, and the quality of RNA samples was assessed using Agilent 2100 Bioanalyzer. RNA was amplified and labeled for hybridization to the Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix). The quality of hybridization data was assessed using normalized unscaled SE and relative log expression statistics (23) and MAS5.0 report quality scores. Normalized unscaled SE and relative log expression statistics were computed using the affyPLM package in Bioconductor. Expression values were computed from the raw (.cel) Affymetrix files and normalized using robust multichip averaging RMA (Bioconductor release 2.4.0). The list of the potential *miR-9-3* targets was generated from the comparison results of the expression array data and the information from miRanda[3] and TargetScan.[4]

The Ingenuity Pathway Analysis version 6.5 (Ingenuity Systems)[5] was used to analyze the list of the potential *miR-9-3* targets. Filtering was done

[3] http://www.microrna.org/microrna
[4] http://www.targetscan.org
[5] http://www.ingenuity.com

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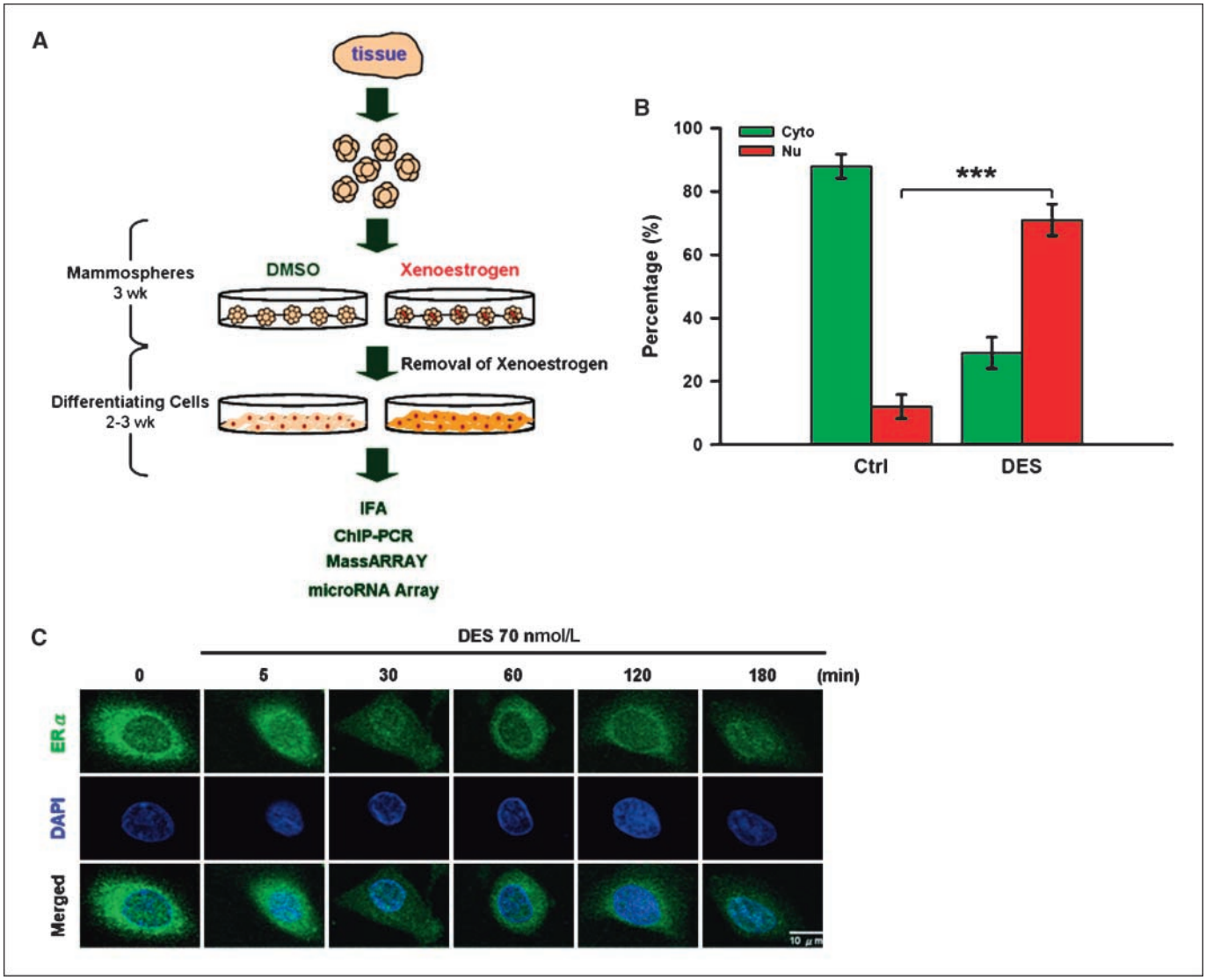

Figure 1. Preexposure of MDECs to diethylstilbestrol and immunofluorescence analysis of nuclear ER-α. *A,* treatment scheme. Breast progenitor cells were propagated as nonadherent spherical colonies, called mammospheres, and treated with 70 nmol/L diethylstilbestrol or DMSO solvent control for 3 wk. To induce differentiation, cells were seeded on a collagen substratum in the absence of diethylstilbestrol for 3 wk. Expression profiling (microRNA microarray), immunofluorescence image (*IFA*), and epigenetic [chromatin immunoprecipitation-PCR (*ChIP-PCR*) and *MassARRAY*] analyses were then done on the progeny epithelial cells. *B,* increased internalization of ER-α in diethylstilbestrol-preexposed MDECs. After the preexposure to diethylstilbestrol (*DES*) or DMSO, MDECs were subjected to immunofluorescence analysis. The percentage of subcellular localization of ER-α–positive cells, independently scored by two researchers, is shown in the bar chart. *Columns,* mean of five independent sets of MDEC samples; *bars,* SE. $P < 0.001$ (Student's t test). *C,* nuclear trafficking of ER-α in MDECs on diethylstilbestrol treatment. DMSO-preexposed MDECs were exposed to 70 nmol/L diethylstilbestrol in the indicated time points. Translocation of ER-α protein (*green*) from the cytoplasm to the nucleus was observed, suggesting functional estrogen signaling. Nuclei were stained with 4′,6-diamidino-2-phenylindole (*DAPI*; *blue*). The turquoise signals (*merged*) highlight the localization of ER-α in the nucleus.

to remove genes with no annotation in Ingenuity Pathway Analysis, resulting in a list of 102 network eligible genes. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. For confirmation studies, total RNA of transfectants was transcribed with the SuperScript III reverse transcriptase (Invitrogen) and then subject to qRT-PCR analyses. Results are displayed as the relative expression level of the given gene to nontreatment samples.

Apoptosis assays. MCF-7 cells were harvested 4 to 7 days after the transfection with *miR-9-3* mimics and stained with propidium iodide to monitor cell cycle distributions (Roche). Results were obtained using BD Flow Cytometer (BD). The amount of G_0-G_1 cells was given as fold changes of transfectants relative to untreated cells.

Statistical analysis. All data were presented as mean ± SE of n independent measurements. Statistical comparisons between two groups were made by Student's t test using SigmaPlot 11. For samples with equal variance, the paired Student's t test was used. For samples with unequal variance, the Mann-Whitney rank-sum test was used.

Supplementary data. The supplementary data include one figure and two tables.

Results and Discussion

We modified a suspension culture protocol (19, 21) to enrich and propagate breast progenitors. These cells (n = 2,000-10,000) formed spherical colonies, called mammospheres, were exposed to a pharmacologically relevant dose (70 nmol/L; ref. 24) of

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diethylstilbestrol for 3 weeks (Fig. 1*A*). After the exposure, mammospheres placed on two-dimensional collagen substratum with the removal of diethylstilbestrol were differentiating into epithelial cells for 2 to 3 weeks (19). Immunofluorescence images were used to determine whether changes of ER-α localization occurred in these MDECs (Fig. 1*B*). Consistent with our previous report on 17β-estradiol-preexposed MDECs (19), nuclear ER-α was preferentially observed in diethylstilbestrol-preexposed MDECs (71%) relative to control cells (12%; $n = 5$; $P < 0.001$). In contrast, this nuclear localization is usually transient and cyclical in normal breast epithelial cells in response to physiologic stimulation (e.g., menstrual cycle; ref. 25). For example, on ligand stimulation of DMSO-preexposed MDECs with 70 nmol/L diethylstilbestrol, translocation of ER-α to the nucleus took place rapidly within 5 min, and ER-α was reshuffled back to the cytoplasm after ~60 min (Fig. 1*C*). More cycles of nuclear translocation occurred and might gradually phase out in the post-stimulation period (25). It is therefore unusual to observe nuclear internalization of ER-α without ligand stimulation, such as in diethylstilbestrol-preexposed MDECs. This

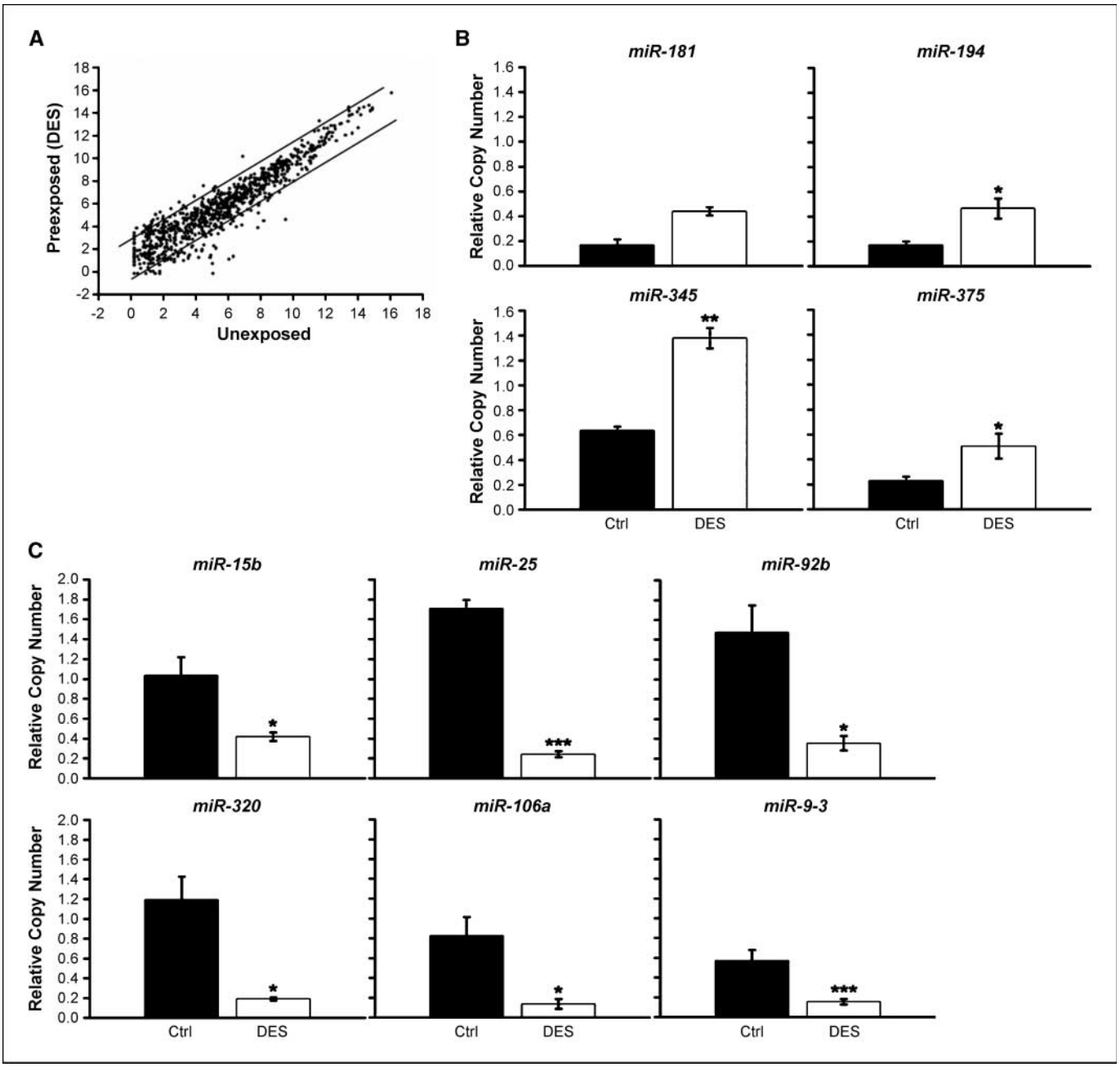

Figure 2. microRNA expression profiling of diethylstilbestrol-preexposed MDECs. *A,* differentially expressed microRNA loci in diethylstilbestrol-preexposed MDECs. A total of 82 differentially expressed loci in control and preexposed cells are shown in the scatter plot (see Supplementary Table S1 for a complete list of the differentially expressed loci). The scatter plot represents the averaged values of three experiments. *B* and *C,* validation of differentially expressed loci by qRT-PCR. Gene-specific qRT-PCR on four independent sets of DMSO- and diethylstilbestrol-preexposed MDECs was done to validate four up-regulated loci (*B*) and six down-regulated loci (*C*). *U6* was used as internal control for microRNA expression. Mean ± SE ($n = 4$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ (Student's *t* test).

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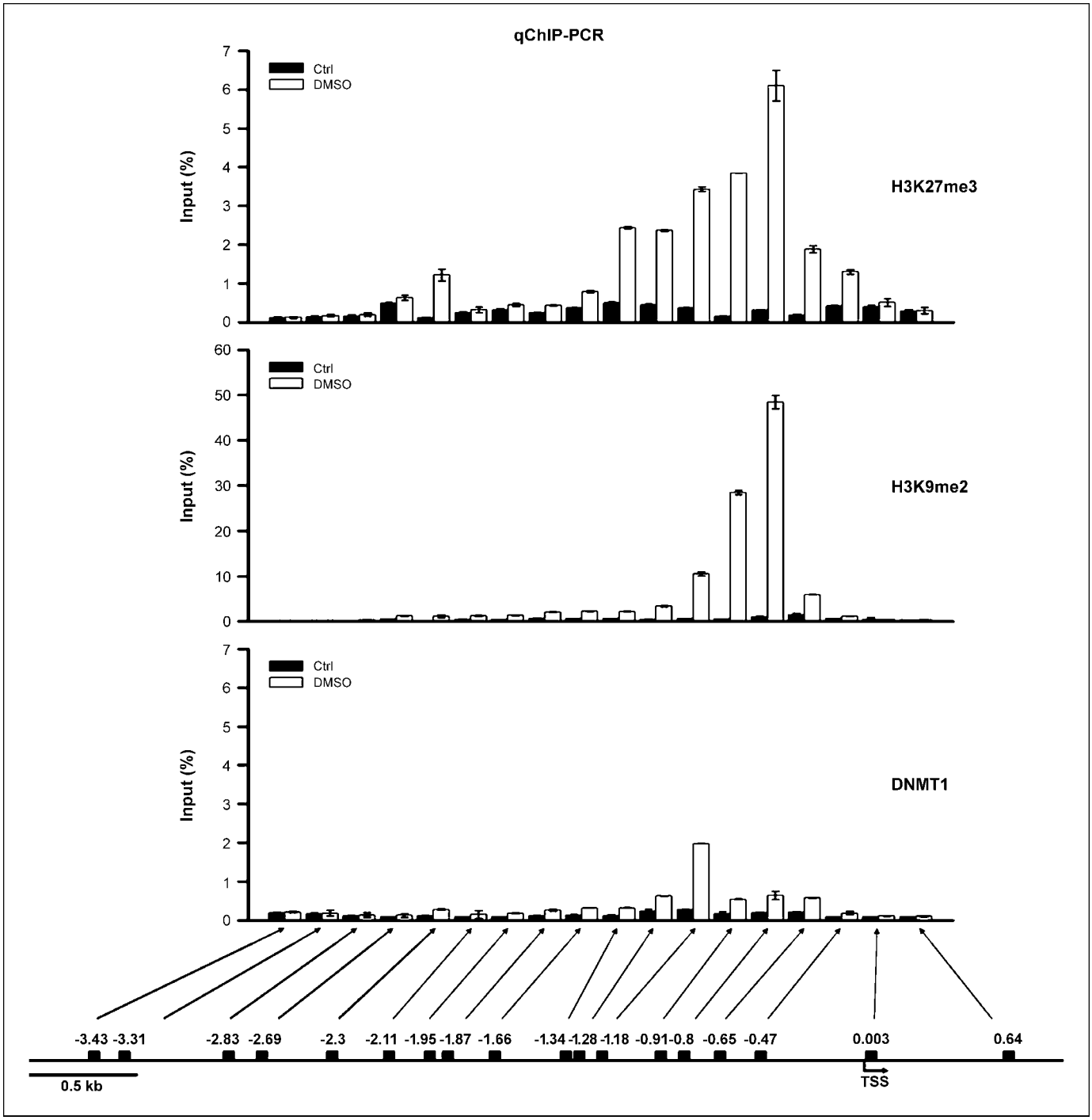

Figure 3. Recruitment of epigenetic markers at the *miR-9-3* locus. DNA from DMSO- or diethylstilbestrol-preexposed MDECs was immunoprecipitated with antibodies specific for H3K27me3, H3K9me2, and DNA methyltransferase 1. Immunoprecipitated DNA was subjected to quantitative PCR to measure enrichment at regions from 3.5 kb upstream to 1 kb downstream of the *miR-9-3* TSS. Mean ± SE ($n = 6$) of two independent experiments. *Bottom,* a diagram of regions surveyed by real-time PCR in *miR-9-3*.

persistent retention of nuclear ER-α could be a heritable event, reminiscent of that observed in ER-α–positive breast cancer cells (e.g., MCF-7; ref. 26). We speculate that, as a result of continuous exposure to xenoestrogen, the dynamics of nucleocytoplasmic shuttling of ER-α could be disrupted due to a defect involving Crm1, a protein regulating the nuclear export of hormone receptors (27). It is likely such a defect was transmitted to the progeny derived from progenitor cells continuously exposed to diethylstilbestrol. However, further studies need to be conducted to support the above hypothesis.

To determine whether this heritable effect altered the transcriptome of microRNA genes, we conducted expression microarray analysis of three sets of primary MDECs with a prior history of diethylstilbestrol exposure. Relative to control cells, 9.1% (82 of

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898 loci) of microRNAs were differentially expressed, including 37 up-regulated and 45 down-regulated loci, in diethylstilbestrol-preexposed MDECs (Fig. 2*A*; Supplementary Table S1). Independent validation by qRT-PCR assays confirmed the findings in 10 randomly selected microRNAs (up-regulated: *miR-181, miR-194, miR-346*, and *miR-376* and down-regulated: *miR-15b, miR-25, miR-92b, miR-320, miR-106a*, and *miR-9-3*) in 4 sets of diethylstilbestrol-preexposed MDECs (Fig. 2*B* and *C*).

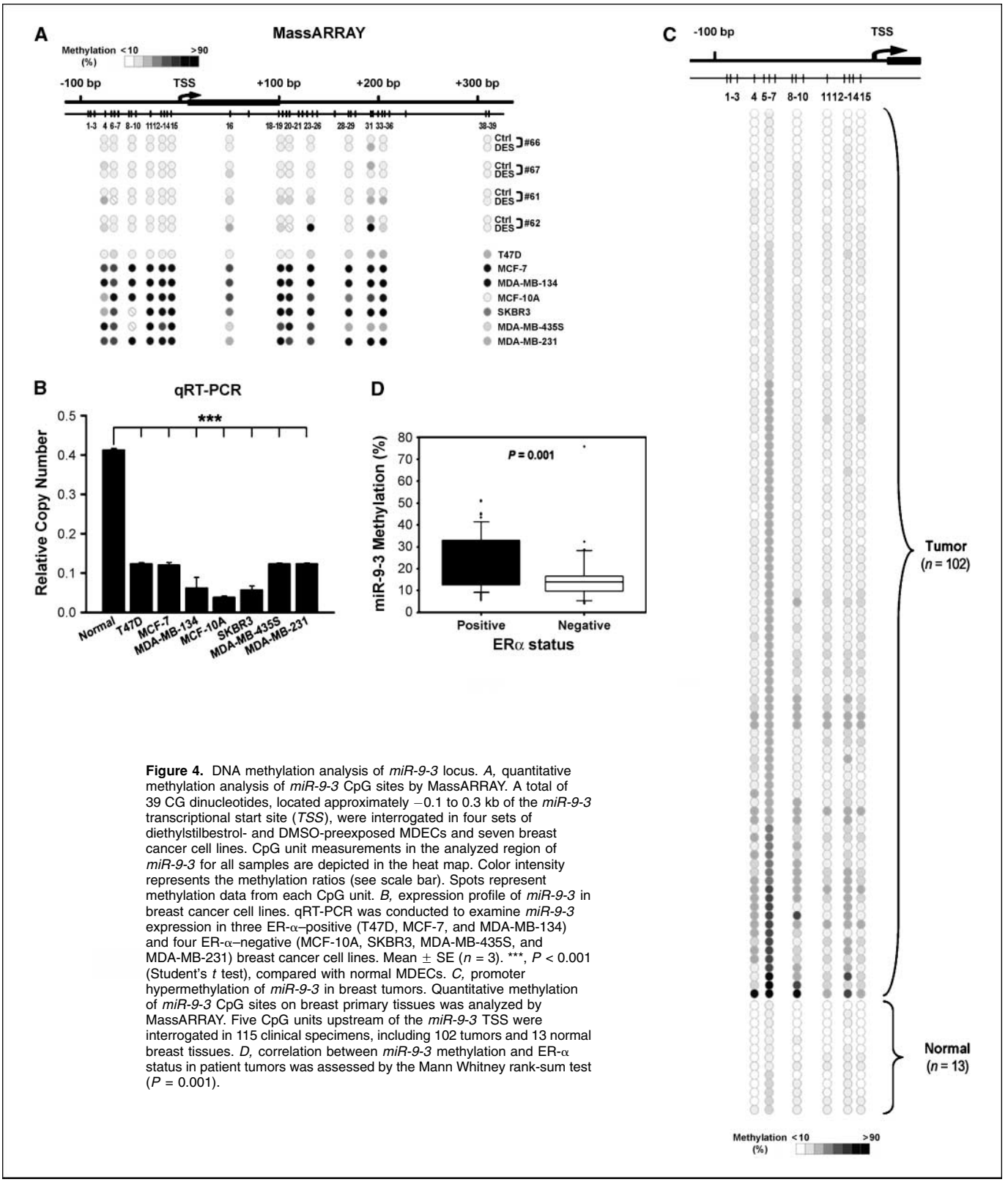

Figure 4. DNA methylation analysis of *miR-9-3* locus. *A,* quantitative methylation analysis of *miR-9-3* CpG sites by MassARRAY. A total of 39 CG dinucleotides, located approximately −0.1 to 0.3 kb of the *miR-9-3* transcriptional start site (*TSS*), were interrogated in four sets of diethylstilbestrol- and DMSO-preexposed MDECs and seven breast cancer cell lines. CpG unit measurements in the analyzed region of *miR-9-3* for all samples are depicted in the heat map. Color intensity represents the methylation ratios (see scale bar). Spots represent methylation data from each CpG unit. *B,* expression profile of *miR-9-3* in breast cancer cell lines. qRT-PCR was conducted to examine *miR-9-3* expression in three ER-α–positive (T47D, MCF-7, and MDA-MB-134) and four ER-α–negative (MCF-10A, SKBR3, MDA-MB-435S, and MDA-MB-231) breast cancer cell lines. Mean ± SE ($n = 3$). ***, $P < 0.001$ (Student's t test), compared with normal MDECs. *C,* promoter hypermethylation of *miR-9-3* in breast tumors. Quantitative methylation of *miR-9-3* CpG sites on breast primary tissues was analyzed by MassARRAY. Five CpG units upstream of the *miR-9-3* TSS were interrogated in 115 clinical specimens, including 102 tumors and 13 normal breast tissues. *D,* correlation between *miR-9-3* methylation and ER-α status in patient tumors was assessed by the Mann Whitney rank-sum test ($P = 0.001$).

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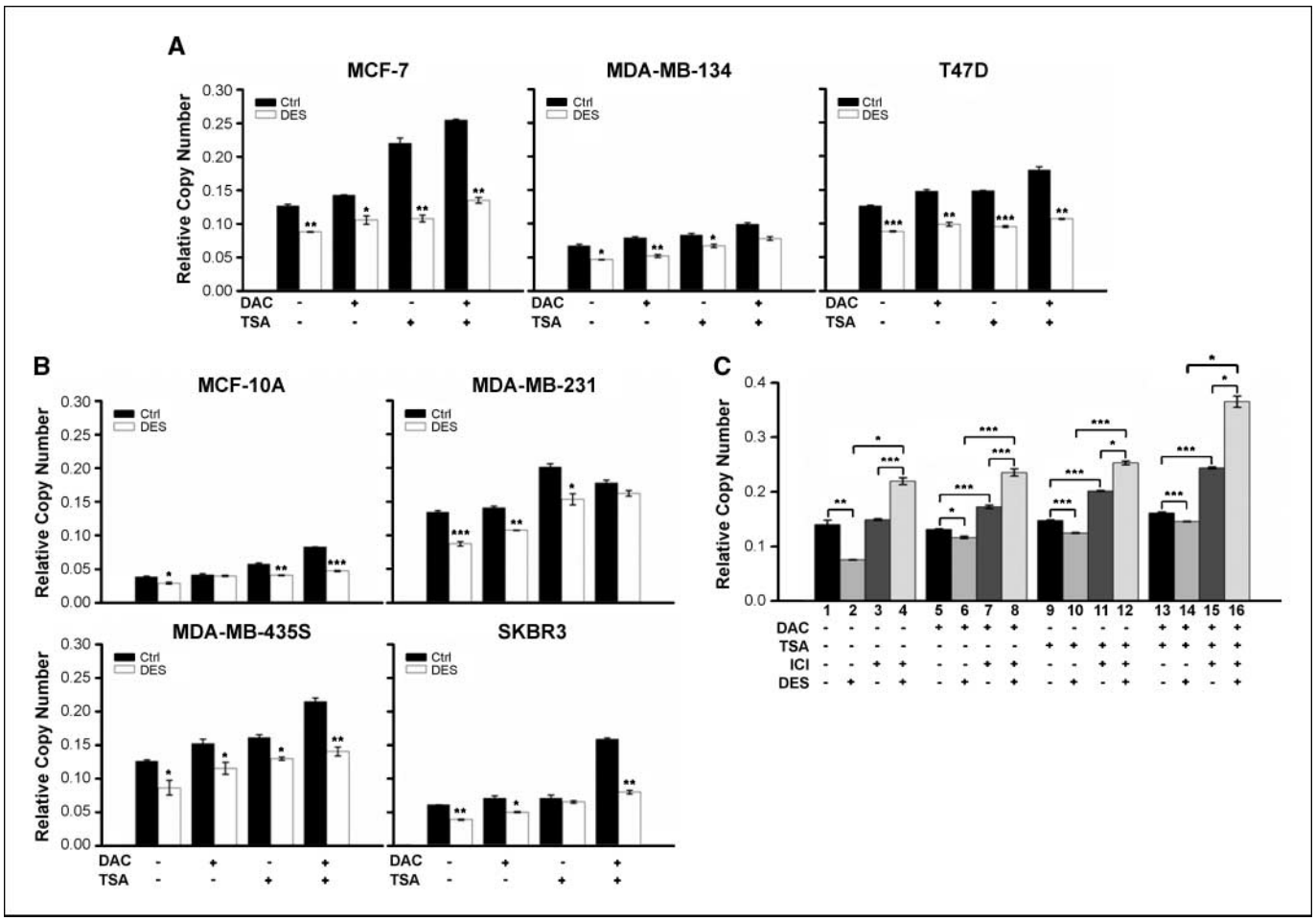

Figure 5. Epigenetic silencing of *miR-9-3* mediated by ER-α–dependent and ER-α–independent pathways. *A* and *B,* effects of epigenetic inhibitors on *miR-9-3* expression of breast cancer cell lines. Four ER-α–positive (*A*) and six ER-α–negative (*B*) breast cancer cell lines were pretreated with 5-aza-2′-deoxycytidine (*DAC*; 1 μmol/L) and/or trichostatin A (*TSA*; 1 μmol/L) and then stimulated with diethylstilbestrol for 6 h. Total RNA was collected for qRT-PCR analysis to monitor *miR-9-3* expression. *U6* was used as internal control. Mean ± SE ($n = 3$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ (Student's *t* test), compared with DMSO-treated cells. *C,* restoration of *miR-9-3* expression by the ER-α antagonist, ICI182780. An ER-α–positive breast cancer cell line, MCF-7, was treated with 5-aza-2′-deoxycytidine (1 μmol/L), trichostatin A (1 μmol/L), and/or ER-α antagonist, ICI182780 (1 μmol/L), before 6 h diethylstilbestrol stimulation. Total RNA was addressed to qRT-PCR analysis of *miR-9-3*. *U6* was the internal control. Mean ± SE ($n = 3$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ (Student's *t* test).

Epigenetic characterization of *miR-9-3* was further studied in detail because this microRNA was previously implicated in breast tumorigenesis (28). Chromatin immunoprecipitation-qRT-PCR assays showed that increased levels (2- to 23-fold; $P < 0.001$) of the polycomb EZH2-mediated chromatin modification, H3K27me3 (29, 30), were observed at the 1.66 to 0.47 kb upstream region of *miR-9-3* transcription start site in diethylstilbestrol-preexposed MDECs relative to control cells (Fig. 3). Enrichment (2- to 48-fold; $P < 0.001$) of another repressive chromatin mark, H3K9me2, was also seen in the 1.28 to 0.65 kb upstream region of the transcription start site. Increased recruitment (2- to 7-fold; $P < 0.001$) of DNA methyltransferase 1 was noted, suggesting that DNA methylation was gradually accumulated in the CpG island of *miR-9-3*. The enrichment area of H3K27me3 is usually more widespread than that of H3K9me2, consistent with the patterns previously reported by MacGarvey

Figure 6. Direct and indirect target genes of *miR-9-3*. *A,* microarray profiles of *miR-9-3* targets. MCF-7 cells were transfected with two forms (*miR-9* or *miR-9** mimics) of *miR-9-3*. After 48 h, total RNA was collected for microarray analysis. Common genes derived microarray profiling and TargetScan database were shown in the heat map (see also Supplementary Table S2 for the list of target genes). The indicated genes are found in signaling network of p53. *B,* signaling network of p53 regulated by *miR-9-3*. Network interaction of the p53 pathway was constructed using the Ingenuity Pathway program, which identified 34 significant genes as the *miR-9-3* targets. Genes in red and green ovals are up-regulated and down-regulated genes, respectively. Genes in blue open rectangles are correlated to the p53 pathway. *C,* confirmation of potential *miR-9-3* targets. The expression level of selected p53 pathway-related genes was examined by qRT-PCR in *miR-9* transfectants (*top*) and diethylstilbestrol-preexposed MDECs (*bottom*). *β-Actin* was used as internal control. *D,* induction of apoptosis by *miR-9-3*. microRNA mimics (*miR-9* and *miR-9**) were separately transfected into MCF-7 cells for 48 h. Apoptosis assay was done, as described in Materials and Methods, on days 4 and 7 after the transfection. Scrambled oligonucleotides were used as negative controls. Mean ± SE ($n = 3$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ (Student's *t* test), compared with control cells.

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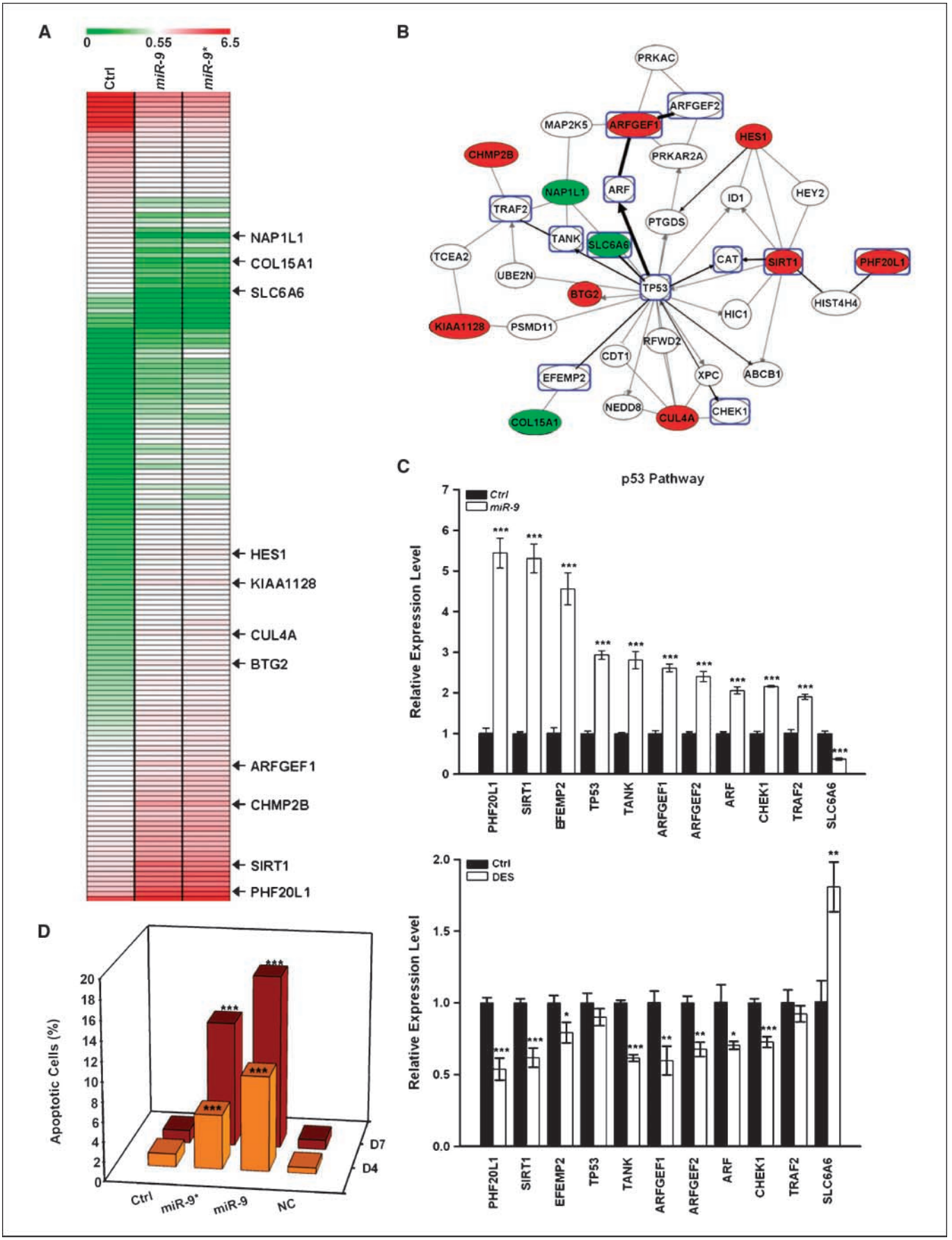

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and colleagues (29). Furthermore, the enrichment of DNA methyltransferase 1 is evidently lower relative to that of H3K27me3 and H3K9me2, supporting the notion that repressive chromatin marks usually take place first and then signal DNA methylation to be further accumulated in the region that undergoes permanent, heritable silencing (30–32).

F4 Methylation profiles of 14 CpG units (1-3 CpG sites per unit) within this island were surveyed by MassARRAY (Fig. 4*A*). Compared with control cells, increased methylation at several CpG units was evident in diethylstilbestrol-preexposed MDECs. However, this level of accumulation (10-50%) was less obvious in diethylstilbestrol-preexposed MDECs compared with high methylation levels (30-97%) observed in six breast cancer cell lines (except T47D) and one immortalized line (MCF10A). Moreover, we observed that, compared with control MDECs, the expression levels of *miR-9-3* were significantly lower in these cell lines ($P < 0.001$; Fig. 4*B*) and in MDECs preexposed to other xenoestrogens (Supplementary Fig. S1). These results support our hypothesis that hypermethylation of *miR-9-3* CpG island is associated with the silencing of this microRNA locus. In addition, we believe that diethylstilbestrol preexposure at the mammosphere stage may attenuate the active transcription of *miR-9-3*. Repressive chromatin of this locus is subsequently established and sets the stage for heritable silencing. This programmed repression during epithelial differentiation leads to an increased susceptibility to stochastic *de novo* methylation, which in turn may act as "seeds" to trigger more methylation accumulation (33). Extending this *in vitro* observation, we found that 32.4% of 102 primary breast tumors analyzed by MassARRAY exhibited extensive promoter hypermethylation in the upstream region of the CpG islands, whereas 13 uninvolved, normal controls showed little or no methylation (Fig. 4*C*). Statistical analysis further revealed this epigenetic event preferentially occurring in ER-α–positive tumors ($P = 0.001$; Fig. 4*D*).

This result implies that promoter hypermethylation of *miR-9-3* is associated with the ER-α signaling pathway in breast cancer cells. We have shown previously that persistent deregulation of the ER-α signaling may lead to permanent silencing of downstream target genes that is maintained by promoter hypermethylation (19, 34, 35). We therefore suggest that diethylstilbestrol, as an estrogenic ligand (see Fig. 1*C*), may activate an ER-α–mediated signaling pathway resulting in the epigenetic repression of *miR-9-3*. To test this hypothesis, we first treated three ER-α–positive (MCF-7, MDA-MB-134, and T47D) breast cancer cell lines with a demethylating agent, 5-aza-2′-deoxycytidine (1 μmol/L), and/or a histone deacetylase inhibitor, trichostatin A (1 μmol/L; Fig. 5*A*). Partial restoration of the *miR-9-3* expression was observed when these cell lines were treated with either agent. The combined treatment of 5-aza-2′-deoxycytidine and trichostatin A synergistically restored the expression of this microRNA gene. When diethylstilbestrol (70 nmol/L) was subsequently added to these treated cells, the repression (2- to 14-fold) of *miR-9-3* occurred again at 6 h of the estrogenic treatment. Surprisingly, when ER-α–negative cell lines (MCF-10A, MDA-MB-231, MDA-MB-435S, and SKBR3; Fig. 5*B*) were similarly treated with these epigenetic agents, we also observed the suppression of *miR-9-3* by diethylstilbestrol. One explanation is that, without the mediation of hormone receptors, diethylstilbestrol might act through nongenomic pathways to epigenetically silence this microRNA gene in ER-α–negative cells (4). In addition, we cannot rule out the possibility that the combined treatment of 5-aza-2′-deoxycytidine and trichostatin A leads to reactivation of ER-α expression in these ER-α–negative breast cancer cells (36). This reactivating ER-α may, in turn, repress the *miR-9-3* expression. To definitely prove that epigenetic repression of *miR-9-3* is modulated via the ER-α–dependent pathway, we conducted an experiment by additionally treating ER-α–positive MCF-7 cells with an ER antagonist, ICI182780, which promotes the degradation of ER-α only (ref. 37; Fig. 5*C*). Again, diethylstilbestrol could repress the reexpression of *miR-9-3* in MCF-7 cells treated with 5-aza-2′-deoxycytidine and/or trichostatin A (*lanes 6, 10,* and *14*). This suppression, however, was partially abrogated in MCF-7 cells when the synthesis of ER-α could be blocked by ICI182780 (e.g. *lanes 4, 8,* and *12* in *C*). Furthermore, we observed that, compared with the expression level of control MDECs (normal) in Fig. 4*B*, diethylstilbestrol-mediated *miR-9-3* repression could be completely restored in cells treated with 5-aza-2′-deoxycytidine, trichostatin A, and ICI182780 (*lane 16*). Although the pharmacologic mechanism among these compounds is complicated, our results suggest that ER-α indeed plays a key role in diethylstilbestrol-induced epigenetic silencing of *miR-9-3*. Taken together, our findings suggest that diethylstilbestrol may act through the ER-α–dependent pathway to repress *miR-9-3*. In the absence of hormone receptors, this epigenetic repression can alternatively be mediated via an ER-α–independent pathway.

To determine the roles of *miR-9-3* silencing in breast tumorigenesis, we assessed the function of this microRNA gene in a knock-in study. MCF-7 cells, which have the epigenetically down-regulated *miR-9-3*, were transiently transfected with a mature mimic, *miR-9*. Microarray expression profiling was used to determine target genes of *miR-9-3* in transfected versus control cells (Fig. 6*A*). A total of 70 up-regulated and 32 down-regulated protein-coding genes were identified (1.5-fold alteration) as putative targets of *miR-9-3* (Supplementary Table S2). A similar expression profile was also obtained when MCF-7 cells was transfected with another mimic, *miR-9**. Of these differentially regulated microRNAs, Ingenuity Pathway Analysis identified 8 up-regulated genes (*CHMP28, ARFGEF1, HES1, KIAA1128, BTG2, SIRT1, PHF20L1*, and *CUL4A*) and 3 down-regulated genes (*NAP1L1, SLC6A6*, and *COL15A1*) that were significantly associated with the p53 signaling pathway ($P = 0.046$; Fig. 6*B*). qRT-PCR assays were extended to analyze in 11 candidate genes associated with this signaling network, including 4 original loci (*SLC6A6, ARFGEF1, SIRT1*, and *PHF20L1*) identified by this microarray analysis (Fig. 6*C, top*). Except for *SLC6A6*, the expression of the other 10 genes was up-regulated in MCF-7 cells transfected with the *miR-9* mimic ($P < 0.001$). In contrast, these 10 genes were down-regulated when *miR-9-3* was epigenetically silenced in MDECs preexposed to diethylstilbestrol (Fig. 6*C, bottom*; $P < 0.01$). Of these genes directly or indirectly regulated by *miR-9-3, ARF* and *SIRT1* are known to be involved in p53-mediated apoptosis (38, 39). Inactivation of *ARF* can reduce apoptosis and enhance proliferation in cancer cells (38). Induction of *SIRT1* contributes to p53-mediated apoptosis by stabilizing p53 (39). However, this apoptotic activity (5- to 10-fold relative to control cells; $P < 0.001$) could be restored when the functional *miR-9-3* was reintroduced to MCF-7 breast cancer cells (Fig. 6*D*).

In conclusion, we have identified a microRNA gene, *miR-9-3*, which is frequently hypermethylated in breast cancer. One potential causal factor leading to this epigenetic repression is the prolonged exposure of breast progenitor cells to xenoestrogens. In our example, diethylstilbestrol acts as an estrogenic ligand that activates either genomic or nongenomic pathways to

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mediate gene transcription through down-regulation of the target microRNA, *miR-9-3*. Persistent activation of this signaling activity may further establish a repressive heterochromatin environment in the promoter CpG island region of this microRNA. Accumulation of DNA methylation later occurs in the region and leaves the heritable hallmark in differentiated progeny. Because *miR-9-3* plays a role in regulating apoptosis, epigenetic silencing of this gene may promote proliferation of cancer cells. Restoration of its expression by the combined epigenetic and microRNA-based therapies, therefore, could be an additional approach for future treatment of breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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