Comprehensive Analysis of MicroRNA (miRNA) Targets in Breast Cancer Cells

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Background: miRNA deregulation contributes to tumor progression. Results: Endogenous miRNA targets were identified in two breast cancer cell lines by integrated analysis of miRNA/mRNA expression and miRNA-mRNA interaction. Conclusion: miRNAs collectively function to promote survival but suppress cell migration/invasion. Significance: The defined endogenous miRNA targets will facilitate future studies to link miRNA deregulation with breast cancer cell properties.

MicroRNAs (miRNAs) regulate mRNA stability and translation through the action of the RNAi-induced silencing complex. In this study, we systematically identified endogenous miRNA target genes by using AGO2 immunoprecipitation (AGO2-IP) and microarray analyses in two breast cancer cell lines, MCF7 and MDA-MB-231, representing luminal and basal-like breast cancer, respectively. The expression levels of ~70% of the AGO2-IP mRNAs were increased by DROSHA or DICER1 knockdown. In addition, integrated analysis of miRNA expression profiles, mRNA-AGO2 interaction, and the 3’-UTR of mRNAs revealed that >60% of the AGO2-IP mRNAs were putative targets of the 50 most abundantly expressed miRNAs. Together, these results suggested that the majority of the AGO2-associated mRNAs were bona fide miRNA targets. Functional enrichment analysis uncovered that the AGO2-IP mRNAs were involved in regulation of cell cycle, apoptosis, adhesion/migration/invasion, stress responses (e.g. DNA damage and endoplasmic reticulum stress and hypoxia), and cell-cell communication (e.g. Notch and Ephrin signaling pathways). A role of miRNAs in regulating cell migration/invasion and stress response was further defined by examining the impact of DROSHA knockdown on cell behaviors. We demonstrated that DROSHA knockdown enhanced cell migration and invasion, whereas it sensitized cells to cell death induced by suspension culture, glucose depletion, and unfolding protein stress. Data from an orthotopic xenograft model showed that DROSHA knockdown resulted in reduced growth of primary tumors but enhanced lung metastasis. Taken together, these results suggest that miRNAs collectively function to promote survival of tumor cells under stress but suppress cell migration/invasion in breast cancer cells.

MicroRNAs (miRNAs) are emerging as key modulators of gene expression at the post-transcriptional level by repressing translation and/or inducing mRNA degradation (1, 2). Most miRNAs are initially transcribed as long primary transcripts (pri-miRNAs) that are processed within the nucleus into short stem-loops (pre-miRNAs) by DROSHA, a member of the ribonuclease III superfamily of double-stranded RNA-specific endoribonucleases (3). The pre-miRNAs are transported to the cytoplasm and further processed by DICER1, another double-stranded RNA-specific ribonuclease, to generate mature miRNAs, which are loaded into the RNA-induced silencing complexes (RISCs) (4). miRNAs recruit mRNA targets to RISCs through Watson-Crick base pairing (2). Computational sequence analysis and experimental evidence suggest that bases 2–8 at the 5’-end of mature miRNAs (termed seed sequences) and their complementary sequences located in the 3’-untranslated region (3’-UTR) of mRNA are the major determinants of miRNA-mRNA interaction (5–8). A single miRNA can target hundreds of mRNAs, and a single mRNA can be coordinately regulated by multiple miRNAs (7, 9). Approximately 60% of mammalian mRNAs have one or more evolutionarily conserved miRNA target sequences (7). However, it is unclear whether a miRNA exerts its effects via regulating its entire repertoire of targets or a subset of specific effectors in a given cell context. The complexity of miRNA function can hardly be depicted by traditional studies that focus on a single miRNA and its predicted targets one at a time. A prerequisite for understanding the collective function of endogenous miRNAs is to determine what mRNAs and signaling pathways are targeted by miRNAs under physiologically relevant conditions.

Argonaute proteins are the catalytic components of the RISCs for mRNA silencing or destruction. All four human argonaute proteins (AGO1, AGO2, AGO3, and AGO4) are able to interact with miRNAs as components of RISCs to inhibit translation, but only AGO2 possesses the endoribonuclease activity.

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2 The abbreviations used are: miRNA, microRNA; pri-miRNA, primary miRNA; IP, immunoprecipitation; AGO2-IP, AGO2 immunoprecipitation; RISC, RNAi-induced silencing complex; KD, knockdown; Ab, antibody; qPCR, quantitative PCR; APA, alternative polyadenylation.
to catalyze small RNA-directed, site-specific mRNA cleavage (10, 11). In addition, AGO2 is the most abundant argonaute protein in the majority of mammalian tissues, including mammary gland (12–14). Therefore, AGO2 probably plays a key role in RNA-induced silencing in mammary gland epithelial cells. Several studies have demonstrated that miRNA targets can be identified from immunopurified AGO2 complexes (6, 15–21). Among the various approaches developed to identify miRNA targets, AGO2 immunoprecipitation (AGO2-IP), combined with mRNA expression microarray analysis, represents a direct and feasible approach to systematically identify miRNA targets in a physiologically relevant manner, which was employed in our study to investigate miRNA targets in breast cancer cells. Such systematic studies will advance our understanding of the complex features of miRNA function.

Deregulation of miRNAs is associated with breast cancer development and progression (22–33). Although several key targets of breast cancer-associated miRNAs have been identified and linked to tumor phenotypes, the gene networks orchestrated by miRNAs in breast cancer cells are largely unknown. In this study, we performed AGO2-IP, followed by expression microarray analysis, to systematically identify miRNA targets in MCF7 and MDA-MB-231, the two widely used cell lines that represent luminal estrogen-dependent and basal-like triple negative breast tumors, respectively. The numbers of mRNAs detected in AGO2-IP from MCF7 and MDA-MB-231 cells were 877 and 703, respectively (false discovery rate = 0.1). In silico analysis of the 3′-UTRs of these AGO2-IP mRNAs as well as their expression in cells with impaired miRNA synthesis suggested that the majority of the AGO2-IP mRNAs were bona fide miRNA targets. Functional enrichment analysis revealed that the endogenous miRNAs predominantly target genes that regulate cell cycle, apoptosis, adhesion/migration/invasion, stress responses (e.g. DNA damage, hypoxia, and endoplasmic reticulum stress), and cell-cell communication (e.g. Notch and Ephrin signaling pathways). Accordingly, inhibiting miRNA processing by DROSHA or DICER1 knockdown enhanced cell ability for migration and invasion but sensitized cells to apoptosis induced by various types of stress.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—MCF7 and MDA-MB-231 were purchased from ATCC (Manassas, VA) and cultured in minimal essential medium supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin-streptomycin. To generate cells that stably express shRNA against DROSHA or DICER1, cells were transduced with lentivirus containing pSicoR-Drosha1 or pSicoR-Dicer1 (Addgene 14766 or 14763) (34) and selected in medium supplemented with 2 μg/ml puromycin.

**AGO2 Immunoprecipitation**—Cells (3 × 10⁶) were suspended in 3 ml of ice-cold hypotonic buffer (10 mM Tris (pH 7.5), 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 100 units/ml RNase OUT, and protease inhibitor mixture) for 15 min. The cytoplasmic fraction was isolated by homogenization with a Dounce homogenizer and centrifugation at 14,000 × g at 4 °C for 10 min and incubated with control IgG (5 μg of Ab/mg of lysate) and anti-mouse IgG-coated magnetic beads for 1 h to eliminate nonspecific binding. The precleared lysates were then mixed with mouse anti-human Ago2 (5 μg of Ab/mg of lysate; clone 2E12-1C9, Abnova (Taipei City, Taiwan)) and anti-mouse IgG-coated magnetic beads. After incubation overnight at 4 °C on a rocking platform, AGO2-IP beads were washed twice with ice-cold wash buffer (hypotonic buffer supplemented with 150 mM NaCl and 0.5% Nonidet P-40) and once with high salt buffer (hypotonic buffer supplemented with 400 mM NaCl and 0.5% Nonidet P-40). RNA and protein were extracted from the AGO2-IP complexes using TRIzol (Invitrogen) and Laemmli buffer, respectively.

**Quantification of mRNA, miRNA, and pri-miRNA Expression Using qPCR**—Total RNA was converted to cDNA by using iScript cDNA synthesis kits (Bio-Rad) or the NCode™ miRNA First-Strand cDNA Synthesis Kit (Life Technologies) for mRNA or miRNA detection, respectively. qPCR was performed on the CFX96™ Real-Time PCR Detection System using SYBR Green supermix (Bio-Rad). Expression data of mRNA and miRNA were normalized to GAPDH and U6 snRNA, respectively, using the 2⁻ΔΔCt method, and presented as mean ± S.E. (n = 3).

**Immunoblotting**—Protein extracts were resolved in SDS-PAGE, transferred to PVDF membrane, and immunoblotted with the indicated antibodies. Antibodies for DROSHA, MAP1LC3, and GAPDH were from Cell Signaling Technologies (Boston, MA), and AGO2 was from Abnova.

**Microarray Analysis**—The purified RNA samples from whole cells (input RNA) and AGO2-IP were submitted to the University of Tennessee Health Science Center Center of Genomics and Bioinformatics (Memphis, TN) for labeling and hybridization to HT-12 expression BeadChips (Illumina Inc.). Three independent AGO2-IP experiments were performed. Hybridization signals were processed (annotation, background subtraction, quantile normalization, and presence call filtering) using Illumina Genome Studio software (Illumina). AGO2-IP-enriched miRNAs were identified using Genespring GX version 9.0 (Agilent Technologies Inc., Santa Clara, CA) with the following cut-offs: false discovery rate = 0.1 (AGO2-IP versus input, n = 3), -fold enrichment (AGO2-IP versus input) ≥ 1.5 in more than 2 of 3 experiments. Functional annotation and pathway mapping of the AGO2-IP mRNAs were performed by Ingenuity pathway analysis (Ingenuity Systems, Inc., Redwood City, CA). The microarray data can be found in the Gene Expression Omnibus database with accession number of GSE48162.

**Apoptosis Assays**—To induce anoikis, cells (5 × 10⁴/well) were seeded in 6-well dishes coated with polyHEMA (Sigma) to prevent cell attachment. To induce endoplasmic reticulum stress, cells were treated with thapsigargin (50 nM). For glucose depletion, cells were seeded in growth medium overnight, washed with PBS twice, and cultured in glucose-free medium for 16 h. The glucose-free medium consists of DMEM (without glucose; Life Technologies), 5% dialyzed FBS (Life Technologies), and 100 units/ml penicillin-streptomycin. Apoptotic cells with compromised membrane integrity were detected with YO-PRO-1 dye according to the manufacturer’s instructions (Life Technologies), followed by flow cytometer analysis.
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Transient Transfection and Luciferase Reporter Assay—CMV-d2eGFP-21 (miR-21 sponge), CMV-d2eGFP-empty (vector control for miR-21 sponge) and pCMV-luc-miR21 (luciferase reporter with miR-21 target sites have been characterized previously (Addgene 21927, 26164, and 20876) (37). To examine the efficiency of the miR-21 sponge to inhibit miR-21 function, MDA-MB-231 cells were transfected with pCMV-luc-miR21, along with CMV-β-galactosidase and various doses of CMV-d2eGFP-21 or vector control, using Lipofectamine 2000 (Life Technologies). Luciferase and β-galactosidase activities were measured 48 h after transfection using the luciferase and β-galactosidase assay system, respectively (Promega, Madison, WI). Luciferase activity was normalized to β-galactosidase activity and expressed as mean ± S.E. (n = 6). To examine the effect of miR-21 inhibition on interaction between AGO2 and miR-21 targets, MDA-MB-231 cells (1 × 10^5) were transfected with 15 μg of CMV-d2eGFP-21 or empty vector using Lipofectamine 2000, followed by AGO2-IP 48 h after transfection. RNA samples prepared from whole cells and AGO2-IP were subjected to qPCR analysis. To examine the effect of miRNA inhibition on mRNA expression, cells (4 × 10^5) were transfected with 50 nM miRCURY LNA miRNA inhibitor (Exiqon) or a control oligonucleotide using Lipofectamine RNAiMAX (Invitrogen). Total RNA was prepared 48 h after transfection and subjected to qPCR analysis. The sequences of the miRNA inhibitors for miR-221 and miR-200a are AACCCAGCAGACAATGTAGC and CATCGTGCTTCTTGCAGTCTC, respectively.

Migration and Invasion Assays—Cells (20,000 cells/0.5 ml/well) were plated onto control membrane inserts with 8-μm pores or Matrigel-coated membrane inserts (BD Biosciences), which are placed in 24-well chambers filled with 0.6 ml of growth medium. Twenty-four hours after plating, cells that remained on the upper surface of the membrane were removed by cotton-tipped swabs, and cells that migrated/invaded to the lower surface of the membrane were fixed with methanol, stained with 0.5% crystal violet, and counted under a microscope. The percent invasion was expressed as follows: % invasion = (mean number of cells invading through Matrigel insert membrane × 100)/mean number of cells migrating through control insert membrane.

Orthotopic Xenograft Model and Lung Metastasis—All animal studies adhered to protocols approved by the Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center. Cells (7.5 × 10^5 cells in 10 μl of PBS) were surgically inoculated into the right inguinal mammary gland fat pads of 4-week-old female NSG mice (NOD.Cg Prkdcscid Il2rgtm1Wjl/SzJ, The Jackson Laboratory). Mice were inspected weekly for tumor appearance by visual observation and palpation. Primary tumor outgrowth was monitored twice a week using digital calipers. Tumor volume was calculated as follows: volume = (width^2 × length)/2. Tumor and lung tissues were extracted 7 weeks after inoculation. The left lung lobes were fixed with 4% paraformaldehyde and subjected to tissue section (10 μm) and H&E staining. Genomic DNA from lung tissues (~20 mg from the right lung lobes) was prepared using the Wizard Genomic DNA Purification Kit (Promega) and subjected to qPCR analysis using primers specific for the human Abl sequences (forward, 5'-ACG CCTGTAATCCAGCACTT-3'; reverse, 5’-TGCCCAGGCTGGATGCA-3') (38).

Statistical Analysis—Data from two or three independent experiments with replicates are presented as means ± S.D. Analysis of variance and post hoc least significant difference analysis or t tests were performed using GraphPad Prism 5 software. p values of <0.05 (*) were considered statistically significant.

RESULTS

Identification of Endogenous miRNA Targets in Luminal and Basal-like Breast Cancer Cells—MCF7 and MDA-MB-231 cells were chosen for study because they are the most frequently used cell lines that represent luminal and basal-like breast cancer, respectively. A better understanding of the regulatory networks of gene expression in these two cell lines is critical to understand changes in breast cancer cell behavior elicited by various types of stress or genetic manipulations. AGO2 is the most abundantly expressed argonaute protein in mammary gland (see the Tissue-specific Gene Expression and Regulation (TiGER) Web site). Therefore, AGO2 probably plays a key role in RNA-induced silencing in mammary gland epithelial cells, and miRNAs coimmunoprecipitated with AGO2 may well represent the majority of endogenous miRNA targets.

First we examined the specificity and AGO2-IP efficiency of a mouse monoclonal AGO2 antibody (clone 2E12-1C9, Abnova). When whole cell lysates were used for immunoblotting, the antibody recognized a single band at ~95 kDa in both MCF7 and MDA-MB-231 cells (Fig. 1A, top). The efficiency of the AGO2 antibody for IP was confirmed by enrichment of AGO2 protein in the IP complexes (anti-AGO2 versus control IgGx) and depletion of AGO2 protein in the IP flow-through (Fig. 1A, bottom). Next, we examined the enrichment of miRNA targets in the AGO2-IP complexes. As shown in Fig. 1B, several miRNAs that have been established as miRNA targets in MCF7 cells (i.e. BTG2, CCNE1, CDC25a, Dicer1, Ezh2, and Runx1) were significantly enriched by AGO2-IP (p < 0.05, AGO2-IP versus IgG-IP).

To systematically identify miRNA targets, total RNA was isolated from AGO2-IP complexes and subjected to microarray analysis using human HT-12 expression BeadChips (Illumina Inc.). Three independent IP and array analysis were conducted. Using a cut-off set that combined false discovery rate and -fold enrichment (false discovery rate = 0.1 (AGO2-IP versus input, n = 3), -fold enrichment (AGO2-IP versus input) ≥ 1.5 in more than 2 of 3 independent biological repeats), 877 and 703 miRNAs were detected in AGO2-IP from MCF7 in MDA-MB-231, respectively (Fig. 1C and supplemental Table S1 and GSE48162). The AGO2-IP miRNAs from the two cell lines shared a marked overlap and also exhibited cell type-specific miRNA-AGO2 interaction, as summarized in Fig. 1C. The differences between these two cell lines may reflect the differential expression of miRNAs and miRNAs as well as the presence of different isoforms of miRNAs due to alternative splicing and/or polyadenylation.

Identification of Signaling Pathways Targeted by miRNAs—To understand the physiological role of these miRNA targets, we performed functional enrichment analysis of AGO2-IP
mRNAs by using the Ingenuity pathway analysis system (Ingenuity Systems, Inc.). The signaling pathways and cellular functions commonly regulated by miRNAs in both MCF7 and MDA-MB-231 cells included cell cycle, apoptosis, adhesion/migration/invasion, lipid metabolism, stress response (e.g. ATM, autophagy, endoplasmic reticulum stress, hypoxia, and mitochondrial dysfunction), and transmembrane receptor signaling (e.g. notch, ephrin, and tumor necrosis factor) (Fig. 1C, right). Several signaling pathways critical for luminal phenotype of breast cancer were found to be targeted by miRNAs in MCF7, including nuclear receptor (e.g. signaling pathways mediated by estrogen, androgen, and retinoic acid receptor receptors), HER-2, and p53 signaling pathways. In contrast, the Wnt/β-catenin signaling pathway that confers phenotypic plasticity to basal-like breast cancer was targeted by miRNAs in MDA-MB-231 cells (39). These results provide an overview of signaling pathways targeted by miRNA in luminal and basal-like breast cancer cells, suggesting that miRNAs play an important role in regulating cell response to extracellular stimuli and transmembrane receptor-mediated cell-cell communications.

Validation of AGO2-IP mRNAs as Bona Fide miRNA Targets—To validate that the identified AGO2-mRNA interactions were indeed mediated by miRNAs, we examined the effect of miR-21 inhibition on mRNA-AGO2 interaction in MDA-MB-231 cells. A construct (CMV-d2eGFP-21) that expresses a sponge RNA with multiple target sites complementary to miR-21 was used to inhibit miR-21 function (37). The efficiency of miR-21 sponge to inhibit miR-21 activity was monitored by expression of a luciferase indicator (pCMV-luc-miR21) that harbors four copies of miR-21 target sites in the 3'UTR (40). In transiently transfected MDA-MB-231 cells, miR-21 sponge increased the expression of the luciferase indicator in a dose-dependent manner (Fig. 2A) but showed no significant effect on the expression of a control luciferase reporter (data not shown). Next, we examined the effect of miR-21 sponge on AGO2 interaction of a panel of established miR-21 targets. As shown in Fig. 2B, miR-21 sponge significantly decreased the amount of miR-21 targets detected in AGO2-IP from MDA-MB-231 cells, including BTG2, COL4A1, DCUN1D3, EIF4EBP2, EPHA4, JAG1, SPRY4, and ZCCHC3.

In addition, we examined the effect of miRNA inhibition on the expression of cell type-specific AGO2-IP miRNAs by using LNA-modified antisense oligonucleotides for miR-221 and miR-200a, which represent cell line-specific miRNAs that are...
highly expressed in MDA-MB-231 and MCF7, respectively. As shown in Fig. 2, miR-221 inhibition in MDA-MB-231 cells increased the expression of a panel of miR-221 targets that were specifically detected in AGO2-IP from MDA-MB-231 cells. The expression of these mRNAs was not significantly affected by anti-miR-221 in MCF7 cells (data not shown). Conversely, miR-200a inhibition in MCF7 cells increased the expression of a panel of miR-200a targets that were specifically found in AGO2-IP from MCF7 (Fig. 2). The expression of these genes was not significantly affected by miR-200a inhibition in MDA-MB-231 (data not shown). Collectively, these results suggest that the AGO2-IP mRNAs are likely targets of endogenous miRNAs.

DROSHA Knockdown Increases Expression of AGO2-IP mRNAs—mRNA destabilization is closely correlated with translation suppression by miRNAs (1). Therefore, we speculated that blocking DROSHA-mediated miRNA synthesis would result in the accumulation of AGO2-IP mRNAs if they are bona fide miRNA targets. To knockdown DROSHA, cells were stably transduced with a lentiviral construct (pSicoR-Drosha1) that expresses DROSHA-specific shRNA (34). Immunoblotting and qPCR showed that DROSHA expression was reduced by ~80% at both the mRNA and protein level in MDA-MB-231 cells expressing the shRNA (designated as DROSHA-KD) compared with control cells that were transduced with a lentiviral construct expressing scramble RNA (designated as MDA-MB-231/C) (Fig. 3A). Because MCF7 showed modest DROSHA knockdown efficiency (~50%), the following studies were conducted in MDA-MB-231 only.

To examine the effect of DROSHA knockdown on mRNA procession, the expression levels of 13 pri-miRNAs were examined using the TaqMan Pri-miRNA assays (Invitrogen), including pri-MIRLET7D, MIR7–3HG, MIR17HG, MIR25, MIR21, MIR22HG, MIR30B, MIR30C2, MIR100HG, MIR106A, MIR125B2, MIR130A, and MIR221). DROSHA knockdown significantly increased the abundance of seven pri-miRNAs, concomitant with a decreased expression of the corresponding mature miRNAs (Fig. 3, B and C). These results demonstrated that DROSHA knockdown abolished processing of some, but not all, of the pri-miRNAs in MDA-MB-231 cells. The various effects of DROSHA knockdown on different pri-miRNAs are probably due to the presence of multiple miRNA processing pathways (41). This finding suggests that DROSHA knockdown could have cell context-dependent effects, dependent on the expression profiles of pri-miRNAs and activities of various miRNA processing pathways.

Having demonstrated that DROSHA knockdown reduced the expression of a subset of miRNAs, we examined its impact on the expression of AGO2-IP miRNAs by microarray analysis using the human HT-12 expression BeadChips. As shown in Fig. 4, the expression levels of the vast majority of AGO2-IP miRNAs (>70%) were increased by DROSHA knockdown. Similarly, the specific increase in expression levels of AGO2-IP miRNAs was also observed in MDA-MB-231 cells with DICER1 knockdown (data not shown and GSE48162). Taken together, these results support the conclusion that the AGO2-IP miRNAs are bona fide miRNA targets.
The Majority of AGO2-IP mRNAs Are Putative Targets of Abundantly Expressed miRNAs—Next, we examined the relationship between AGO2-IP mRNAs and putative targets of miRNAs that are abundantly expressed in MCF7 and MDA-MB-231 cells. miRNA expression in MCF7 and MDA-MB-231 cells has been extensively studied, and several global miRNA targets in breast cancer cells.
expression data sets are publicly available (see the Array-Express, Gene Expression Omnibus, and the Cell Catalogue of Somatic Mutations in Cancer web sites). The reported expression levels of individual miRNAs appear to vary greatly among these data sets, presumably due to differences in RNA sample processing and the platforms used for miRNA profiling. To compile a reliable list of abundantly expressed miRNAs in these two cell lines, we performed a meta-analysis of a total of seven data sets, as indicated in Fig. 5A. The expression levels of individual miRNAs in each data set were ranked according to Z scores (42), and the average Z scores of seven data sets were used to identify the 50 most abundantly expressed miRNAs in each cell line. The relative expression levels of these miRNAs were presented in Fig. 5A, among which 35 miRNAs exhibited comparable expression levels in both cell lines (Group C), whereas 28 miRNAs were differentially expressed (Groups A and B).

Among the abundantly expressed miRNAs grouped in Fig. 5A, ~50% of the miRNAs in each group were randomly chosen to examine their association with AGO2 in MCF7 and MDA-MB-231 cells. As shown in Fig. 5B, 27 miRNAs were detected in AGO2-IP. The relative abundance of the 27 miRNAs detected in AGO2-IP from MCF7 and MDA-MB-231 cells was consistent with the result from meta-analysis of their overall expression levels. For example, higher levels of Group A miRNAs were detected in AGO2-IP from MCF7 than in that from MDA-MB-231, whereas higher levels of Group B miRNAs were detected in AGO2-IP from MDA-MB-231 than in that from MCF7. This result suggests that AGO2 binding is well correlated with the expression levels of most miRNAs.

These abundantly expressed miRNAs in MCF7 (Groups A and C in Fig. 5A) and MDA-MB-231 (Groups B and C in Fig. 5A) harbor a total of 35 different seed sequences (Table 1). The putative targets of these miRNAs were identified by using the

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**FIGURE 5.** Identification of the 50 most abundantly expressed miRNAs in MCF7 and MDA-MB-231 cells. A, heat map of miRNAs abundantly expressed in MCF7 and MDA-MB-231 cells. The relative expression levels miRNA were calculated according to Z scores from seven publicly available data sets. B, relative miRNA levels detected in AGO2-IP from MCF7 and MDA-MB-231 cells. The results are presented as mean ± S.E. (error bars) (n = 3).
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To investigate whether APA plays a role in cell type-specific miRNA-miRNA interactions in breast cancer cells, we examined the expression ratio of the extended 3'-UTR regions (between the proximal and distal polyadenylation site) relative to the coding region of a panel of mRNAs. The cell type-specific AGO2-interacting mRNAs selected for this study are putative targets of miRNAs commonly expressed in both MCF7 and MDA-MB-231 cells and harbor APA sites according to the AREsite and xPAD Expression & Poly(A) Database (44, 48). As shown in Fig. 6, PTPRK, a MCF7-specific AGO2-IP mRNA, showed a higher ratio of the extended 3'-UTR relative to coding region in MCF7 than in MDA-MB-231 cells. This result suggests that an RTRPK isoform with a long 3'-UTR is preferentially produced in MCF7 cells. Conversely, PMAIP1 and MAPK6, two mRNAs that were detected in AGO2-IP specifically in MDA-MB-231 cells, exhibited a higher ratio of their extended 3'-UTRs relative to coding regions in MDA-MB-231 than in MCF7 cells. In addition, we also detected a preferential expression of the extended 3'-UTRs of SIAH1, SLC35A1, SPRY4, UBE2N, and APITD1 in MBA-MB-231 cells, where they were found to be associated with AGO2. However, 13 mRNAs (from a total of 21 examined) showed no difference in the expression of their extended 3'-UTRs, despite their differential interaction with AGO2, which included Ccdc25, CLASp1, DGR8, FXR1, MMD, PPRA, RUNX1, TARBP2, TFDP1, TRAM2, UBE2N, XP05, and ZC3HC1C1. These results suggest that APA accounts for the cell type-specific miRNA interaction of some, but not all, mRNAs.

**DROSHA Knockdown Increases Cell Migration and Invasion**—Because genes encoding proteins involved in adhesion, migration, and invasion (Fig. 1C) were overrepresented in AGO2-IP mRNAs, we speculated that blocking miRNA processing may alter cell migration and invasion. Boyden chamber migration and invasion assays showed that DROSHA knockdown significantly enhanced migration and invasion of MDA-MB-231 cells (Fig. 7A). These observations suggest that the endogenous miRNAs of MDA-MB-231 function collectively to suppress migration and invasion.

**DROSHA Knockdown Promotes Cell Death in Response to Various Types of Stress**—Genes involved in various stress signaling pathways were significantly enriched in AGO2-IP mRNAs in both MCF7 and MDA-MB-231 cells, implicating a role of miRNAs in cell damage control and adaptation. Intriguingly, among the AGO2-IP mRNAs are several critical components of the autophagy pathway (e.g., CTS1, DDIT4, ERN1, HSPA5, IDUA, LAMTOR1, RAC3, ULK3, and VTI1B). Autophagy is a catabolic process that delivers cellular components through double-membrane vesicles (autophagosomes) to lysosomes for degradation. Autophagy plays an important role in eliminating damaged cellular components and recycling cellular materials for macromolecular and organelle biosynthesis and nutrient and energy homeostasis (49). Given the prominent cytoprotective roles of autophagy, we hypothesized that blocking miRNA processing may alter autophagy activity and consequently cell sensitivity to stress.

First, we examined the effect of DROSHA knockdown on autophagy activity by measuring the conversion of MAP1LC3A from cytosolic (LC3A-I) to membrane-bound lipidated form

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### TABLE 1

| Seed sequence | miRNA Target Filter of the Ingenuity pathway analysis system | Cell line specificity |
|---------------|-------------------------------------------------------------|-----------------------|
| AAAGCGU       | hsa-miR-320a                                                | Common                |
| AAAGUGC       | hsa-miR-17-5p/20b-5p/93-5p/106a-5p/106b-5p                  | Common                |
| AACACGUC      | hsa-miR-212-5p                                              | Common                |
| ACACUCCUC     | hsa-miR-181a-5p/181b-5p                                      | Common                |
| ACCGCACUC     | hsa-miR-100a-5p                                             | Common                |
| AGCGCAAUC     | hsa-miR-49a-5p/49b-5p/49c-5p                                 | Common                |
| AGCGGCAAUC    | hsa-miR-32-5p/30b-5p/30c-5p/30d-5p                          | Common                |
| UCACUUAGUC    | hsa-miR-26a-5p                                              | Common                |
| UCAACGUC      | hsa-miR-27a-5p                                              | Common                |
| UCACUCAUC     | hsa-miR-22a-3p/23b-3p/28b-3p                                 | Common                |
| ACCGACUC      | hsa-miR-105a-5p                                             | MDA-MB-231            |
| AGCCACAUC     | hsa-miR-29a-5p/29b-5p/29c-5p                                 | MDA-MB-231            |
| AGCGGCCUC     | hsa-miR-22-5p                                               | MDA-MB-231            |
| AGGCAUCAUC    | hsa-miR-146a-5p/146b-5p                                     | MDA-MB-231            |
| GCCAUCUC      | hsa-miR-202a-5p                                             | MDA-MB-231            |
| GGCAGGUC      | hsa-miR-20b-5p/20c-5p/429                                   | MCF7                  |
| AGGGAGUC      | hsa-miR-96a-5p                                              | MCF7                  |
| GGGGACUC      | hsa-miR-342-5p                                              | MCF7                  |
| UCAAGGUC      | hsa-miR-376a-5p                                             | MCF7                  |
| UGAAAGUAGC    | hsa-miR-203a                                                | MCF7                  |
| UUGGGAGCA     | hsa-miR-182                                                 | MCF7                  |
| UUGGACUC      | hsa-miR-96-5p                                               | MCF7                  |
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Table 2: Distribution of miRNA targets

| MDA-MB-231          | Top 48 miRNAs of MDA-MB-231 |
|----------------------|-----------------------------|
| targets | non-targets | Total | targets/total, % | p value* |
| all expressed genes | 4431 | 5933 | 10364 | 42.75 | 0.0001 |
| AGO2-IP             | 433  | 269  | 702   | 61.68 | <0.0001 |

| MCF7                 | Top 50 miRNAs of MCF7       |
|----------------------|-----------------------------|
| targets | non-targets | Total | targets/total, % | p value* |
| all expressed genes | 4652 | 5964 | 10616 | 43.82 | 0.0001 |
| AGO2-IP             | 583  | 293  | 876   | 66.55 | <0.0001 |

DROSHA Knockdown Reduced Growth of Primary Tumors but Enhanced Spontaneous Lung Metastasis in an Orthotopic Xenograft Model—During initiation and the continuous expansion of solid tumors, cells are subjected to various types of stress that activate autophagy, including hypoxia, nutrient deprivation, and alteration of extracellular matrix. In order to further characterize the protective role of miRNAs in cells under stress, we examined the effect of DROSHA knockdown on tumor growth in vivo. Control and DROSHA knockdown MDA-MB-231 cells (7.5 × 10⁵ cells in PBS) were surgically inoculated into the fourth inguinal mammary gland fat pads of 4-week-old female NSG (NOD.Cg Prkdcscid Il2rgtm1Wjl/SzJ) mice. All animals developed palpable tumors within 2 weeks after inoculation. However, a slower growth rate of tumors derived from DROSHA-KD cells was observed (Fig. 8A, top). The difference in tumor growth was further confirmed by tumor weights at 7 weeks after inoculation (Fig. 8A, bottom). H&E staining of tissue sections revealed the presence of necrotic loci in tumors derived from DROSHA-KD cells but not in tumors from control cells (Fig. 8B). This result, together with the observation that DROSHA-KD increased cell apoptosis in response to various types of stress, suggests that DROSHA-dependent miRNAs support tumor cell survival.

To investigate the effect of DROSHA knockdown on metastatic potential, we examined the presence of tumor cells in lung sections from mice 7 week after inoculation of DROSHA-KD or control cells in the mammary gland fat pads. As shown in Fig. 8C, small lung metastases were observed in...
mice inoculated with control cells \(\text{(bottom left)}\). In contrast, large areas of lung parenchyma were replaced by tumor cells in mice received DROSHA-KD cells \(\text{(bottom right)}\). We further quantified metastatic burden in the lungs by qPCR using primers specific to human Alu sequences \(38\). DROSHA-KD increased the amount of tumor cells in lungs by \(6\)-fold \(\text{(Fig. 8C)}\). These results implicate a role of DROSHA-mediated miRNA synthesis in suppressing tumor metastasis.

**DISCUSSION**

Cancer-related miRNAs have emerged as promising therapeutic targets and intervention tools. However, a comprehensive understanding of cellular signaling pathways regulated by miRNAs, which depends on identifying miRNA targets under biologically relevant conditions, is greatly needed. In this study, we examined endogenous miRNA targets in breast cancer cells by an integrated analysis of AGO2-mRNA interaction, miRNA expression, gene expression, and cell behavior changes in response to inhibition of miRNA processing. To our knowledge, this is the first study aimed to systematically identify miRNA targets in MCF7 and MDA-MB-231, two widely used breast cancer cell lines that represent luminal and basal-like breast cancer, respectively.

The AGO2-IP mRNAs identified in this study probably represent \textit{bona fide} miRNA targets based on the following findings: 1) \(\sim 70\%\) of AGO2-associated mRNAs exhibited increased expression in response to inhibition of miRNA processing by DROSHA or DICER1 knockdown, suggesting that the majority of AGO2-IP mRNAs were targeted by endogenous miRNAs; 2) \textit{in silico} analysis revealed that putative targets of the 50 most abundantly expressed miRNAs were significantly overrepresented by AGO2-IP mRNAs, implicating a role of these miRNAs in mediating AGO2-mRNA association; 3) \(\sim 30\%\) of the AGO2-IP miRNAs were previously identified as miRNA targets in cells of various origins \(6,15–21\); 4) most of the signaling pathways that were overrepresented in AGO2-IP mRNAs have been reported to be regulated by miRNAs, such as cell cycle control, apoptosis, and adhesion/migration/invasion; and 5) we experimentally confirmed that a subset of the signaling pathways overrepresented in AGO2-IP mRNAs were significantly affected by DROSHA knockdown. However, one limitation of our experimental approach is that it preferentially detects mRNAs that stably bound to AGO2 and have intact poly(A) tails, which may be biased against mRNAs that are targeted by miRNAs for rapid deadenylation and degradation.

Signaling pathway and function mapping of the AGO2-IP miRNAs revealed that miRNAs predominantly target genes that regulate cell cycle, apoptosis, autophagy, adhesion/migration/invasion, membrane receptor-mediated cell-cell communication (e.g. Ephrin and Notch signaling pathways), and stress responses (e.g. DNA damage, endoplasmic reticulum stress, hypoxia, and mitochondria dysfunction) in both luminal and basal-like breast cancer cells. Regulation of cell cycle and apoptosis by miRNAs has been well documented \(54–60\). Notably, more antiproliferation and proapoptotic genes were identified.
as miRNA targets than proproliferation and antiapoptotic genes, indicating that miRNAs, in general, support cell proliferation and protect cells against apoptosis. Consistent with this hypothesis, global miRNA elevation due to increased activity of XPO5 was found to be critical for cell G1/S entry, whereas global miRNA inhibition by DROSHA knockdown in human colon adenocarcinoma HT29 cells has been shown to enhance apoptosis induced by 5-fluorouracil treatment (61, 62).

miRNA deregulation has been frequently described in metastatic tumors, implicating a role of miRNAs in regulating cell properties associated with metastasis (63). We found that genes involved in cell adhesion/migration/invasion were overrepresented in AGO2-IP miRNAs, and DROSHA knockdown in human colon adenocarcinoma HT29 cells has been shown to enhance apoptosis induced by 5-fluorouracil treatment (61, 62).

miRNA deregulation has been frequently described in metastatic tumors, implicating a role of miRNAs in regulating cell properties associated with metastasis (63). We found that genes involved in cell adhesion/migration/invasion were overrepresented in AGO2-IP miRNAs, and DROSHA knockdown significantly enhanced cell migration and invasion in vitro and enhanced spontaneous lung metastasis in an orthotopic xenograft model. These results suggest that miRNAs collectively function to inhibit cell migration and invasion, which is consistent with the observation that miRNA down-regulation rather than up-regulation occurs frequently in metastatic tumor cells (63). In support, a recent high throughput study showed that over 20% of the 904 human miRNAs have regulatory activity on migration and invasion of cancer cells from diverse origins, and most of these miRNAs exhibited suppressive impact (60). In addition, DICER1 down-regulation has been shown to enhance tumor metastasis (64).

One intriguing finding of our study is that a large number of genes critically involved in cell stress response were miRNA targets. In solid tumors, cells must adapt continuously to fluctuations in their microenvironment, including hypoxia, nutrient deprivation, therapeutic insults, and alteration of extracellular signals (e.g. extracellular matrix, cytokines, and hormones). Cell response to environment changes involves concerted action of diverse signaling pathways to eliminate damage and facilitate adaptation. Recent studies suggest that autophagy is a common downstream event of various types of cellular stress and plays an important role in promoting tumor cell survival and adaptation (49, 51–53, 65, 66). Autophagy is a catabolic process that delivers cellular components through double-membrane vesicles (autophagosomes) to lysosomes for degradation, allowing cells to eliminate damaged components and recycle cellular materials for macromolecular and organelle biosynthesis and nutrient and energy homeostasis (49). We

![Figure 7](https://example.com/figure7.png)

**FIGURE 7.** Global miRNA inhibition by DROSHA knockdown in MDA-MB-231 cells enhances cell migration and invasion, but promotes cell death in response to various types of stress. A, DROSHA knockdown increases cell potential for migration and invasion, which were detected by Boyden chamber assays with uncoated or Matrigel-coated membrane, respectively. The results are presented as mean number of cells/field ± S.E. (error bars) (n = 3). C, control. B, DROSHA knockdown impairs autophagy flux, indicated by the lack of response of MAP1LC3A to glucose depletion (GD) or chloroquine (CQ) treatment. In control cells with normal autophagy activity, glucose depletion induces conversion of MAP1LC3A from cytosolic (LC3A-I) to membrane-bound lipidated form (LC3A-II) due to increased autophagosome assembly, whereas chloroquine causes accumulation of LC3A-II by inhibiting autophagosome degradation by lysosome. C, DROSHA knockdown sensitizes cells to apoptosis induced by various types of stress. Apoptotic cells with compromised membrane integrity were detected with YO-PRO-1 dye, followed by flow cytometer analysis.
found that blocking DROSHA-mediated miRNA synthesis led to impaired autophagy flux and sensitized cells to apoptosis induced by various stressors that activate autophagy. Our data from in vivo studies provided further evidence supporting a protective role of miRNAs against cell death. Our results suggest that miRNAs collectively function to maintain proper autophagy flux and protect cells against stress-induced cell death. Given the critical roles of miRNA and autophagy in cell homeostasis, the interaction between these two pathways warrants further investigation.

Cell type-specific effects of miRNAs have been recognized, but the underlying mechanism is not clear. One potential mechanism is the presence of mRNA isoforms with various lengths of 3'-UTRs due to the usage of alternative polyadenylation sites. By comparing mRNA-AGO2 interaction, miRNA expression, and mRNA expression in MCF7 and MDA-MB-231 cells, we identified a panel of mRNAs that were targeted by miRNAs in a cell type-specific manner. We provided experimental evidence suggesting that cell type-specific usage of alternative polyadenylation may be responsible for differential regulation by miRNAs of some mRNAs, such as RTPRK, PMAIP1, and MAPK6.

In conclusion, we conducted a genome-wide analysis of miRNA targets in luminal and basal-like breast cancer cells, followed by experimental validation in cells with impaired miRNA function at the level of single miRNA or global miRNA processing. Our results suggest that miRNAs play an important role in protecting cells against cell death and repressing metastasis. We also provided experimental evidence supporting the possibility that alternative polyadenylation contributes to cell type-specific regulation of certain mRNAs by miRNA. These data provide an overview of the function of endogenous miRNAs in two major subtypes of breast cancer and a base of future studies to link breast cancer cell properties with individual miRNAs.

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