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MicroRNA-145 suppresses cell invasion and metastasis by directly targeting mucin 1

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Abstract

MicroRNAs (miRNAs) 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.

Keywords

breast cancer; invasion; metastasis; miRNA; miR-145; posttranscriptional regulation

Cell migration and invasion are the major features of metastatic tumor cells which are responsible for the most of cancer related death. It is well known that the potential of a tumor cell to metastasize depends on numerous factors. Accumulating evidence suggests that microRNAs (miRNAs) could be key players in regulation of tumor cell invasion and metastasis (1). miRNAs are small non-coding 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 of targets (7) and thus a large number of protein-coding genes could be under control of miRNAs. As a result, miRNAs play a fundamental role in 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 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 repressing 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 demonstrated that *miR-145* is a tumor suppressor impacting invasion and metastasis in part by targeting mucin 1 (MUC1).

Materials and Methods

Reagents

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

Cell culture

All cell lines were purchased from American Tissue Culture Collection (ATCC) (Manassas, VA) except for LM2-4142 (16) which was a generous gift from Dr. Joan Massagué (Sloan-Kettering Institute). Breast cancer cell lines BT-549, MDA-MB-231 and LM2-4142 were grown in RPMI 1640 (Cambrex, Walkersville, MD) supplemented with 10% FBS (Sigma-Aldrich). HEK-293T cells were cultured in DMEM (Cambrex) supplemented with 10% FBS. All media contained 2 mM glutamine, 100 units of penicillin/ml, and 100 μ g of streptomycin/ml. Cells were incubated at 37 °C and supplemented with 5% CO₂ 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 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-Not1-3.1 (5'GCGGCCGCCTACAAGTTGGCAGAAGTGGC; for MUC1 with UTR we used primers MUC1-R1-5.1 and MUC1-UTR-Not1-3.1 (see below). The PCR product was first cloned into a PCR cloning vector (pCR8) and then subcloned into pCMV-Myc at EcoR1 and Not1 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, Madison, WI). We first amplified the MUC1 3'-UTR sequence by PCR using primers MUC1-UTR-5.1 (5'TCTGCCAACTTG TAGGGGCAC) and MUC1-UTR-Not1-3.1 (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 EcoR1 and Not1 sites were introduced into the original Xba1 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-Not13.2 (5'GCGGCCGCCAGGATCCCCGCTATCTCAGG), and then cloned into the modified pGL3 control vector at EcoR1 and Not1 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'CAGCTGCCCGTAGTTCCTTC); 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 [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays according to standard methods, as previously described (18).

Invasion assay

Matrigel chambers (BD Biosciences, San Jose, CA) 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/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 while 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 (IHC)

Immunohistochemistry 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 horse radish peroxidase (HRP). Signals were revealed 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 immunohistochemical staining.

Experimental metastasis assay

Female athymic nude (nu/nu) mice (4–5 weeks old) were purchased from Harlan Sprague Dawley (Indianapolis, IN) 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 SIU 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, 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 less than 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 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 like MDA-MB-231 and LM2-4142 (S-Fig. 1), suggesting cell type-specific function of *miR-145*. Of note, we observed changes in morphology of the *miR-145* infected cells which revealed a less invasive morphological phenotype, i.e., round and flat compared to 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* impacts cell invasion by matrigel chamber assays. As expected, *miR-145* significantly impaired invasion (Fig. 1A). For example, in MDA-MB-231 cells, *miR-145* caused a reduction of invasive cells by over 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 about 75%) in LM2-4142 whereas anti-*miR-145* increased their invasiveness (Fig. 1C). To determine whether *miR-145* suppresses cell invasion *in vivo*, we used an experimental metastasis model by tail vein injection. As shown in Fig 1B, while 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. 1D). 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 demonstrated 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 (not shown), which is not comparable to 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 which is upregulated in several types of tumors (22,23). Therefore, we further characterized how *miR-145* suppresses MUC1.

As shown in Fig. 2A, *miR-145* suppressed over 55% activity for Luc-MUC1-UTR compared to 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. 2A, top panels), 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. 2A, bottom).

Next, we determined whether ectopic expression of *miR-145* can suppress the endogenous MUC1 at the protein level by Western blot. It has been reported of several MUC1 isoforms in the lysates of MUC1-positive cultured cancer cells: high molecular weight isoforms (150–300 kDa) presumably due to glycosylation and a low molecular weight isoform (20–35 kDa) (24). As shown in Fig. 2B (top panels), *miR-145* suppressed both large and small isoforms of MUC1. Since the small isoform is the predominant form in cancer cell lines and tumor specimens, and 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 less than 40% of vector control cells, whereas anti-*miR-145* increased its protein level by over 200% (S-Fig. 2). In contrast, *miR-224* or anti-*miR-224* had no effect on MUC1 expression (Fig. 2B, bottom panels). 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 S-Fig. 3. Similar to the result in MDA-MB-231 cells, while *miR-145* suppressed, anti-*miR-145* increased level of MUC1 in these cells (S-Fig. 4). Finally, we confirmed this *miR-145*-mediated repression of MUC1 by immunofluorescence staining. The red signal of (MUC1) in the *miR-145* transduced MDA-MB-231 (Fig. 2C) and LM2-4142 cells (S-Fig. 5) were visibly low compared to that of the cells infected with vector control, especially compared to the cells transfected with anti-*miR-145* (Fig. 2C, middle of right panels and S-Fig. 5), 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 to the vector control in MDA-MB-231 or LM2-4142 cells, but this difference was not significant (S-Fig. 6), 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 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. 3A, left panel). Co-transfection 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. 3A, right panel).

Having demonstrated 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 which can be reversed by *miR-145* (Fig. 3B, top right panels). In contrast, about 30% *miR-145* cells were round and more epithelial characteristic compared to <5% round cells for vector control (Fig. 3B, top left panels). Consistent with the morphological changes, MUC1 enhanced cell invasiveness (Fig. 3C), 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 to 13 nodules from the MUC1 plus *miR-145* cells (Fig. 3D), 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. 4A, left panels). In contrast, anti-*miR-145* increased β -catenin and cyclin D1 (Fig. 4A, right panels). Since *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* (not shown), thus 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 down-regulation 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. While *miR-145* suppressed, anti-*miR-145* increased cadherin 11 (Fig. 4B). We further confirmed by immunohistochemistry that cadherin 11 was repressed in MDA-MB-231 cells expressing *miR-145* (Fig. 4C). 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. 5A, top panels). This suppression of MUC1 significantly reduced invasiveness (Fig. 5B). At the same time, MUC1 siRNA also caused downregulation of β -catenin and cadherin 11 as well as cyclin D1 (Fig. 5A), 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. 6A, MUC1 level was visibly elevated in 4 of 6 cases of

advanced breast cancer with average value of 2.4 compared to the matched normal tissue as 1.0 (Fig. 6B), which inversely correlated with *miR-145* expression (Fig. 6C). We then determined whether metastasis status affects MUC1 expression by immunohistochemical staining breast cancer tissue microarrays and found that, in general, MUC1 level was higher in metastatic tumors than in non-metastatic tumors (Fig. 6D and S-Table 1), 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 and 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 in 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 demonstrates 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 7 variants which differ mainly in the VNTR region. Since all variants share the same 3'-UTR, *miR-145* is expected to be able to suppress all of the variants. Our western data supports this notion (Fig. 2B)

The role of MUC1 in invasion and metastasis has been demonstrated 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-3 β 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-3 β -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. 4A). 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 TGF- α signaling pathway (34).

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 C-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 to 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 tumors 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 and 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. 4A and B). Moreover, suppression of MUC1 by RNAi also downregulates β -catenin and cadherin 11 (Fig. 5A). Therefore, further characterization of this pathway will provide new insight into *miR-145*-mediated suppression of invasion and metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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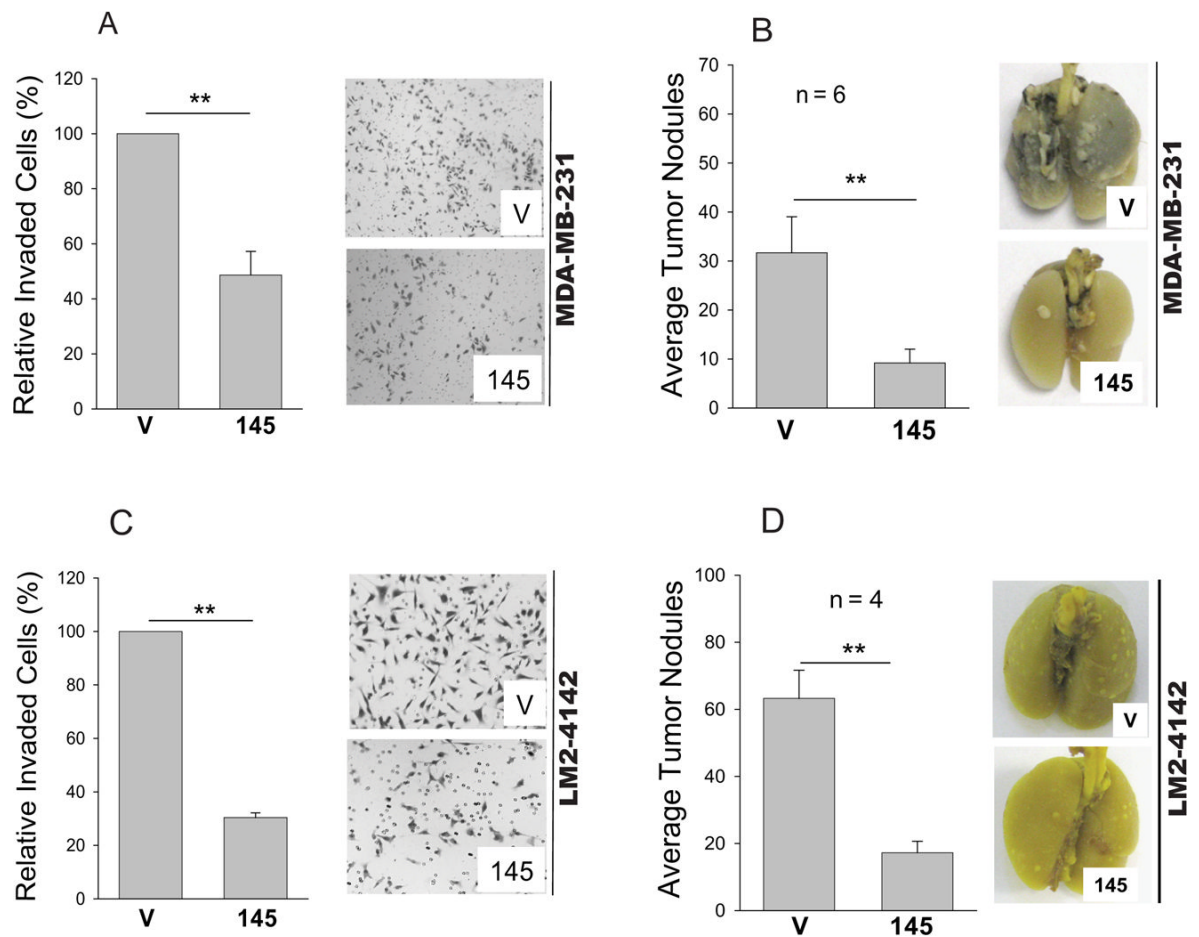


Fig. 1. Effect of *miR-145* on cell invasion and lung metastasis

A & B, Stably transduced MDA-MB-231 or LM2-4142 cells ectopically expressing *miR-145* (145) or vector alone (V) were subject to matrigel chamber assay as detailed in Materials and Methods. Invaded cells on the membrane were counted as follows. Since 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 in a computer screen with grids so that all of the cells on the entire membrane were counted. **B & 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 tail vein as described in Materials and Methods. Values **A** and **C** are means \pm SE of three independent experiments. **, $p < 0.01$; SC, scrambled oligo; anti-145, anti-*miR-145*.

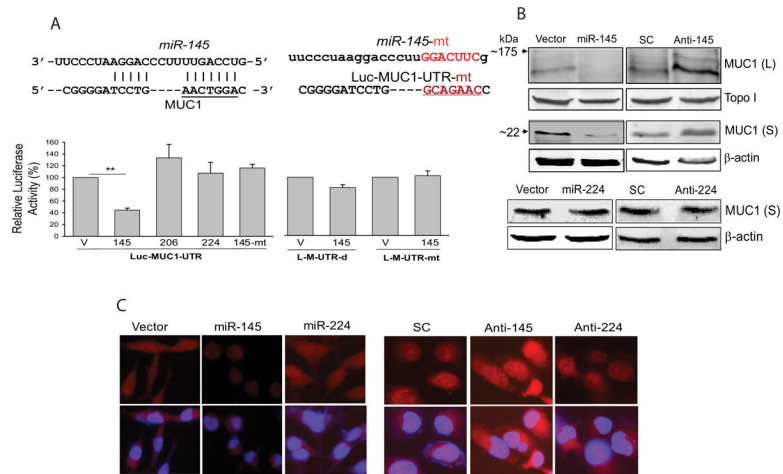


Fig. 2. *miR-145* directly targets MUC1

A, Effect of *miR-145* on MUC1-3'-UTR luciferase reporters. Top panels: 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 panels: 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 that 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) and 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 panels: Effect *miR-145* or anti-*miR-145* on large (L) and small (S) isoforms of MUC1. Bottom panels: 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 subject 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). Values in **A** are means ± SE of three independent experiments. **, $p < 0.01$; SC, scrambled oligo; anti-145, anti-*miR-145*.

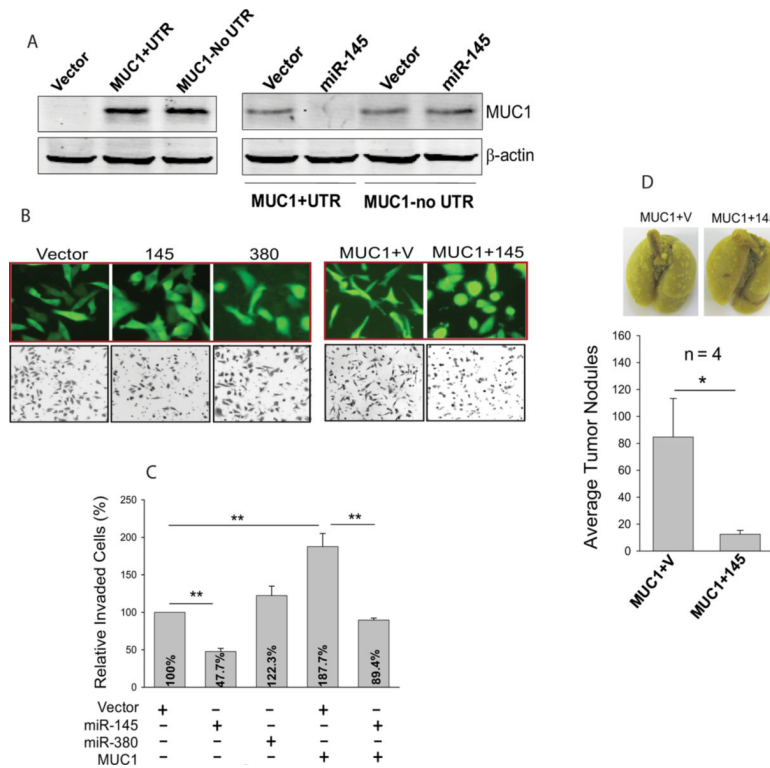


Fig. 3. Ectopically expressed MUC1 promotes cell invasion which can be reversed by *miR-145*
A, Left panel: expression vector carrying Myc-tagged MUC1 with or without the 3'-UTR was introduced into 293T cells and western blot was performed 24 h after transfection. The membrane was probed with Myc tag antibody. Right panel: *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 panels: 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 (Zhu et al unpublished). 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 panels: 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 tail vein as detailed in Materials and Methods. Values in **C** are means \pm SE of three independent experiments. **, $p < 0.01$; *, $p < 0.05$.

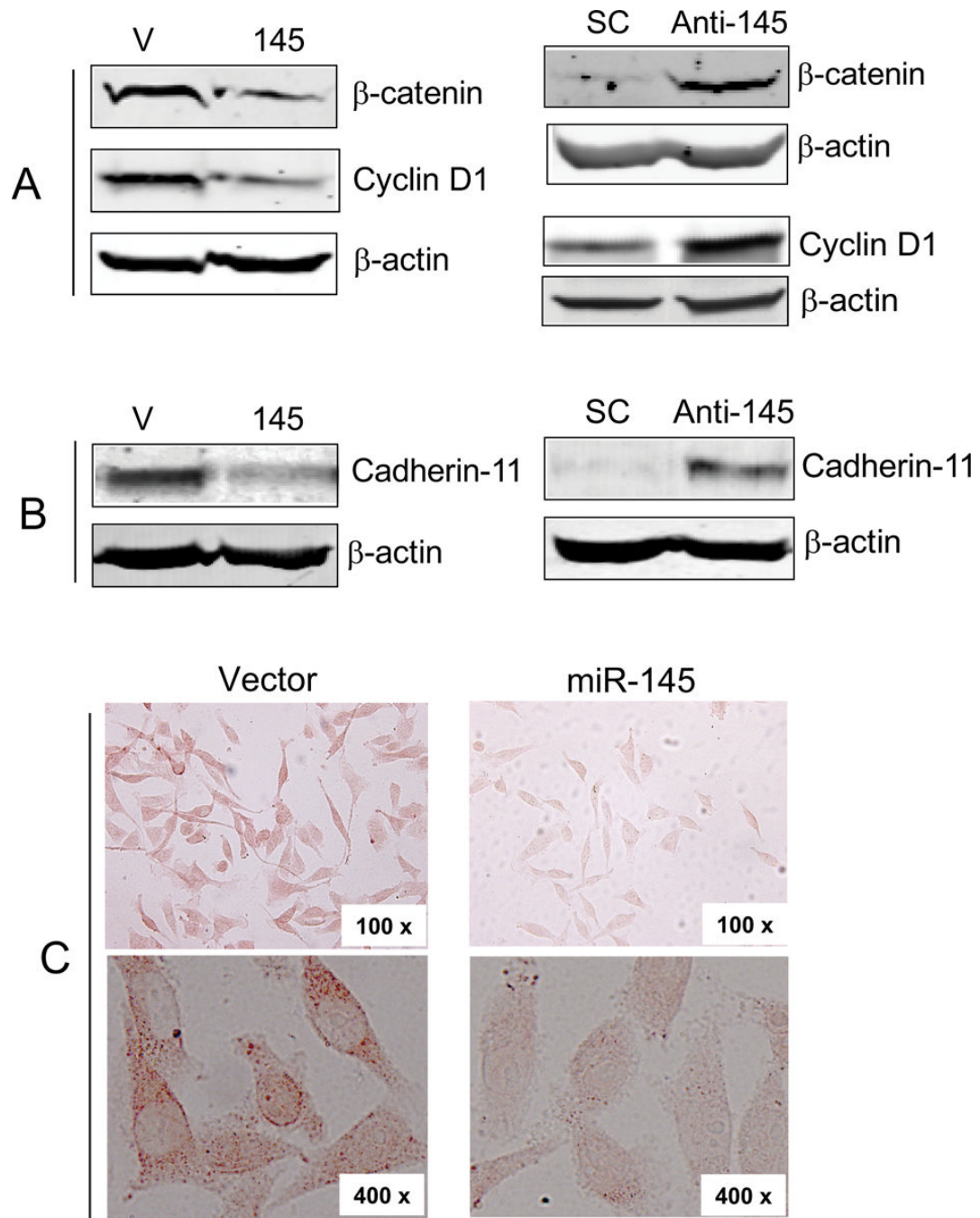


Fig. 4. Effect of *miR-145* on β -catenin and cadherin 11

A and **B**, *miR-145* suppresses, while 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 immunohistochemistry. Stably transduced MDA-MB-231 cells ectopically expressing vector alone or *miR-145* were used for immunohistochemistry analysis as detailed in Materials and Methods.

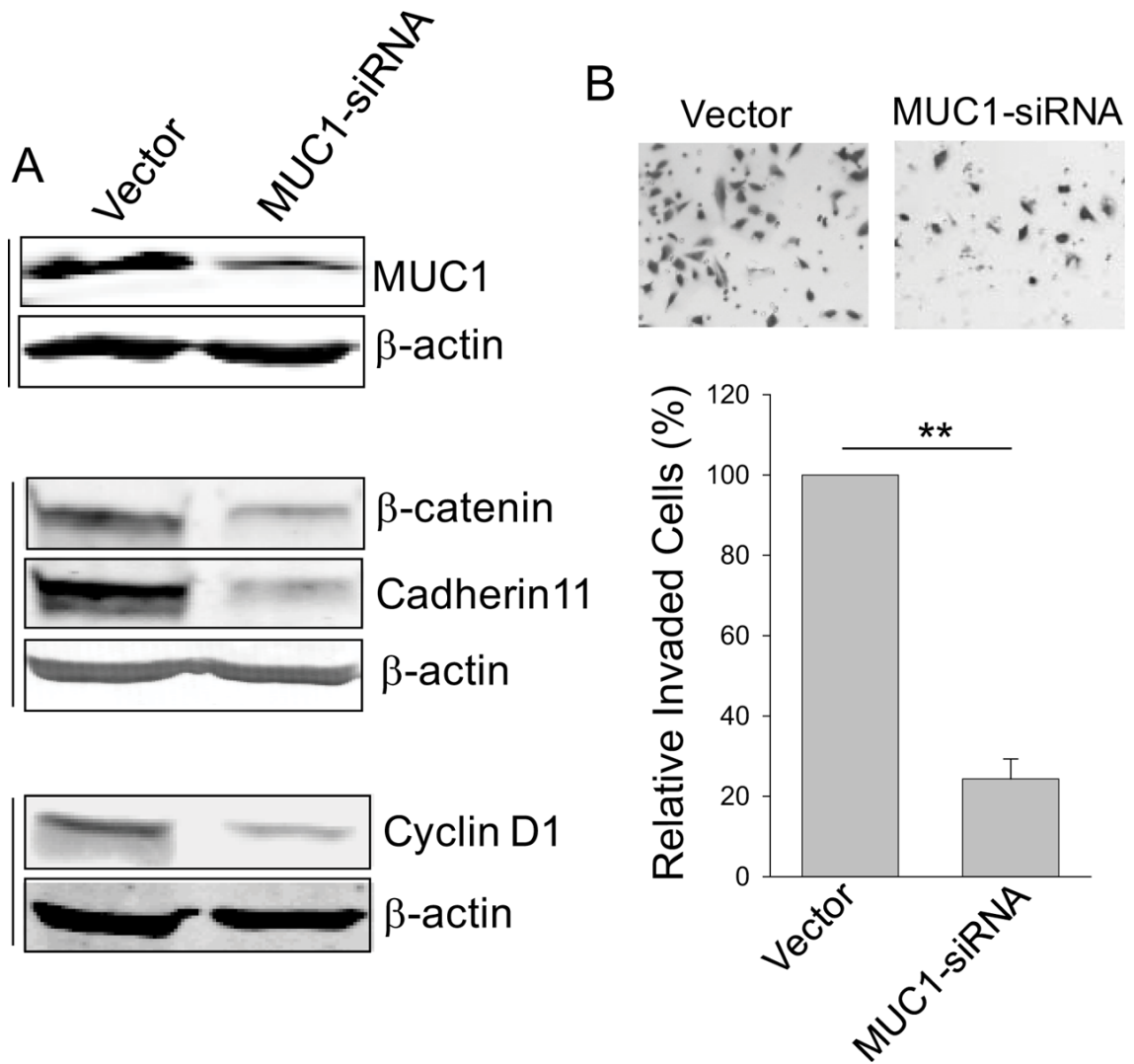


Fig. 5. Suppression of MUC1 by RNAi reduces levels of β -catenin, cyclin D1 and cadherin 11 as well as invasiveness

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. Values are means \pm SE of three separate experiments. **, $p < 0.01$.

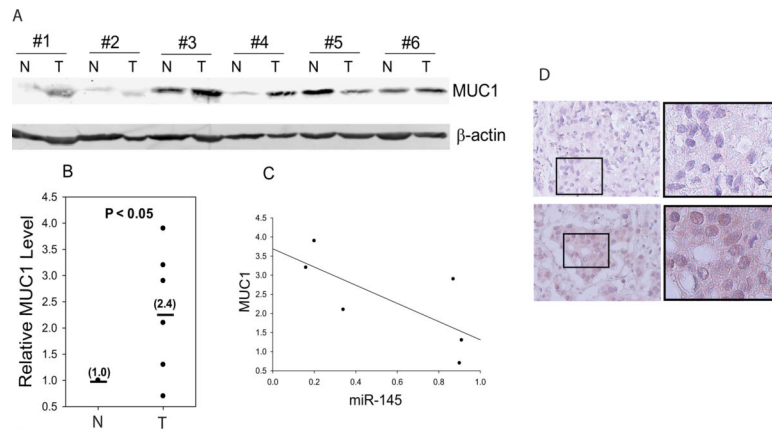


Fig. 6. Expression of MUC1 in breast tumor specimens

A and **B**, Western blot reveals upregulation of MUC1 in breast tumors (T) compared to 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 immunohistochemistry, **a**: infiltrating ductal carcinoma without metastasis, and **b**: infiltrating ductal carcinoma with metastasis.