IIncRNA-RoR and miR-145 Regulate Invasion in Triple-Negative Breast Cancer via Targeting ARF6

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Abstract

Triple-negative (ER\textsuperscript{-}, HER2\textsuperscript{-}, PR\textsuperscript{-}) breast cancer (TNBC) is an aggressive disease with a poor prognosis with no available molecularly targeted therapy. Silencing of microRNA-145 (miR-145) may be a defining marker of TNBC based on molecular profiling and deep sequencing. Therefore, the molecular mechanism behind miR-145 downregulation in TNBC was examined. Overexpression of the long intergenic noncoding RNA regulator of reprogramming, lincRNA-RoR, functions as a competitive endogenous RNA sponge in TNBC. Interestingly, lincRNA-RoR is dramatically upregulated in TNBC and in metastatic disease and knockdown restores miR-145 expression. Previous reports suggest that miR-145 has growth-suppressive activity in some breast cancers; however, these data in TNBC indicate that miR-145 does not affect proliferation or apoptosis but instead, miR-145 regulates tumor cell invasion. Investigation of miR-145-regulated pathways involved in tumor invasion revealed a novel target, the small GTPase ADP-ribosylation factor 6 (Arf6). Subsequent analysis demonstrated that Arf6, a known regulator of breast tumor cell invasion, is dramatically upregulated in TNBC and in breast tumor metastasis. Mechanistically, Arf6 regulates E-cadherin localization and affects cell-cell adhesion. These results reveal a lincRNA-RoR/miR-145/ARF6 pathway that regulates invasion in TNBCs.

Implications: The lincRNA-RoR/miR-145/ARF6 pathway is critical to TNBC metastasis and could serve as biomarkers or therapeutic targets for improving survival. Mol Cancer Res; 13(2): 330–8. ©2014 AACR.
ceRNA networks have been identified as key regulators of muscle differentiation (16) and in the PTEN tumor-suppressor pathway (17).

Recently, lncRNAs were implicated in stem cell pluripotency. Loewer and colleagues (18) identified lncRNA-RoR (regulator of reprogramming) as a major regulator of pluripotency by examining lncRNA expression, following fibroblast reprogramming into induced pluripotent stem cells (iPSC). lncRNA-RoR was dramatically upregulated in pluripotent cells. Furthermore, they found that lncRNA-RoR was essential for iPSC derivation. Wang and colleagues (19) also examined the role of lncRNA-RoR in ESCs and found that lncRNA-RoR is essential for ESC pluripotency. Furthermore, they found that lncRNA-RoR functions as ceRNA for miR-145, thereby protecting core pluripotency factors from miR-mediated silencing. This group found that this interaction led to loss of mature miR-145 expression. Using RNA immunoprecipitation experiments they validated the interaction of miR-145 with lncRNA-RoR, which they found could be disrupted by mutating bases in the target sites for miR-145 seed pairing.

ARF proteins (ARF1-6) are small GTPases that regulate membrane protein trafficking and endocytosis (20). ARF6 was previously implicated in tumor cell invasion in breast (21), brain (22), and skin (23, 24) cancer. In breast cancer, ARF6 was found to be essential for tumor cell invasion (21), brain (22), and skin (23, 24) cancer. ARF6 was found to be essential for tumor cell invasion in breast cancer and was previously implicated in tumor cell invasion in breast cancer (21). Hyperactivation of ARF6 was able to impart metastatic characteristics to nonmetastatic breast phenotype (21). ARF6 was found to be essential for tumor cell invasion. Furthermore, we found that lncRNA-RoR results in ARF6 overexpression. We examine the function of ARF6 in breast cancer cells and find that ARF6 can affect cell–cell adhesion (via localization of E-cadherin) and tumor cell invasion. Furthermore, we find that in clinical samples ARF6 protein levels are higher in lymph node metastasis compared with primary tumors, suggesting that this protein may play an important role in the metastatic process.

**Materials and Methods**

**Cell culture**

HEK293T, MCF-7, HS578T, and MDA-MB-231 cells were maintained in DMEM with 5% FBS and 1% glutamine (Invitrogen). MCF10A were grown in DMEM/F-12 medium supplemented with 10 ng/mL insulin, 100 ng/mL cholera toxin, 0.5 μg/mL hydrocortisone (Sigma), 20 ng/mL EGF, and 5% horse serum (Invitrogen). Cells were grown at 37°C in an atmosphere containing 5% CO₂.

**Human tissue array**

Immunostaining of paraffin-embedded breast tumor tissue microarray (TMA; BR932; US Biomax) was performed to detect ARF6 protein expression. Additional paraffin-embedded ductal carcinoma in situ (DCIS) samples were obtained from the University of Maryland Pathology Biorepository and Research Core. Sections were deparaffinized and rehydrated using xylene and gradient ethanol. Antigens were retrieved by boiling in sodium citrate (10 mmol/L, pH 6.0), which was proceeded by blocking in 10%, goat serum in PBS for 1 hour. This was followed by overnight incubation (at 4°C) with mouse anti-ARF6, 1:200 in blocking buffer (Santa Cruz Biotechnology; sc7971) followed with biotin goat anti-mouse secondary antibody (1:200). The Avidin-Biotin Peroxidase Substrate Kit (Vector Laboratories) was used to develop brown precipitate. Hematoxylin was used for nuclei staining. Using light microscopy, cores were scored on a 0 to 3 scale (none, light, moderate, and intense) for staining intensity of ARF6.

**RNA quantification**

Total RNA was extracted with TRIzol reagent (Invitrogen). Small RNA was converted to complimentary DNA using the poly-A polymerase–based First-Strand Synthesis Kit (SABioscience). Subsequent miR analysis was performed by real-time PCR with miR-145 primer assays (SABioscience) normalizing to control U6 snRNA levels. Total RNA was converted to cDNA by first treating with DNase I to remove genomic DNA and then using M-MLV reverse transcriptase (Invitrogen) and oligoDT12-18 or random hexamer primers. The following primers were used for qRT-PCR.

**Human tissue array**

Western blotting and proliferation assay

Western blotting was performed as previously described (25) using ARF6 (Santa Cruz Biotechnology 3A-1), E-cadherin (BD Transduction), or N-cadherin (Santa Cruz Biotechnology H-63) antibodies. Data were normalized to β-actin (Sigma). For proliferation assays, 1 × 10⁴ MDA-MB-231 cells (control and overexpressing miR-145) were plated in 96-well plates. After 3 days, MITT solution was added to wells [final (0.5 mg/mL)]. Cells were incubated for 4 hours at 37°C. Media were removed and MITT formazan crystals were solubilized in DMSO. Absorbance was measured at 560 nm in a microplate reader (Bio-Rad).

Plasmids, transfections, and luciferase assay

pCMV-miR-145 expression vector and pCMV-MIR control vectors were obtained from Origene. pBabe–lncRNA-RoR was obtained from Addgene (plasmid 45763) shRNA for lncRNA-RoR and scramble control shRNA was purchased from Origene using pGFP-C-shLenti backbone and the following target sequence: GGAAGGCTAGTGGCGGATATG and loop: TC-AAGAG. Constitutively active ARF6 (Q67L) expression vector was obtained from Addgene (plasmid 10835). ARF6 3’ untranslated region (UTR) was amplified using the following
Primers: F: CAGTACGCTGAGACTTGCTGCACTGCAAAATG; R: CAGTACCGACAGAACTTAG CCCACACATGGCA. PCR product was cloned downstream luciferase ORF into Nhel and XhoI sites in pSGG 3'UTR reporter (Switegear Genomics). For pSGG–ROR luciferase reporter construct, ROR cDNA was amplified using the primers 5′-AACATGCTGAGTTTATTTTTTGAGGAACTGTCATA-3′ and 5′-ACAATGGCACTGCCGTTAATTTAAACAGCCGTTGCTGCT-3′ and cloned into the EcoRI sites of pCMV-24xms2 vector (Addgene 40651) was digested with BamHI, treated with T4 DNA polymerase and then digested with AgeI. The vector backbone was recovered. The ROR cDNA was amplified by PCR using primers 5′-AAATGGAATTCTTTATTTTTTGAGGAACTGACCGGTGGTGAAATTAACACA-3′ and 5′-AACATGCTGAGTTTATTTTTTGAGGAACTGCATGCTGCT-3′ and cloned into the dual luciferase assay system (Promega) normalizing to Renilla luciferase activity (26).

tet-ON–lincRNA-RoR. The ROR cDNA was amplified by PCR using primers 5′-AACATGCGATCCTTATTTTGGACAGTGCATGCGAAGTGTATAGTCATA-3′ and 5′-AACATGCGATCCTTATTTTGGACAGTGCATGCGAAGTGTATAGTCATA-3′ and cloned into the EcoRI sites of FUW-tet vector (Addgene: 20326). Cells were cotransfected with FUW-M2rtTA (Addgene 20342) at the 1:8 ratio.

Fluorescence in situ hybridization of lincRNA-RoR

Cy3-labeled lincRNA-RoR probe was obtained from Exiqon. Cells were fixed in 4% formaldehyde and permeabilized using 0.5% Triton-X-100 in PBS, followed by blocking with 3% BSA in 4× saline–sodium citrate buffer. Cells were hybridized for 1 hour at 60°C with lincRNA-RoR probes (2 ng/mL dilution in buffer containing 10% dextran sulfate in 4× saline–sodium citrate buffer). Cells were washed in 4×, 2×, 1× saline–sodium citrate buffer and slides mounted in Prolong Gold.

MS2-binding assay

Twenty-four MS2-binding sites were cloned downstream of pBABE–lincRNA-RoR (ROR–MS2). The Phage-cmv–cFP–24xms2 vector (Addgene 40651) was digested with BamHI, treated with T4 DNA polymerase and then digested with AgeI. The vector backbone was recovered. The ROR cDNA was amplified by PCR using the primers 5′-AACATGCGATCCTTATTTTGGACAGTGCATGCGAAGTGTATAGTCATA-3′ and 5′-AACATGCGATCCTTATTTTGGACAGTGCATGCGAAGTGTATAGTCATA-3′, treated with T4 DNA polymerase and Agel sequentially and cloned into the phage-cmv–24xms2 vector prepared as described above. phage-ubc–nlsl–had MCP–gfp (MS2–GFP) fusion construct was obtained from Addgene (plasmid 40649). Cells were cotransfected with ROR–MS2 and MS2–GFP constructs and GFP localization was monitored with confocal microscopy.

Three-dimensional cell culture and immunofluorescence

MCF-10A cells were dissociated into single cells and cultured with DMEM/F-12 containing 5% Matrigel. 5% heat-inactivated FBS, 0.5 µg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 µg/mL insulin, 10 ng/mL EGF, and 5 µM/L Y-27632. Cells were embedded into Matrigel-coated chamber-slides and grown for 7 to 14 days with replacement of fresh assay medium every 4 days. MDA-MB-231 cells transfected with control or miR-145 expression vectors were grown in chamber slides for 48 hours. Fluorescence was visualized using an Olympus IX81 spinning disk confocal microscope. Immunofluorescence staining was performed as previously described (27), by co-staining with ARF6 and E-cadherin antibodies or kit® antibody (Sigma; SAB4501880) followed by Alexa Fluor–conjugated secondary antibodies (Life Technologies) and DAPI counterstaining.

Transwell invasion assay

Invasion assays were carried out using Transwell migration chambers (8-µm pore size; Costar) coated with 0.5 mg/mL Matrigel (BD Biosciences) on top of the membrane. A total of 0.5 × 10^5 cells/mL were seeded in the upper chamber in serum-free medium. The lower chamber contained 15% FBS. The cells were allowed to migrate toward the 15% FBS gradient overnight. Nonmigrated cells on the top of the membrane were removed with cotton swabs. The migrated cells were stained with 1% crystal violet in methanol/PBS and counted using a light microscope.

The Cancer Genome Atlas

Data from The Cancer Genome Atlas (TCGA) were analyzed using the UCSC Genome Browser (http://genome-cancer. soe.ucsc.edu/). The publicly available dataset analyzed was the TCGA breast–invasive carcinoma exon expression by RNAseq (IlluminaHiSeq) N = 1,160, ER, PR, and HER2 status were analyzed using UCSC Cancer Browser tools.

Statistical analysis

Statistical analysis was performed using the Student t test and P values of < 0.05 were considered significant. Data were represented as mean ± SE. GraphPad Prism 4.0 software was used for all data analysis.

Results

miR-145 is downregulated in TNBC in which miR-145 regulates breast tumor cell invasion

The molecular underpinnings of TNBC are poorly understood and as such, there are no available molecularly targeted therapies. To gain a better understanding of the pathways driving tumorigenesis in TNBC, we began by examining the unique miR signatures of this breast cancer subtype. Analysis of breast tumor deep sequencing data had previously revealed that miR-145 loss is a hallmark of TNBC (4). We began our study by using publicly available databases to verify previous observations regarding miR-145 expression. We examined RNAseq data from TCGA, which contained data from 1,106 breast cancer patient samples. As shown in Fig. 1A, miR-145 is downregulated in nearly every breast tumor sample compared with normal breast tissue (fold Δ and P value shown in Supplementary Fig. S1; ref. 28). Furthermore, we examined ER, PR, and HER2 status in patient data from TCGA and found that patients with TNBC clustered with the tumors having the lowest miR-145 expression. We also examined miR-145 expression in several samples of invasive ductal carcinoma (IDC) and matched normal tissue and again identified miR-145 loss in all samples of breast tumor tissue (Fig. 1B). Next, we examined TNBC cell models MDA-MB-231 and HS578T compared with the enontumorigenic mammary epithelial cell line MCF10A and the ER-positive breast cancer cell line MCF-7. We observed the strongest repression of miR-145 in TNBC models compared with

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nontumorigenic mammary epithelial cells or ER-positive breast cancer cells (Fig. 1C). Combined with previous studies, these data strongly indicate that miR-145 is dramatically silenced in TNBC.

Next, we began investigating what function miR-145 may play in TNBC. In ER-positive breast cancer, miR-145 was previously shown to regulate tumor cell proliferation (29). We examined the impact of miR-145 overexpression on the proliferation of MDA-MB-231 cells. We found that in TNBC cells miR-145 activation failed to significantly affect cell proliferation as examined via MTT assay (Fig. 1D). Furthermore, we also examined cell proliferation by performing Ki67 staining. We found that nearly 100% of control MDA-MB-231 cells and miR-145-overexpressing cells positively stained for nuclear Ki67, indicating active proliferation (Supplementary Fig. S2A).

We next examined what impact miR-145 might have on tumor cell invasion in TNBC. We grew cells on Matrigel-coated Transwell inserts and tested the impact of miR-145 overexpression. We found that miR-145-overexpressing TNBC cells demonstrated a significant decrease in tumor cell invasion compared with control cells (Fig. 1E and F). These data suggest that miR-145 may regulate invasion-related gene expression in TNBC. We also confirmed these results in a separate TNBC cell line, HS578T, again finding that miR-145 affects invasion but not proliferation in TNBC cells (Supplementary Fig. S2B and S2C).

lincRNA-RoR is overexpressed in TNBC in which it serves as competitive endogenous RNA for miR-145

We next wanted to identify the molecular mechanisms responsible for miR-145 downregulation in TNBC. It was recently shown that in ESCs miR-145 is subjected to posttranscriptional regulation via competitive endogenous RNA (19). lincRNA-RoR was found to contain miR-145-binding elements and function as a competitive sponge for miR-145 binding. To test whether lincRNA-RoR might regulate miR-145 in TNBC, we began by examining the expression profile of lincRNA-RoR in normal breast tissue, early-stage tumors (DCIS), and腺癌 (TNBC) cells.
invasive breast tumors (IDC) using qRT-PCR. We found that lincRNA-RoR was significantly upregulated in DCIS and IDC tumor tissues, showing the highest expression in invasive tumor tissues (Fig. 2A). Next, we examined lincRNA-RoR in breast cancer cell lines. We found that lincRNA-RoR was significantly overexpressed in MDA-MB-231 and HS578T TNBC cells when compared with normal tissue (Fig. 2B). Subsequently, we wanted to examine cellular localization of lincRNA-RoR to probe its potential to interact with cytoplasmic miR-145. We performed in situ hybridization and were able to detect lincRNA-RoR in the cytoplasm and nucleus of breast cancer cells (Supplementary Fig. S3A). For further confirmation, we performed MS2-binding assays, in which we cloned 24 MS2-binding sites downstream of lincRNA-RoR, and tested the ability of MS2 to probe its potential to interact with cytoplasmic miR-145. We performed in situ hybridization and were able to detect lincRNA-RoR in the cytoplasm and nucleus of breast cancer cells (Supplementary Fig. S3A). For further confirmation, we performed MS2-binding assays, in which we cloned 24 MS2-binding sites downstream of lincRNA-RoR, and tested the ability of a GFP–MS2 fusion protein with a nuclear localization signal to sequester lincRNA-RoR–MS2 in the cytoplasm. In the absence of lincRNA-RoR–MS2, all the GFP–MS2 is localized in the nucleus (Supplementary Fig. S3B). However, in the presence of lincRNA-RoR–MS2, there are foci of GFP–MS2 in the cytoplasm. These results also support the cytoplasmic localization of lincRNA-RoR in which it may interact with miR-145.

We next wanted to confirm the interaction of miR-145 with the sites predicted by Miranda (30) targeting algorithms (Fig. 2C). We began by testing the impact of lincRNA-RoR overexpression on miR-145 levels in HEK-293T cells (which lack lincRNA-RoR expression). We found that lincRNA-RoR overexpression resulted in a significant decrease in miR-145 levels (Fig. 2D). Next, we examined the impact of cotransfection of miR-145 and lincRNA-RoR and found that cotransfection overcame the negative repression of endogenous miR-145 and resulted in decreasing lincRNA-RoR levels. This suggests a tug of war between these two molecules with some threshold where either the miR or the lincRNA gains the upper hand and silences its partner. We also cloned lincRNA-RoR sequence into a luciferase miR reporter construct to examine the ability of miR-145 to bind sequences in lincRNA-RoR. We found that miR-145 overexpression resulted in decreased luciferase activity compared with control cells (Fig. 2F), indicating miR-145 binding to sites in lincRNA-RoR. Next, we examined this interaction in breast cancer cell lines. We found that lincRNA-RoR overexpression resulted in decreasing mature, but not primary or precursor miR-145 levels in MDA-MB-231 TNBC cells (Fig. 2G). Finally, using lentiviral shRNA, we knocked down lincRNA-RoR in MDA-MB-231 cells and found that this lead to an increase in miR-145 expression. Taken together, these results suggest that in TNBC cells lincRNA-RoR can function as a sponge and repress miR-145 expression.

Finally, we examined whether lincRNA-RoR regulation of miR-145 could affect breast cancer cell invasion. We transfected MCF7

![Figure 2](image-url)

Figure 2.

LincRNA-RoR is overexpressed in TNBC where it serves as competitive endogenous RNA for miR-145. A, lincRNA-RoR expression in breast tumor tissues as measured by qRT-PCR. B, lincRNA-RoR expression in breast cancer cell lines. C, miR-145 response element in lincRNA-RoR as predicted by MiRanda 4.0 algorithm. D, miR-145 expression analysis following lincRNA-RoR overexpression in HEK-293T cells. E, lincRNA-RoR expression analysis following miR-145 overexpression in HEK-293T cells. F, luciferase reporter assay for lincRNA-RoR in HEK-293T cells overexpressing miR-145. G, miR-145 expression analysis in MDA-MB-231 breast cancer cells following lincRNA-RoR overexpression. H, fluorescent images of MDA-MB-231 cells stably infected with lincRNA-RoR shRNA. I, expression analysis of lincRNA-RoR and miR-145 following lincRNA-RoR knockdown via qRT-PCR; **P < 0.05.
cells with tetON-lincRNA-RoR + rtTA and after 24 hours induced lincRNA-RoR expression with 100 ng/mL doxycycline. Induced cells were grown atop Matrigel-coated Transwell inserts and cells were allowed to migrate for 24 hours. Following 24 hours, we observed a significant increase in invasive cells, whereas control MCF7 cells showed little to no invasive activity (Supplementary Fig. S4A).

miR-145 directly targets the 3′UTR of ARF6 mRNA

In addition to understanding the molecular mechanisms underlying the regulation of miR-145, we wanted to probe what invasive pathways miR-145 might regulate in TNBC cells. We examined computationally predicted targets of miR-145 using TargetScan algorithm (31). Among the highest scoring predicted mRNA targets of miR-145 was ADP-ribosylation factor 6 (ARF6). ARF6 is a small GTPase that has been previously implicated as a critical regulator of tumor cell invasion in metastatic breast cancer (21). A previous proteomics study revealed that miR143/145 modulation altered ARF6 protein levels in colon cancer cells, suggesting that ARF6 might be a direct target for miR-145 (32). ARF6 mRNA 3′UTR contains an impressive five predicted miR-145-binding elements (Fig. 3A). We began by examining ARF6 expression in TNBC. We found that ARF6 demonstrated inverse expression relative to miR-145. ARF6 was dramatically overexpressed in TNBC cells compared with nontumorigenic mammary epithelial cells (Fig. 3B and C). To test the predicted binding of miR-145 to ARF6 mRNA, we cloned the ARF6 3′UTR downstream a luciferase ORF and performed luciferase reporter assays for ARF6 3′UTR. We found that overexpression of miR-145 resulted in a significant decrease in luciferase activity compared with control cells (Fig. 3D). Next, we examined ARF6 expression in MDA-MB-231 cells following miR-145 overexpression. We detected a small but significant decrease in ARF6 mRNA levels following miR-145 overexpression (Fig. 3E). However, following miR-145 overexpression, we detected a dramatic decrease in ARF6 protein levels (Fig. 3F). These data suggest that miR-145 inhibition of ARF6 is mostly occurring by interfering with ARF6 translation. Finally, to test whether our previously

Figure 3.

miR-145 directly targets the 3′UTR of ARF6 mRNA. A, table of the predicted miR-145-binding sites in the ARF6 mRNA 3′UTR as predicted by TargetScan algorithm. B, ARF6 mRNA expression in breast cancer cell lines via qRT-PCR. C, ARF6 protein expression in MDA-MB-231 cells. D, luciferase reporter for ARF6 mRNA 3′UTR following miR-145 overexpression. E, ARF6 mRNA levels in MDA-MB-231 cells following miR-145 overexpression. F, ARF6 protein levels in MDA-MB-231 cells following miR-145 overexpression. G and H, MCF-7 cells overexpressing constitutively active ARF6. A loss of E-cadherin demonstrated via Western blotting and a more invasive phenotype as evidenced by Matrigel-coated invasion assays; *, P < 0.05.
As suggested by a previous study, ARF6 might contribute to breast cancer invasion via regulating invadopodia or by regulating cell–cell adhesion through controlling E-cadherin localization (33, 34). This group found that in the presence of EGF ligand (activating EGFR signaling) ARF6 was able to repress E-cadherin at the protein level. We examined the impact of overexpression of constitutively active ARF6 on the invasive activities of nonmetastatic MCF-7 breast cancer cells. As confirmation of the earlier observations, we found that in the presence of EGF, MCF-7 cells overexpressing ARF6 showed a decrease in E-cadherin protein levels (Fig. 3G). Furthermore, MCF-7 cells overexpressing ARF6 were capable of invading Matrigel in Transwell invasion assays as evidenced by the staining of invasive protrusions on the bottom of Transwell inserts, whereas control MCF-7 cells demonstrated no invasive capabilities in this assay (Fig. 3H).

**ARF6 overexpression alters E-cadherin localization and disrupts cell–cell junctions**

To further examine the potential importance of ARF6 overexpression in breast cancer, we performed gain-of-function studies in MCF10A nontumorigenic mammary epithelial cells. We performed three-dimensional (3D) cell culture experiments with MCF10A cells overexpressing ARF6 and examined E-cadherin localization using immunofluorescence. 3D cell culture can be used to recapitulate mammary organogenesis in which control MCF10A cells grow into hollow acinar structures with polarized luminal and basolateral surfaces. Control MCF10A cells formed hollow acini structures with E-cadherin localization to cell–cell junctions (Fig. 4A). In ARF6-overexpressing MCF10A cells, there was an obvious loss of E-cadherin expression and localization to cell–cell junctions. Furthermore, morphology of ARF6-overexpressing acini was altered with greater spacing between nuclei as evidenced by DAPI staining, suggesting a disruption in cell–cell junctions.

**The miR-145 target ARF6 is overexpressed in lymph node metastasis**

As there is previously no clinical data suggesting that ARF6 expression is associated with breast tumor invasiveness, we examined ARF6 expression using IHC in a TMA with core samples from matched normal breast, primary tumor, and lymph node metastasis core samples. Normal tissue n = 5, DCIS n = 6, IDC n = 26, MET n = 9.

![Figure 4. The miR-145 target ARF6 is overexpressed in lymph node metastasis. A, 3D cell culture of MCF10A cells stably infected with ARF6. Cells are grown in EGF supplemented media (10 ng/mL) for 7 days followed by fixation and staining with ARF6 and E-cadherin antibodies followed by DAPI counterstaining. Acinii were examined via confocal microscopy. B, IHC for ARF6 was performed on a TMA of matched normal and breast tumor tissue, including matched primary and lymph node metastasis core samples. Normal tissue n = 5, DCIS n = 6, IDC n = 26, MET n = 9.](image-url)
staining in lymph node metastasis cores (P < 0.03). These data offer support for the clinical relevance of ARF6s in breast cancer metastasis.

**Discussion**

Deep sequencing studies have previously shown that miR-145 downregulation is a hallmark of TNBC (4). Here, using Transwell invasion assays, we have found that miR-145 regulates tumor cell invasion in TNBC and not apoptosis or proliferation. We examined the molecular mechanism responsible for miR-145 downregulation in TNBC and found that the linRNA-RoR regulates mature miR-145 by serving as a competitive endogenous RNA sponge. This is the first report of this ceRNA network in human cancer.

To better understand the function of miR-145 in TNBC, we examined the predicted targets of miR-145 and identified ARF6, a small GTPase known to regulate endocytic recycling and previously implicated in breast tumor invasion (21). ARF6 mRNA 3’UTR contains five predicted miR-145-binding sites. Using a 3’UTR luciferase reporter, qRT-PCR, and Western blotting, we validated miR-145 targeting of the 3’UTR of ARF6 mRNA. Next, we found that ARF6 overexpression in MCF-7 cells promoted a more invasive phenotype as evidenced by Transwell invasion assays. We examined ARF6 function via 3D cell culture and found that overexpression of ARF6 results in loss of E-cadherin localization and disruption of cell–cell junctions. Finally, we examined ARF6 expression in a breast tumor tissue array and found that ARF6 levels were significantly higher in lymph node metastasis, suggesting a role of ARF6 in breast cancer metastasis. On the basis of our in vitro findings, it is important to next examine whether miR-145 and ARF6 can regulate TNBC metastasis in vivo.

Previously, miR-145 and linRNA-RoR have been implicated in embryonic and adult stem cells (10). These molecules may also be critical regulators of cancer stem cell biology, as it was previously reported that miR-145 is silenced in breast cancer stem cells. There is mounting evidence of a powerful connection between epithelial–mesenchymal transition and breast cancer stem cell (12). Here, we have demonstrated a connection between miR-145 and invasion/metastasis that involves altered cell morphology and loss of epithelial adherens junction protein E-cadherin. In future studies, it will be interesting to test whether miR-145 and linRNA-RoR play important roles in regulating the cancer stem cell phenotype in TNBC, which has previously been shown to play a critical role in drug resistance and metastasis.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: G. Eades, Q. Zhou

Development of methodology: Y. Zhang, Y. Yao

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Wolfson, Y. Zhang, Y. Yao

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Zhang, Y. Yao

Writing, review, and/or revision of the manuscript: G. Eades, Q. Zhou

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Q. Li, Q. Zhou

Study supervision: Q. Zhou

**Grant Support**

This work was supported by NCI F31 CA183522 (to G. Eades) and by grants from ACS, and the NCI R01 (to Q. Zhou).

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Received May 7, 2014; revised August 11, 2014; accepted September 12, 2014; published OnlineFirst September 24, 2014.


Molecular Cancer Research

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doi:10.1158/1541-7786.MCR-14-0251

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