Tumor and Stem Cell Biology

Cancer Research

MicroRNA-145 Suppresses Cell Invasion and Metastasis by Directly Targeting Mucin 1

Mohit Sachdeva and Yin-Yuan Mo

Abstract

MicroRNAs are important gene regulators that could play a profound role in tumorigenesis. Our previous studies indicate that *miR-145* is a tumor suppressor capable of inhibiting tumor cell growth both *in vitro* and *in vivo*. In this study, we show that *miR-145* exerts its function in a cell-specific manner. Although *miR-145* inhibits cell growth in MCF-7 and HCT-116 cells, it has no significant effect on cell growth in metastatic breast cancer cell lines. However, *miR-145* significantly suppresses cell invasion in these cells; in contrast, the antisense oligo against *miR-145* increases cell invasion. *miR-145* is also able to suppress lung metastasis in an experimental metastasis animal model. This *miR-145*–mediated suppression of cell invasion is in part due to the silencing of the metastasis gene *mucin 1* (*MUC1*). Using luciferase reporters carrying the 3′-untranslated region of MUC1 combined with Western blot and immunofluorescence staining, we identify MUC1 as a direct target of *miR-145*. Moreover, ectopic expression of MUC1 enhances cell invasion, which can be blocked by *miR-145*. Of interest, suppression of MUC1 by *miR-145* causes a reduction of β-catenin as well as the oncogenic cadherin 11. Finally, suppression of MUC1 by RNAi mimics the *miR-145* action in suppression of invasion, which is associated with downregulation of β-catenin and cadherin 11. Taken together, these results suggest that as a tumor suppressor, *miR-145* inhibits not only tumor growth but also cell invasion and metastasis. *Cancer Res; 70(1); 378–87. ©2010 AACR.*

Introduction

Cell migration and invasion are the major features of metastatic tumor cells that are responsible for most cancer-related deaths. It is well known that the potential of a tumor cell to metastasize depends on numerous factors. Accumulating evidence suggests that microRNAs (miRNA) could be key players in regulation of tumor cell invasion and metastasis (1). miRNAs are small noncoding RNAs that serve as negative regulators of gene expression (2–5). Through interactions with the 3′-untranslated region (3′-UTR) of mRNA by partial sequence homology, miRNAs cause gene silencing either by mRNA degradation or translation repression (6). Given the unique feature of their targeting, each single miRNA could have over a hundred targets (7), and, thus, a large number of protein-coding genes could be under the control of miRNAs. As a result, miRNAs play a fundamental role in the regulation of diverse cellular functions, and deregulation of miRNA expression is often associated with a variety of disorders, including human malignancy.

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Increasing evidence indicates that miRNAs may function as either oncogenes or tumor suppressors (8). For example, *miR-145* is a putative tumor-suppressive miRNA that is underexpressed in several types of tumors (9–11) and causes cell growth inhibition by targeting c-Myc (12) and IRS-1 (13). In addition, *miR-145* is able to target the pluripotency factors OCT4, SOX2, and KLF4 and functions as a key regulator of human stem cells (14) or promotes differentiation and represses proliferation of smooth muscle cells (15). We have previously shown that *miR-145* plays an important role in p53-mediated repression of c-Myc (12). During the further characterization of *miR-145* in different cancer cell lines, we found that *miR-145* functions as a tumor suppressor in a cell type–specific manner. We showed that *miR-145* is a tumor suppressor affecting invasion and metastasis in part by targeting mucin 1 (MUC1).

Materials and Methods

Reagents. Primary antibodies were purchased from the following vendors: MUC1 (small isoform), β-catenin, and cyclin D1 from Epitomics; cadherin 11 from Invitrogen; Myc-tag from Applied Biological Materials; and the antibody specific to large isoforms of MUC1 from Santa Cruz Biotechnology. Secondary antibodies conjugated with IRDye 800CW or IRDye 680 were purchased from LI-COR Biosciences. PCR primers and anti–*miR-145* LNA oligo were purchased from IDT. MUC1 siRNA was purchased from Open Biosystems. Freshly frozen breast tumor specimens and their matching normal breast specimens were obtained from Cooperative Human Tissue Network (Midwestern Division).

Cell culture. All cell lines were purchased from the American Tissue Culture Collection except for LM2-4142 (16), which was a generous gift from Dr. Joan Massagué (Sloan-Kettering Institute, New York, NY). Breast cancer cell lines BT-549, MDA-MB-231, and LM2-4142 were grown in RPMI 1640 (Cambrex) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich). HEK-293T cells were cultured in DMEM (Cambrex) supplemented with 10% FBS. All media contained 2 mmol/L glutamine, 100 units of penicillin/mL, and 100 μg of streptomycin/mL. Cells were incubated at 37°C and supplemented with 5% CO_2 in the humidified chamber.

Transfection. MDA-MB-231, LM2-4142, or BT-549 cells were transfected with anti–*miR-145* using RNAfectin reagent (Applied Biological Materials) following the manufacturer's protocol.

Plasmids. The plasmid expressing *miR-145* in pCMV or in lentiviral vector pCDH-CMV-MCS-EF1-copGFP (System Biosciences) or a mutant *miR-145* expression vector has been described previously (12). Expression of the mature *miR-145* was verified by TaqMan real-time reverse transcription-PCR (RT-PCR; 17).

To ectopically express MUC1, we cloned the MUC1 coding region in pCMV-Myc. The PCR product for MUC1 without UTR was obtained by primers MUC1-R1-5.1 (5′-GAATTCTGACACCGGGCACCCAGTCTC) and MUC1-*Not*1-3.1 [5′-GCGGCCGCCTACAAGTTGGCAGAAGTGGC; for MUC1 with UTR, we used primers MUC1-R1-5.1 and MUC1-UTR-*Not*1-3.1 (see below)]. The PCR product was first cloned into a PCR cloning vector (pCR8) and then subcloned into pCMV-Myc at *Eco*R1 and *Not*1 sites.

The luciferase-UTR reporter constructs were generated by introducing the MUC1 3′-UTR carrying a putative *miR-145* binding site into pGL3 control vector (Promega). We first amplified the MUC1 3-UTR sequence by PCR using primers MUC1-UTR-5.1 (5′-TCTGCCAACTTGTAGGGGCAC) and MUC1-UTR-*Not*1-3.1 (5′-GCGGCCGCTTTTTTGGCGCAGTGGGAGAC) and MCF10A cDNA as a template. The PCR product was also first cloned into a PCR cloning vector (pCR8) and then subcloned into a modified pGL3 control vector where *Eco*R1 and *Not*1 sites were introduced into the original *Xba*1 site. To delete the putative *miR-145* binding site in the MUC1 3′-UTR, we amplified the UTR by using primers MUC1-UTR-5.1 and MUC1-UTR-*Not*1-3.2 (5′-GCGGCCGCCAGGATCCCCGCTATCTCAGG), and then cloned into the modified pGL3 control vector at the *Eco*R1 and *Not*1 sites. Site-directed mutagenesis of the *miR-145* binding site in the MUC1 3′-UTR was carried out by the two-step PCR approach as described previously (12). All PCR products were verified by DNA sequencing.

Luciferase assay. Luciferase assays were carried out in 293T cells. First, cells were transfected with appropriate plasmids in 12-well plates. Then, the cells were harvested and lysed for luciferase assay 24 h after transfection. Luciferase assays were performed using a luciferase assay kit (Promega) according to the manufacturer's protocol. β-Galactosidase or renilla luciferase was used for normalization.

PCR/RT-PCR and real-time RT-PCR. PCR reactions were performed to amplify the MUC1 with or without 3′-UTR according to the standard three-step procedure. To detect MUC1 mRNA, we used the SYBR green method with primers MUC1-5.1 (5′-ACAGCTACCACAGCCCCTAA) and MUC1-3.1 (5′-CAGCTGCCCGTAGTTCTTTC); average levels of 5s RNA and β-actin were used as an internal control.

Cell proliferation assay. Cell growth assays were carried out by MTT assays according to standard methods, as previously described (18).

Invasion assay. Matrigel chambers (BD Biosciences) were used to determine the effect of *miR-145* or MUC1 on invasiveness per the manufacturer's protocol. In brief, infected cells were harvested, resuspended in serum-free medium, and then transferred to the hydrated Matrigel chambers (~25,000 cells per well). The chambers were then incubated for 24 h in culture medium with 10% FBS in the bottom chambers before examination. The cells on the upper surface were scraped and washed away, whereas the invaded cells on the lower surface were fixed and stained with 0.05% crystal violet for 2 h. Finally, invaded cells were counted under a microscope and the relative number was calculated.

Western blot. Cells were harvested and protein was extracted from transfected cells as previously described (12, 18).

Immunofluorescence microscopy. To determine the effect of *miR-145* on the protein level of MUC1, we also performed immunofluorescence staining using the MUC1 antibody using the previously described procedure (19).

Immunohistochemistry. Immunohistochemistry (IHC) was used to detect MUC1 in paraffin-embedded breast tumor tissue or to detect cadherin 11 in cell culture. For paraffin-embedded breast tumor tissue, slides were pretreated at 65°C for 2 h, followed by deparaffinization using standard procedures. After antigen retrieval, MUC1 antibody was applied to slides, followed by the secondary antibody conjugated with horseradish peroxidase. Signals were revealed by using the Histostain Plus kit (Invitrogen) according to the manufacturer's instruction. To detect cadherin 11 expression in stably transduced MDA-MB-231 cells, the cells were grown on the coated coverslips overnight before IHC staining.

Experimental metastasis assay. Female athymic nude (nu/nu) mice (ages 4–5 wk) were purchased from Harlan Sprague-Dawley and were maintained in the Southern Illinois University School of Medicine's accredited animal facility. All animal studies were conducted in accordance with NIH animal use guidelines and a protocol approved by Southern Illinois University Animal Care Committee. In brief, 1.5×10^6 exponentially growing MDA-MB-231 or LM2-4142 cells with ectopic expression of appropriate genes were injected into nude mice through tail vein. Four weeks after injection, the animals were sacrificed. The lungs were harvested and fixed in Bouin's solution, and tumor nodules were counted.

Statistical analyses. Statistical significance of the studies was analyzed by Student's *t* test. Differences with *P* values of <0.05 are considered significant. The linear correlation coefficient (Pearson's *r*) was calculated to estimate the correlation between *miR-145* values and MUC1 levels in the matched breast tumor specimens.

Results

miR-145 suppresses cell invasion in metastatic breast cancer cell lines. We have previously shown that as a

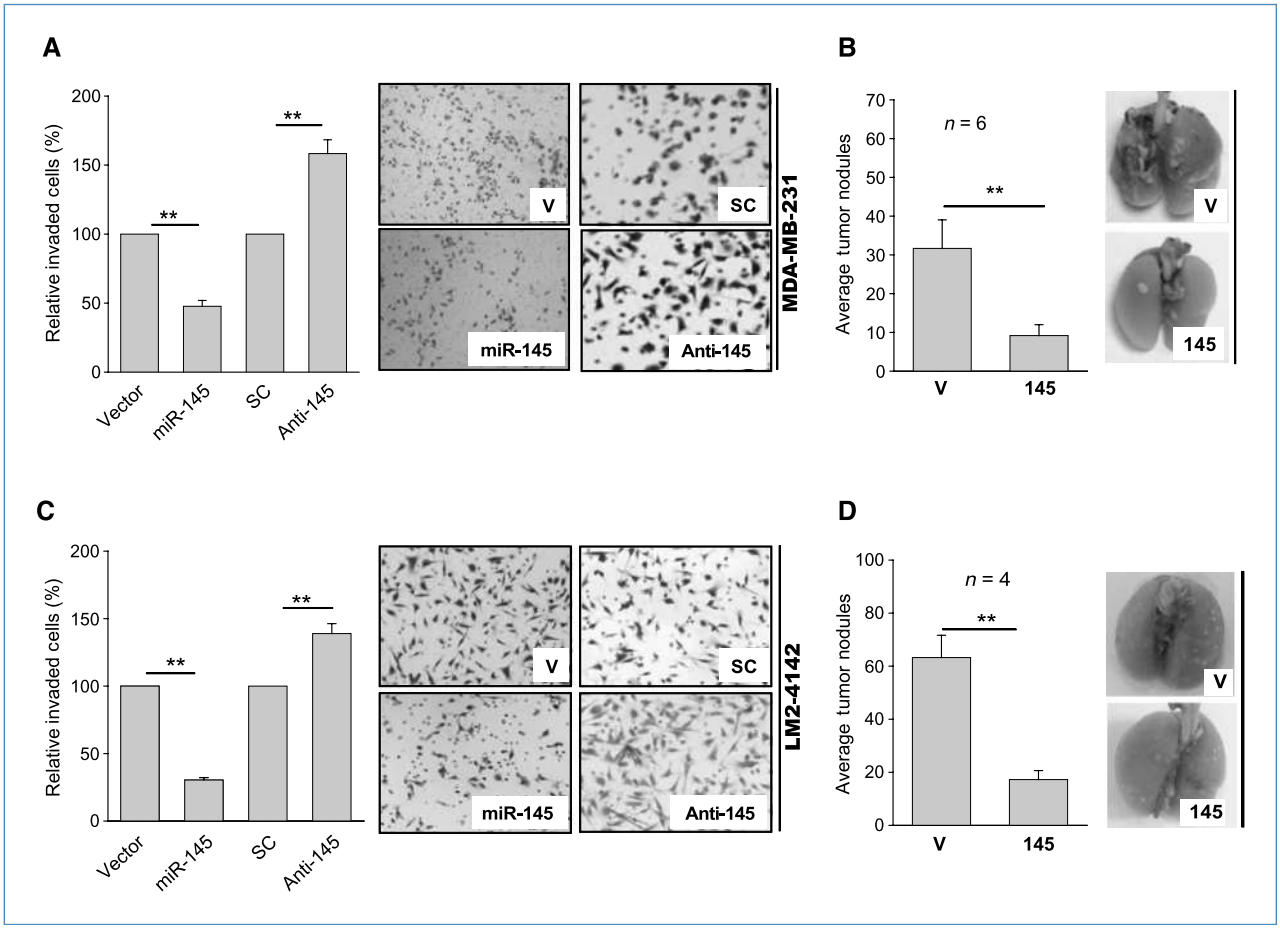

Figure 1. Effect of *miR-145* on cell invasion and lung metastasis. *A* and *C,* stably transduced MDA-MB-231 or LM2-4142 cells ectopically expressing *miR-145* (*145*) or vector alone (*V*) were subjected to a Matrigel chamber assay as detailed in Materials and Methods. In addition, MDA-MB-231 or LM2-4142 cells were transiently transfected with scrambled oligo or anti-*miR-145* and were then subject to matrigel chamber assay. Invaded cells on the membrane were counted as follows: as the distribution of cells on the membrane was not always even, we first took a picture at a low magnification and then enlarged the image on a computer screen with grids so that all of the cells on the entire membrane were counted. *B* and *D,* lung metastasis as revealed by the experimental metastasis animal model. Stably transduced MDA-MB-231 (*B*) or LM2-4142 (*D*) cells ectopically expressing *miR-145* (*145*) or vector alone (*V*) were introduced into female nude mice through the tail vein as described in Materials and Methods. Values in *A* and *C* are means ± SE of three independent experiments. **, $P < 0.01$; *SC,* scrambled oligo; *anti-145,* anti–*miR-145*.

tumor suppressor, *miR-145* inhibited cell growth in MCF-7 and HCT-116 cells (12). However, further characterization indicated that *miR-145* had no significant effect on cell growth in other cell lines such as MDA-MB-231 and LM2-4142 (Supplementary Fig. S1), suggesting a cell type–specific function of *miR-145*. Of note, we observed changes in morphology of the *miR-145*–infected cells that revealed a less invasive morphologic phenotype, i.e., round and flat compared with vector control. These results suggest that *miR-145* may play a suppressive role in cell invasion in these cells because both MDA-MB-231 and LM2-4142 are metastatic breast cancer cell lines.

Therefore, we determined whether *miR-145* affects cell invasion by Matrigel chamber assays. As expected, *miR-145* significantly impaired invasion (Fig. 1*A*). For example, in MDA-MB-231 cells, *miR-145* caused a reduction of invasive cells by more than 50%. In contrast, anti–*miR-145* enhanced cell invasion up to 50%, suggesting that this suppression is specific to *miR-145*. We found more reduction of invasion by *miR-145* (by ~75%) in LM2-4142, whereas anti–*miR-145* increased their invasiveness (Fig. 1*C*). To determine whether *miR-145* suppresses cell invasion *in vivo*, we used an experimental metastasis model by tail vein injection. As shown in Fig. 1*B*, whereas the average lung tumor nodules for the vector control were 32, there were only 9 nodules for *miR-145*. A similar result was obtained for LM2-4142 cells (Fig. 1*D*). These results further support the role of *miR-145* in suppression of invasion and metastasis.

miR-145 directly targets MUC1 by interaction with the 3′-UTR. To understand the mechanism of the *miR-145*–mediated inhibition of cell invasion, we first examined the effect of c-Myc on cell invasion because we previously showed that *miR-145* directly targets c-Myc (12). As a well-known oncogene, c-Myc has been implicated in the involvement of cell invasion indirectly (20). However, we found that ectopic expression of c-Myc gave rise to a modest increase in cell

invasion (data not shown), which is not comparable with the suppressive effect of *miR-145*, suggesting that some other *miR-145* targets may play a more important role in this aspect.

Hence, we searched for additional *miR-145* targets using computer-aided miRNA target prediction programs, such as TargetScan4 (21) and miRBase targets, and found several putative *miR-145* target genes that might play a role in cell invasion, including *ATF1, ATF3, MMP11, ANGPT2*, and *MUC1*. Luciferase reporter assays showed that two of them, *MUC1* and *MMP11*, met the arbitrary 35% reduction cutoff. We were particularly interested in MUC1 because MUC1 is a well-known metastasis-promoting gene that is upregulated in several types of tumors (22, 23). Therefore, we further characterized how *miR-145* suppresses MUC1.

As shown in Fig. 2*A*, *miR-145* suppressed more than 55% activity for Luc-MUC1-UTR compared with vector control; neither *miR-206* nor *miR-224* suppressed Luc-MUC1-UTR. Furthermore, the mutant *miR-145* had no suppressive effect. We then deleted this site from the MUC1-UTR or made a site-directed mutant (Fig. 2*A, top*), and this suppression was abolished in the construct without this *miR-145* binding site (Luc-MUC1-UTR-d) or with a mutant site (Luc-MUC1-UTR-mt; Fig. 2*A, bottom*).

Next, we determined whether ectopic expression of *miR-145* can suppress the endogenous MUC1 at the protein level by Western blot. Several MUC1 isoforms in the lysates of MUC1-positive cultured cancer cells have been reported: high molecular weight isoforms (150–300 kDa), presumably due to glycosylation, and a low molecular weight isoform (20–35 kDa; ref. 24). As shown in Fig. 2*B* (*top*), *miR-145* suppressed both large and small isoforms of MUC1. Because the small isoform is the predominant form in cancer cell lines and tumor specimens, and because it mediates tumor cell growth (24), this study focused on this small isoform. We further showed that the level of small isoform of MUC1 in the *miR-145* cells was <40% of vector control cells, whereas anti–*miR-145* increased its protein level by more than 200% (Supplementary Fig. S2). In contrast, *miR-224* or anti–*miR-224* had no effect on MUC1 expression (Fig. 2*B, bottom*).

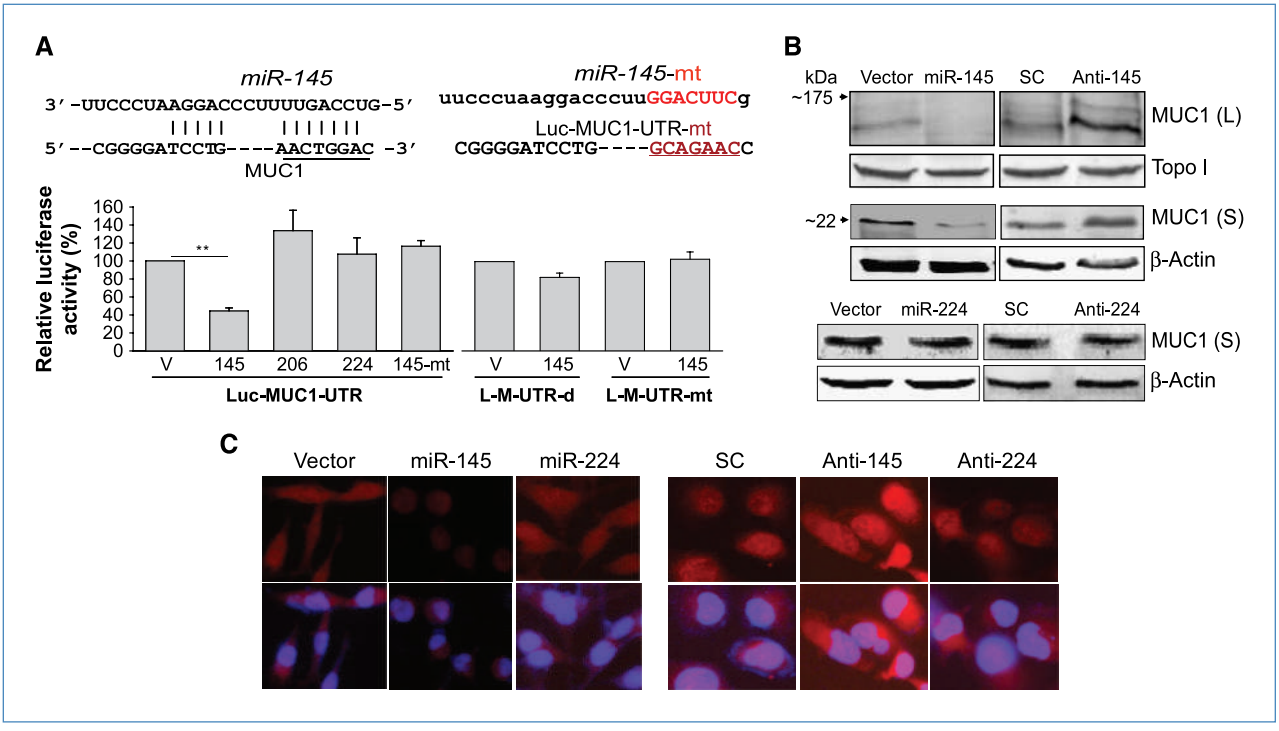

Figure 2. *miR-145* directly targets MUC1. *A,* effect of *miR-145* on MUC1 3′-UTR luciferase reporters. *Top,* alignment of MUC1 3′-UTR and *miR-145*, along with a mutant *miR-145* or mutant MUC1 3′-UTR in which the sequences in red were deleted or mutagenized. *Bottom,* a luciferase reporter carrying the 3′-UTR of MUC1 (*Luc-MUC1-UTR*), deletion of *miR-145* binding site at the 3′-UTR (*Luc-MUC1-UTR-d*), or mutant 3′-UTR in which the *miR-145* binding site was mutated (*Luc-MUC1-UTR-mt*) was introduced into 293T cells along with *miR-145* (*145*), *miR-206* (*206*), *miR-224* (*224*), mutant *miR-145* (145-mt), or vector control (*V*). The cells were harvested 24 h later for luciferase assays. *miR-206* and *miR-224* serve as a negative control. *B, miR-145* suppresses, whereas anti–*miR-145* enhances, the endogenous protein levels of MUC1, as detected by Western blot. Stably transduced MDA-MB-231 cells ectopically expressing *miR-145* or transiently transfected with anti–*miR-145* were used for Western blot analysis. *Top,* effect of *miR-145* or anti–*miR-145* on large (*L*) and small (*S*) isoforms of MUC1. *Bottom,* stably transduced MDA-MB-231 cells with *miR-224* reveal no suppression of MUC1. Topoisomerase I (*topo I*) and β-actin serve as loading controls. *C,* immunofluorescence staining further confirms suppression of MUC1 by *miR-145* in stably transduced MDA-MB-231 cells. The cells were grown on coverslips overnight and then subjected to immunostaining with anti-MUC1 antibody, as described in Materials and Methods. All images with red signals (MUC1) were taken at the same fixed time. Merged pictures are overlays of both MUC1 red signals and nuclear staining by Hoechst dye (*blue*). *Columns,* mean of three independent experiments; *bars,* SEM. **, $P < 0.01$; *SC,* scrambled oligo; *anti-145,* anti–*miR-145*.

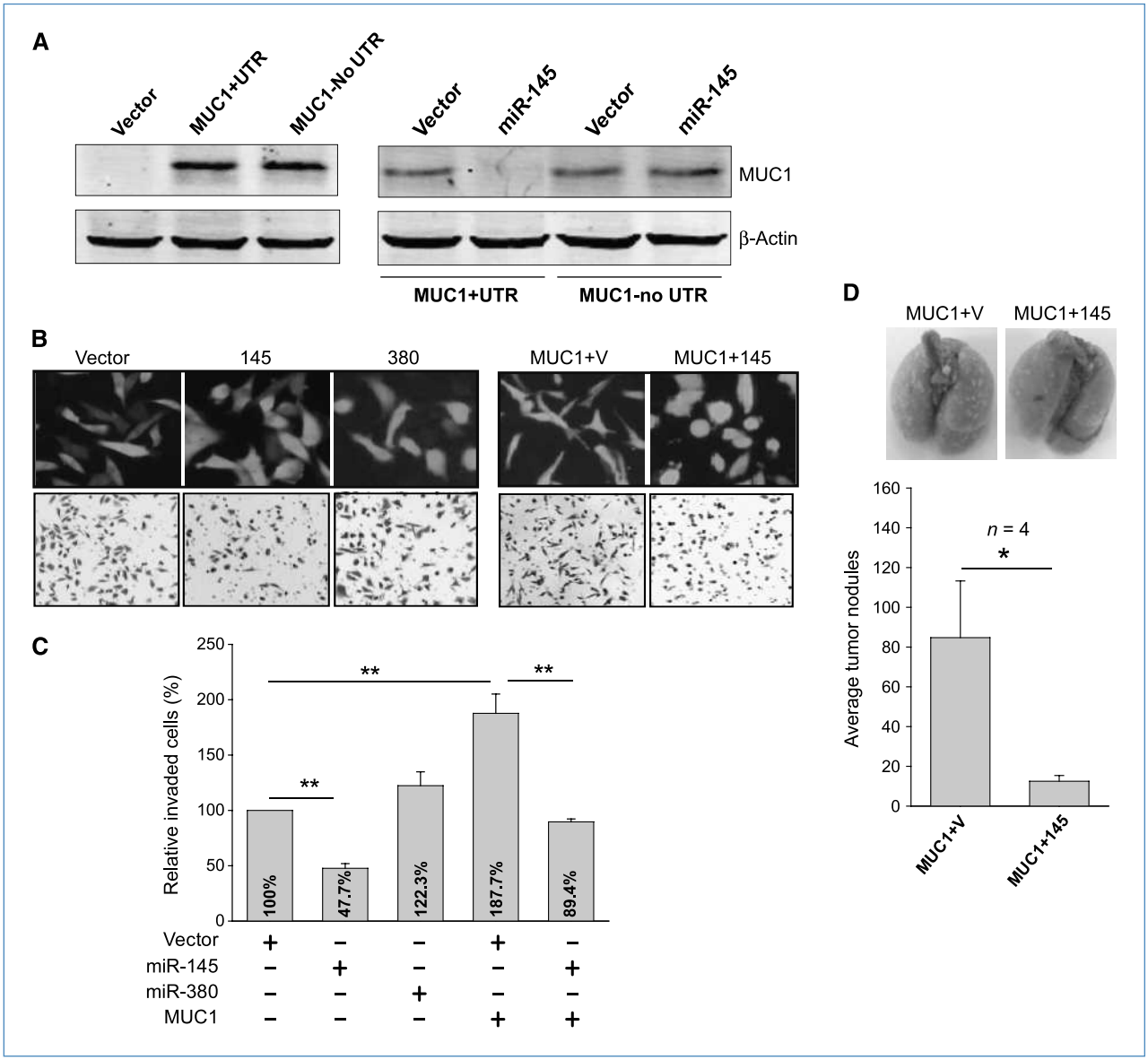

Figure 3. Ectopically expressed MUC1 promotes cell invasion that can be reversed by *miR-145*. *A, left,* expression vector carrying Myc-tagged MUC1 with or without the 3′-UTR was introduced into 293T cells, and Western blot was performed 24 h after transfection. The membrane was probed with a Myc tag antibody. *Right, miR-145* suppresses MUC1 with the corresponding 3′-UTR. 293T cells were transfected with UTR (*MUC1+UTR*) or without UTR (*MUC1-no UTR*) along with vector alone or *miR-145*. The cells were harvested for extraction of protein 24 h after transfection. *B, top,* effect of *miR-145* on morphology changes. *Left three pictures,* stably transduced MDA-MB-231 cells with vector pCDH, *miR-145* (*145*), or *miR-380* (*380*). We used *miR-380* as a negative control here instead of *miR-224* because our separate studies suggested that *miR-224* is able to suppress invasion by targeting other metastatic genes (43). *Right two pictures,* MDA-MB-231 cells infected with MUC1 along with vector control (*V*) or *miR-145* (*145*). Images were taken before invasion assays. *Bottom,* representative fields of invaded cells on the membranes after invasion assays. *C,* quantitative analysis of invasion assays. *D,* suppression of MUC1-mediated metastasis by *miR-145* in experimental metastasis assays. MDA-MB-231 cells ectopically expressing MUC1 and vector (*MUC1+V*) or MUC1 and *miR-145* (*MUC1+145*) were injected into female nude mice through the tail vein as detailed in Materials and Methods. Values in *C* are means of three independent experiments; *bars,* SEM. **, $P < 0.01$; *, $P < 0.05$.

We also tested two additional metastatic breast cancer cell lines, LM2-4142 and BT-549. The relative expression levels of *miR-145* in these cells were shown in Supplementary Fig. S3. Similar to the result in MDA-MB-231 cells, whereas *miR-145* suppressed the level of MUC1, anti–*miR-145* increased the level of MUC1 in these cells (Supplementary Fig. S4). Finally, we confirmed this *miR-145*–mediated repression of MUC1 by immunofluoroscence staining. The red signal of (MUC1) in the *miR-145*–transduced MDA-MB-231 (Fig. 2*C*) and LM2-4142 cells (Supplementary Fig. S5) were visibly low compared with that of the cells infected with vector control, especially compared with the cells transfected with

anti–*miR-145* (Supplementary Fig. S5; Fig. 2*C, middle of right panel*), supporting the suppressive role of *miR-145* in MUC1 expression.

Real-time RT-PCR analysis detected a slight reduction of MUC1 mRNA in *miR-145* cells compared with the vector control in MDA-MB-231 or LM2-4142 cells, but this difference was not significant (Supplementary Fig. S6), suggesting that *miR-145* silences MUC1 mainly at the translational level.

Ectopic expression of MUC1 promotes invasiveness. To confirm the role of *miR-145*–mediated MUC1 suppression in invasion, we cloned the coding region of MUC1 with or without the 3′-UTR and tagged them with the Myc epitope. We first verified the ectopic expression for these two constructs. About the same amount of the exogenous MUC1 was expressed with or without the 3′-UTR (Fig. 3*A, left*). Cotransfection with *miR-145* caused a marked reduction of MUC1 protein level for the clone carrying the 3′-UTR, but not with the construct carrying no 3′-UTR (Fig. 3*A, right*).

Having shown that MUC1 is functionally expressed, we next determined the invasive potential of MUC1. We found notable morphology changes in these cells; they appeared more elongated, a more invasive phenotype that can be reversed by *miR-145* (Fig. 3*B, top right*). In contrast, ~30% *miR-145* cells were round and with more epithelial characteristics compared with <5% round cells for vector control (Fig. 3*B, top left*). Consistent with the morphologic changes, MUC1 enhanced cell invasiveness (Fig. 3*C*), which was inhibited by *miR-145*. Experimental metastasis assays further supported the role of *miR-145* in suppressing invasion and metastasis by targeting MUC1. For example, MUC1 plus vector produced an average of 85 tumor nodules compared with

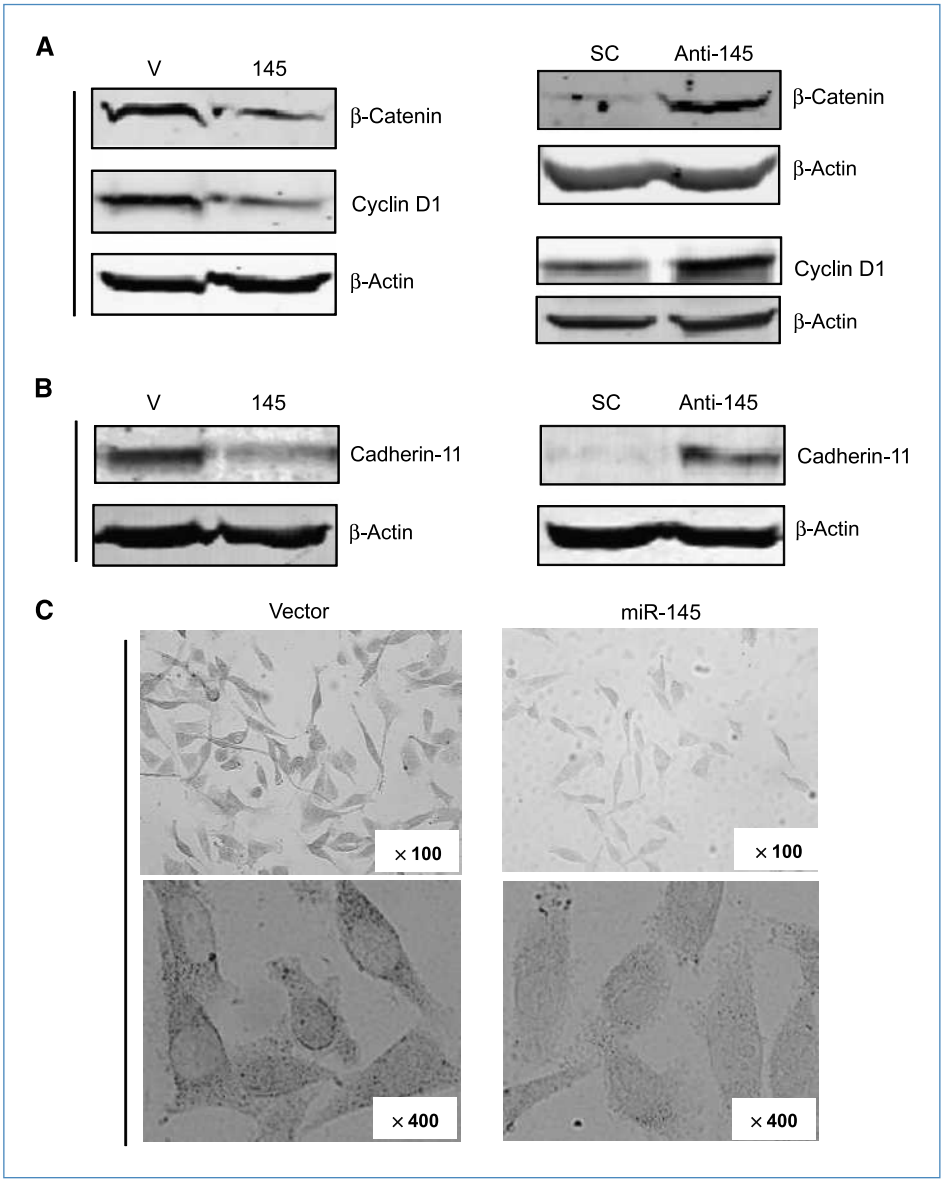

Figure 4. Effect of *miR-145* on β-catenin and cadherin 11. *A* and *B, miR-145* suppresses, whereas anti–*miR-145* enhances, β-catenin, cyclin D1, and cadherin 11. Stably transduced MDA-MB-231 cells ectopically expressing vector alone (*V*) or *miR-145* (*145*), and MDA-MB-231 cells transfected with scrambled oligo (*SC*) or anti–*miR-145* were used for Western blot analysis. *C,* suppression of cadherin 11 by *miR-145* in MDA-MB-231 cells as detected by IHC. Stably transduced MDA-MB-231 cells ectopically expressing vector alone or *miR-145* were used for IHC analysis as detailed in Materials and Methods.

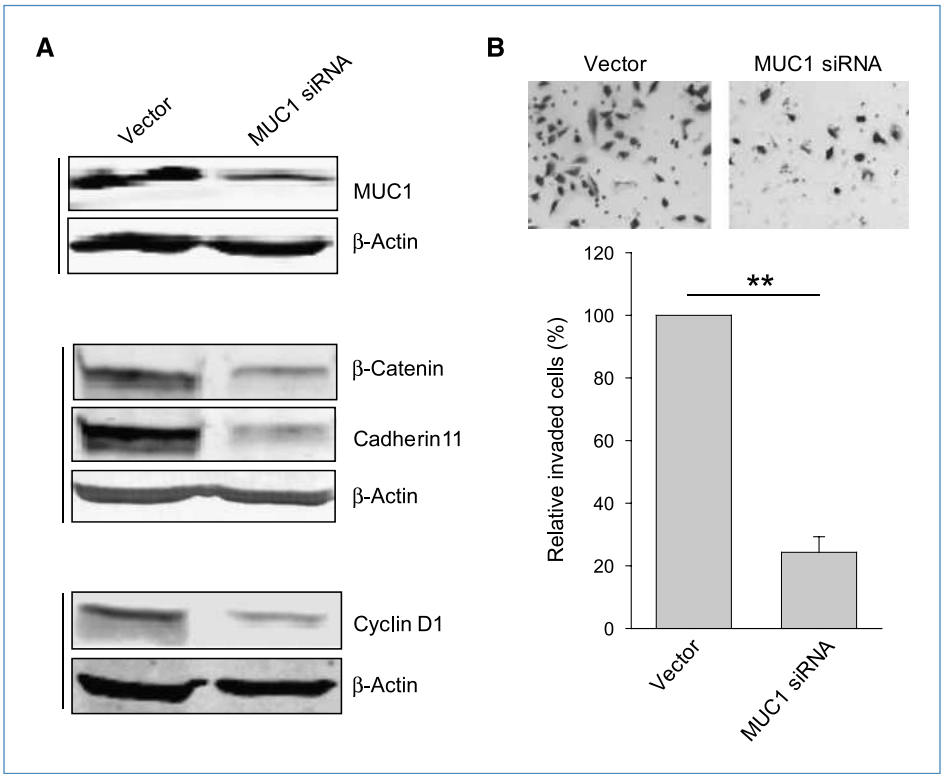

Figure 5. Suppression of MUC1 by RNAi reduces levels of β-catenin, cyclin D1, and cadherin 11 as well as invasiveness. *A,* effect of MUC1 siRNA on MUC1 protein as well as β-catenin, cadherin 11, and cyclin D1. *B,* suppression of cell invasion by MUC1 siRNA. MDA-MB-231 cells were transfected with MUC1 siRNA, followed by invasion assay as detailed in Materials and Methods. *Columns,* means of three separate experiments; *bars,* SEM. **, $P < 0.01$.

13 nodules from the MUC1 plus *miR-145* cells (Fig. 3*D*), similar to the invasion results.

Effect of miR-145 on cadherin 11. MUC1 promotes cell invasion and metastasis possibly through interactions with different cell signaling molecules, causing stabilization of β-catenin (25). Thus, suppression of MUC1 by *miR-145* leading to the reduction of cell invasion is of particular interest because this may suggest that *miR-145* also affects the level of β-catenin. Therefore, we first determined the effect of *miR-145* on β-catenin. As expected, suppression of MUC1 by *miR-145* decreased the level of β-catenin and its downstream gene cyclin D1 (Fig. 4*A, left*). In contrast, anti–*miR-145* increased β-catenin and cyclin D1 (Fig. 4*A, right*). *In silico* analysis did not find any *miR-145* binding site in the 3′-UTRs of β-catenin and cyclin D1; furthermore, luciferase reporters carrying the corresponding UTR revealed no effect by *miR-145* (data not shown). Thus, the suppression of β-catenin and cyclin D1 by *miR-145* is possibly an indirect effect through MUC1. It has been reported that β-catenin forms a complex with E-cadherin and downregulation of MUC1 in cancer cells inhibits cell migration by promoting the E-cadherin/catenin complex formation (26). However, there is no detectable E-cadherin in MDA-MB-231 cells, which instead express a high level of cadherin 11 (27). Evidence further suggests that cadherin 11 plays an oncogenic role and it also interacts with β-catenin (27–29). Therefore, we examined the effect of *miR-145* on cadherin 11. Whereas *miR-145* suppressed cadherin 11, anti–*miR-145* increased cadherin 11 (Fig. 4*B*). We further confirmed by IHC that cadherin 11 was repressed in MDA-MB-231 cells expressing *miR-145* (Fig. 4*C*). Therefore, suppression of cadherin 11 by *miR-145* through targeting MUC1 is likely to contribute to the *miR-145*–mediated reduction of cell invasion.

To further determine the role of MUC1 in *miR-145*–mediated suppression of invasion and metastasis, we suppressed MUC1 by RNAi (Fig. 5*A, top*). This suppression of MUC1 significantly reduced invasiveness (Fig. 5*B*). At the same time, MUC1 siRNA also caused downregulation of β-catenin and cadherin 11 as well as cyclin D1 (Fig. 5*A*), similar to the effect of *miR-145* on these proteins (Fig. 4*A* and *B*). These results suggest that *miR-145* targets MUC1, which in turn causes downregulation of β-catenin, cyclin D1, and cadherin 11, leading to suppression of invasion and metastasis.

MUC1 is upregulated in breast cancer specimens. Finally, we examined MUC1 expression in matched breast tumor specimens to determine its clinical relevance. As shown in Fig. 6*A*, MUC1 level was visibly elevated in four of six cases of advanced breast cancer with an average value of 2.4 compared with the matched normal tissue with a value of 1.0 (Fig. 6*B*), which inversely correlated with *miR-145* expression (Fig. 6*C*). We then determined whether metastasis status affects MUC1 expression by IHC staining breast cancer tissue microarrays and found that, in general, MUC1 level was higher in metastatic tumors than in nonmetastatic tumors (Supplementary Table S1; Fig. 6*D*), which is consistent with the previous reports that MUC1 correlates with poor survival in colorectal cancer (30) and breast cancer (31). Downregulation of *miR-145* could derepress MUC1 as tumor cells progress and metastasize; thus, modulation of *miR-145* may provide a therapeutic strategy for metastatic breast cancer.

Discussion

We have previously shown that *miR-145* is able to suppress tumor cell growth both *in vitro* and *in vivo* in part through targeting the oncogene c-Myc (12). Although c-Myc has been implicated in indirect regulation of invasion and metastasis, our study suggests that factors other than c-Myc may play a more important role in *miR-145*–mediated suppression of cell invasion. The present study shows that MUC1 is a direct target for *miR-145*. Ectopic expression of MUC1 and siRNA knockdown confirmed its invasion-promoting activity. Moreover, *miR-145*–mediated suppression of MUC1 is dependent on the 3′-UTR. Finally, MUC1-induced cell invasion can be reversed by *miR-145*. Therefore, these results highlight the significance of *miR-145* as a tumor suppressor in cell invasion and metastasis by targeting MUC1.

Increasing evidence suggests that MUC1 plays a role in invasion and metastasis (30). For example, MUC1 promotes invasion in breast cancer by interacting with β-catenin (29); moreover, MUC1 and β-catenin are coexpressed at the invasion front of colorectal carcinomas, which correlates with poor prognosis in colorectal cancer (30). However, the precise role of MUC1 in invasion and metastasis as well as its regulation is not well understood. MUC1 is a member of a large mucin family, which are characterized by a variable number of tandem repeats (VNTR) responsible for its glycosylation. MUC1 belongs to type I membrane glycoprotein subfamily and possess a single membrane–spanning domain and a short cytoplasmic tail in addition to the extensive extracellular domain (22). MUC1 has seven variants that differ mainly in the VNTR region. Because all variants share the same 3′-UTR, *miR-145* is expected to be able to suppress all of the variants. Our Western blot data support this notion (Fig. 2*B*),

The role of MUC1 in invasion and metastasis has been shown in different models. For example, the cytoplasmic tail of MUC1 was reported to enhance the invasion of MDA-MB-468 breast cancer cells expressing wild-type GSK-3β and β-catenin (32), suggesting possible interactions between these proteins. Moreover, MUC1 expression is associated with increased steady-state levels of β-catenin in the cytoplasm and nucleus of breast carcinoma cells by blocking the GSK-3β–mediated phosphorylation of β-catenin, thus preventing proteosomal degradation (29). It is possible that the cytoplasmic tail of MUC1 serves as a scaffold protein, enabling interaction between different regulators or alternatively might compete for or sequester β-catenin. In some cell types, the MUC1 cytoplasmic tail is also involved in the transcriptional activation of β-catenin–TCF binding sites and transcriptional activation of cyclin D1 (25). Consistent with this finding, we detected decreased levels of β-catenin and cyclin D1 in the *miR-145* cells (Fig. 4*A*). Moreover, MUC1 may play an antiapoptotic role in response to cellular stresses by stimulating Akt and the antiapoptotic protein Bcl-X to attenuate genotoxin-induced apoptosis (33). A recent report suggests that this MUC1-mediated tumorigenesis is likely through the transforming growth factor-α signaling pathway (34).

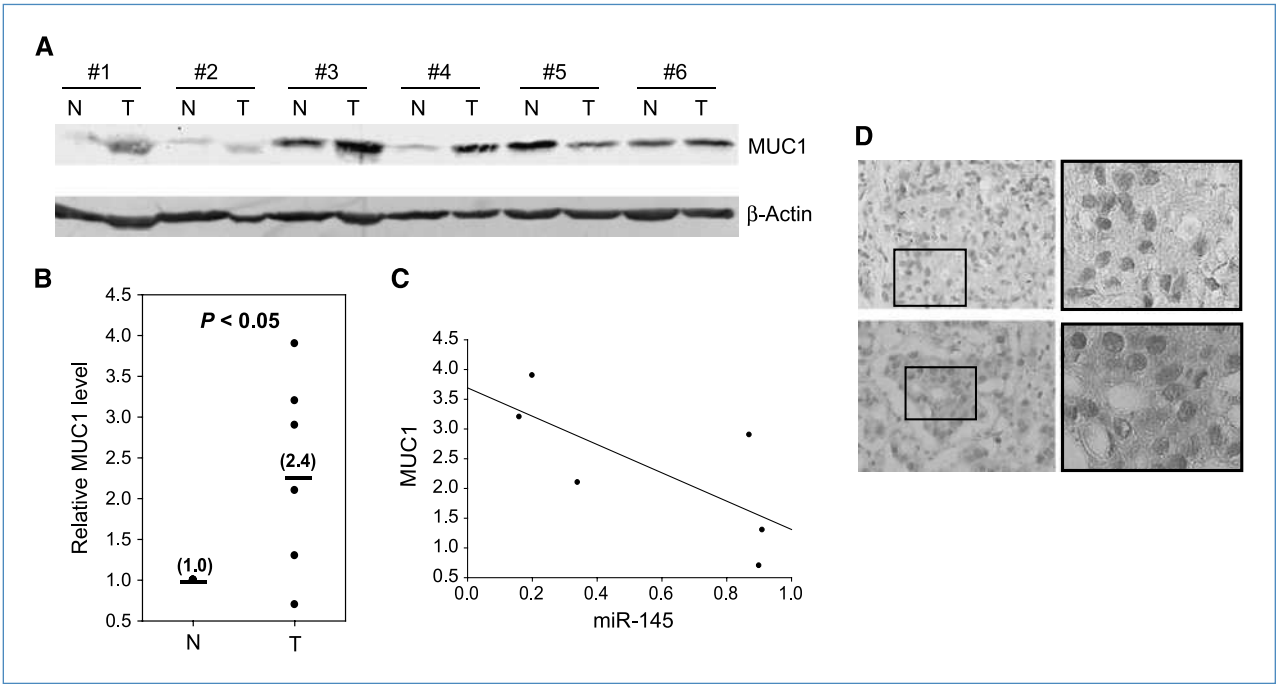

Figure 6. Expression of MUC1 in breast tumor specimens. *A* and *B*, Western blot reveals upregulation of MUC1 in breast tumors (*T*) compared with matched normal breast tissue (*N*). *C*, a negative correlation between MUC1 and *miR-145* in breast tumor specimens after normalization with normal tissue. *D*, expression of MUC1 in breast tumor specimens with different metastasis status by IHC. *Top*, infiltrating ductal carcinoma without metastasis; *bottom*, infiltrating ductal carcinoma with metastasis.

Although MUC1 is often deregulated in tumors, information about its regulation is limited. In one report, MUC1 was shown to be induced by hypoxia in a lung adenocarcinoma cell line (35); a recent report indicates that MUC1 and galectin-3 oncoproteins function in a miRNA-dependent regulatory loop (36). N-glycosylated MUC1 COOH-terminal subunit increases galectin-3 mRNA levels by suppressing expression of *miR-322* and thereby stabilizing galectin-3. However, it is not clear whether MUC1 itself is subject to miRNA regulation. Our study establishes the posttranscriptional regulation of MUC1 by *miR-145*.

Clinically, MUC1 overexpression occurs frequently in many types of cancer, including breast, ovarian, lung, colon, and pancreatic carcinomas (22, 23); its expression tends to increase as tumors progress (37, 38). Consistent with these reports, we also observed an increased level of MUC1 in more metastatic breast tumors compared with less metastatic breast tumors. However, the precise mechanism of MUC1-mediated metastasis remains to be elucidated. Mucin plays an important role in protecting normal cells from pathogen but tumor cells may use this protective shield to evade from immune system (39). In addition, MUC1 may enhance metastasis in part through interaction with adhesion molecules such as the endothelial protein ICAM-1 (40) or by serving as a ligand for galectin-3 (41).

Our study suggests that MUC1-mediated invasion may involve the oncogenic cadherin 11 because *miR-145* also causes downregulation of cadherin 11 in MDA-MB-231 cells. It has been previously shown that MDA-MB-231 has lost the epithelial marker type I cadherins but it expresses an oncogenic cadherin 11 instead (27). Further evidence suggests that cadherin 11 is expressed in several types of cancers, including breast cancer; moreover, cadherin 11 expression promotes the metastasis of prostate cancer cells to bone (42). Although the underlying mechanism remains to be determined, this downregulation of cadherin 11 by *miR-145* is possibly through MUC1 and β-catenin. Given that MUC1 interacts with β-catenin and enhances the β-catenin level (29), it would be reasonable to speculate that this MUC1-mediated β-catenin expression also affects cadherin 11; thus, a likely consequence of ectopic expression of *miR-145* is a reduction of cadherin 11 through suppression of MUC1. In support of this, we found that *miR-145* suppresses MUC1, β-catenin, and cadherin 11 (Fig. 4*A* and *B*). Moreover, suppression of MUC1 by RNAi also downregulates β-catenin and cadherin 11 (Fig. 5*A*). Therefore, further characterization of this pathway will provide a new insight into *miR-145*–mediated suppression of invasion and metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Nicoloso MS, Spizzo R, Shimizu M, Rossi S, Calin GA. MicroRNAs—the micro steering wheel of tumour metastases. Nat Rev Cancer 2009;9:293–302.
2. Pillai RS. MicroRNA function: multiple mechanisms for a tiny RNA? RNA 2005;11:1753–61.
3. Zamore PD, Haley B. Ribo-gnome: the big world of small RNAs. Science 2005;309:1519–24.
4. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–97.
5. Ambros V. The functions of animal microRNAs. Nature 2004;431:350–5.
6. Esquela-Kerscher A, Slack FJ. Oncomirs—microRNAs with a role in cancer. Nat Rev Cancer 2006;6:259–69.
7. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009;136:215–33.
8. Chen CZ. MicroRNAs as oncogenes and tumor suppressors. N Engl J Med 2005;353:1768–71.
9. Sempere LF, Christensen M, Silahtaroglu A, et al. Altered MicroRNA expression confined to specific epithelial cell subpopulations in breast cancer. Cancer Res 2007;67:11612–20.
10. Iorio MV, Ferracin M, Liu CG, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer Res 2005;65:7065–70.
11. Schepeler T, Reinert JT, Ostenfeld MS, et al. Diagnostic and prognostic MicroRNAs in stage II colon cancer. Cancer Res 2008;68:6416–24.
12. Sachdeva M, Zhu S, Wu F, et al. p53 represses c-Myc through induction of the tumor suppressor miR-145. Proc Natl Acad Sci U S A 2009;106:3207–12.
13. Shi B, Sepp-Lorenzino L, Prisco M, et al. Micro RNA 145 targets the insulin receptor substrate-1 and inhibits the growth of colon cancer cells. J Biol Chem 2007;282:32582–90.
14. Yu T, Wang XY, Gong RG, et al. The expression profile of microRNAs in a model of 7,12-dimethyl-benz[*a*]anthrance-induced oral carcinogenesis in Syrian hamster. J Exp Clin Cancer Res 2009;28:64.
15. Cordes KR, Sheehy NT, White MP, et al. miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. Nature 2009;460:705–10.
16. Minn AJ, Kang Y, Serganova I, et al. Distinct organ-specific metastatic potential of individual breast cancer cells and primary tumors. J Clin Invest 2005;115:44–55.
17. Chen C, Ridzon DA, Broomer AJ, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 2005;33:e179.
18. Zhu S, Si ML, Wu H, Mo YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). J Biol Chem 2007;282:14328–36.
19. Wu F, Chiocca S, Beck WT, Mo YY. Gam1-associated alterations of drug responsiveness through activation of apoptosis. Mol Cancer Ther 2007;6:1823–30.
20. Zhang XY, DeSalle LM, Patel JH, et al. Metastasis-associated protein 1 (MTA1) is an essential downstream effector of the c-MYC oncoprotein. Proc Natl Acad Sci U S A 2005;102:13968–73.
21. Grimson A, Farh KK, Johnston WK, et al. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell 2007;27:91–105.
22. Thornton DJ, Rousseau K, McGuckin MA. Structure and function of

the polymeric mucins in airways mucus. Annu Rev Physiol 2008;70: 459–86.
23. Ren J, Agata N, Chen D, et al. Human MUC1 carcinoma-associated protein confers resistance to genotoxic anticancer agents. Cancer Cell 2004;5:163–75.
24. Mahanta S, Fessler SP, Park J, Bamdad C. A minimal fragment of MUC1 mediates growth of cancer cells. PLoS ONE 2008;3:e2054.
25. Huang L, Chen D, Liu D, et al. MUC1 oncoprotein blocks glycogen synthase kinase 3β-mediated phosphorylation and degradation of β-catenin. Cancer Res 2005;65:10413–22.
26. Huang L, Ren J, Chen D, et al. MUC1 cytoplasmic domain coactivates Wnt target gene transcription and confers transformation. Cancer Biol Ther 2003;2:702–6.
27. Pishvaian MJ, Feltes CM, Thompson P, et al. Cadherin-11 is expressed in invasive breast cancer cell lines. Cancer Res 1999;59:947–52.
28. Shibata T, Ochiai A, Gotoh M, Machinami R, Hirohashi S. Simultaneous expression of cadherin-11 in signet-ring cell carcinoma and stromal cells of diffuse-type gastric cancer. Cancer Lett 1996;99:147–53.
29. Schroeder JA, Adriance MC, Thompson MC, Camenisch TD, Gendler SJ. MUC1 alters β-catenin-dependent tumor formation and promotes cellular invasion. Oncogene 2003;22:1324–32.
30. Baldus SE, Monig SP, Huxel S, et al. MUC1 and nuclear β-catenin are coexpressed at the invasion front of colorectal carcinomas and are both correlated with tumor prognosis. Clin Cancer Res 2004;10: 2790–6.
31. Baldus SE, Engelmann K, Hanisch FG. MUC1 and the MUCs: a family of human mucins with impact in cancer biology. Crit Rev Clin Lab Sci 2004;41:189–231.
32. Lillehoj EP, Han F, Kim KC. Mutagenesis of a Gly-Ser cleavage site in MUC1 inhibits ectodomain shedding. Biochem Biophys Res Commun 2003;307:743–9.
33. Raina D, Kharbanda S, Kufe D. The MUC1 oncoprotein activates the anti-apoptotic phosphoinositide 3-kinase/Akt and Bcl-xL pathways in rat 3Y1 fibroblasts. J Biol Chem 2004;279:20607–12.
34. Pochampalli MR, Bitler BG, Schroeder JA. Transforming growth factor α dependent cancer progression is modulated by Muc1. Cancer Res 2007;67:6591–8.
35. Mikami Y, Hisatsune A, Tashiro T, Isohama Y, Katsuki H. Hypoxia enhances MUC1 expression in a lung adenocarcinoma cell line. Biochem Biophys Res Commun 2009;379:1060–5.
36. Ramasamy S, Duraisamy S, Barbashov S, et al. The MUC1 and galectin-3 oncoproteins function in a microRNA-dependent regulatory loop. Mol Cell 2007;27:992–1004.
37. Kohlgraf KG, Gawron AJ, Higashi M, et al. Contribution of the MUC1 tandem repeat and cytoplasmic tail to invasive and metastatic properties of a pancreatic cancer cell line. Cancer Res 2003;63: 5011–20.
38. Nakamori S, Ota DM, Cleary KR, Shirotani K, Irimura T. MUC1 mucin expression as a marker of progression and metastasis of human colorectal carcinoma. Gastroenterology 1994;106: 353–61.
39. Carraway KL, Ramsauer VP, Carraway CA. Glycoprotein contributions to mammary gland and mammary tumor structure and function: roles of adherens junctions, ErbBs and membrane MUCs. J Cell Biochem 2005;96:914–26.
40. Rahn JJ, Chow JW, Horne GJ, et al. MUC1 mediates transendothelial migration *in vitro* by ligating endothelial cell ICAM-1. Clin Exp Metastasis 2005;22:475–83.
41. Yu LG, Andrews N, Zhao Q, et al. Galectin-3 interaction with Thomsen-Friedenreich disaccharide on cancer-associated MUC1 causes increased cancer cell endothelial adhesion. J Biol Chem 2007;282: 773–81.
42. Chu K, Cheng CJ, Ye X, et al. Cadherin-11 promotes the metastasis of prostate cancer cells to bone. Mol Cancer Res 2008;6: 1259–67.
43. Zhu S, Sachdeva M, Wu F, Lu Z, Mo YY. Ubc9 promotes breast cell invasion and metastasis in a sumoylation-independent manner. Oncogene. In press, 2009.