Comprehensive Analysis of miRNA Targets in Breast Cancer Cells*

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*Running title: miRNA targets in breast cancer cells

Key words: miRNA/RISC/DROSHA/DICER1/breast cancer

Background: miRNAs 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.

ABSTRACT

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 fifty 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, while sensitized cell 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 mRNAs 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 the bases 2 to 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 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 likely 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, two most 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 (FDR = 0.1). In silico analysis of the 3′-UTRs of these AGO2-IP mRNAs,
as well 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 (3x10^7) were suspended in 3 ml ice cold hypotonic buffer [10 mM Tris (pH 7.5), 10 mM KCl, 2 mM MgCl2, 1mM DTT, 100U/ml RNase OUT and protease inhibitor cocktail] for 15 min. The cytoplasmic fraction was isolated by homogenization with Dounce homogenizer, centrifugation at 14000g at 4°C for 10 min, and incubated with control IgGκ (5 μg Ab per mg lysate) and anti-mouse IgG coated magnetic beads for 1 h to eliminate non-specific binding. The pre-cleaned lysates were then mixed with mouse anti-human Ago2 (5 μg Ab per mg 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% NP-40) and once with high salt buffer (hypotonic buffer supplemented with 400 mM NaCl and 0.5% NP-40). RNA and protein were extracted from the AGO2-IP complexes using Trizol (Invitrogen) and Laemmli buffer, respectively.

**Quantitation of mRNA, miRNA and pri-miRNA expression using qPCR** - Total RNA was converted to cDNA by using iScript cDNA Synthesis Kits (BioRad, Hercules, CA) or 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 supernimix (BioRad). Expression data of mRNA and miRNA were normalized to GAPDH and U6 snRNA, respectively, using the 2^(-ΔΔCT) method, and presented as mean ± SE (n=3). qPCR primers were obtained from PrimerBank or designed using Primer3Plus (35,36). The expression levels of Pri-miRNA were examined by using TaqMan Pri-miRNA Assays according to manufacturer's instructions (Invitrogen).

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

**Microarray analysis** - The purified RNA samples from whole cells (input RNA) and AGO2-IPs submitted to the UTHSC 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 mRNAs were identified using Genespring GX version 9.0 (Agilent Technologies Inc., Santa Clara, USA) with the following cutoffs: FDR=0.1 (AGO2-IP vs. input, n=3), fold enrichment (AGO2-IP vs. input) ≥ 1.5 in more than two out of three experiments. Functional annotation and pathway mapping of the AGO2-IP mRNAs were performed by Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Inc. Redwood City, CA). All expression microarray data were deposited in Gene Expression Omnibus with access number GSE48162.

**Apoptosis Assays** - To induce anoikis, cells (5x10⁴/well) were seeded in 6-well dishes coated with polyHEMA (Sigma, St Louis, MO, USA) 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 for overnight, washed with PBS twice, and cultured in Glucose-free medium for 16h. The glucose free medium consists of DMEM (without glucose, Life technologies, Grand Island, NY), 5% dialyzed FBS (Life technologies) and 100 units/ml penicillin-streptomycin. Apoptotic cells with comprised membrane integrity were detected with YO-PRO-1 dye according to manufacturer's instructions (Life technologies), followed by flow cytometer analysis.

**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 48h 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 ± SE (n=6). To examine the effect of miR-21 inhibition on interaction between AGO2 and miR-21 targets, MDA-MB-231 cells (1x10⁶) were transfected with 15μg CMV-d2eGFP-21 or empty vector using lipofectamine 2000, followed by AGO2-IP 48h after transfection. RNA samples prepared from whole cells and AGO2-IPs were subjected to qPCR analysis. To examine the effect of miRNA inhibition on mRNA expression, cells (4x10⁵) were transfected with 50 nM miRCURY LNA miRNA inhibitor (Exiqon) or a control oligonucleotides 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 CATCGTTACCAGACAGTGTT, respectively.

**Migration and invasion assays** - Cells (20,000 cells/0.5 ml/well) were plated onto control membrane inserts with 8 micron pores or Matrigel-coated membrane inserts (BD Biosciences, Bedford, MA), which are placed in 24-well chambers filled with 0.6 ml 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 microscope. The percent invasion was expressed as: % invasion = (mean number of cells invading through Matrigel insert membrane x 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 University of Tennessee Health Science Center. Cells (7.5x10^5 cells in 10 μl 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: Volume = (width^2 x 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 Alu sequences (forward: 5′-ACGCCTGTAATCCCAGCACTT-3′; reverse: 5′-TCGCCCAGGCTGGAGTGCA-3′) (38).

Statistical analysis – Data from two or three independent experiments with replicates are presented as means ± sd. ANOVA and post-hoc least significant difference analysis or t-tests were performed using GraphPad Prism 5 software. P values < 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 (39). Therefore, AGO2 likely plays a key role in RNA-induced silencing in mammary gland epithelial cells and mRNAs communoprecipitated 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 (clone2E12-1C9, Abnova). When whole cell lysates were used for immunoblotting, the antibody recognized a single band at ~95 kD in both MCF7 and MDA-MB-231 cells (Fig. 1A upper panel). The efficiency of the AGO2 antibody for IP was confirmed by enrichment of AGO2 protein in the IP complexes (anti-AGO2 vs. control IgG) and depletion of AGO2 protein in the IP flow through (Fig. 1A, lower panel). Next, we examined the enrichment of miRNA targets in the AGO2-IP complexes. As shown in Fig. 1B, several mRNAs 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 vs. IgG-IP).

To systematically identify mRNA 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 cutoff set that combined FDR and fold-enrichment (FDR = 0.1 (AGO2-IP vs. input, n=3), fold enrichment
miRNA targets in breast cancer cells

(AGO2-IP vs. input) ≥ 1.5 in more than 2 out of three independent biological repeats), 877 and 703 mRNAs were detected in AGO2-IPs from MCF7 in MDA-MB-231, respectively (Fig. 1C, Supplementary Table S1 and GSE48162). The AGO2-IP mRNAs from the two cell lines shared a marked overlap, and also exhibited cell-type specific mRNA-AGO2 interaction, as summarized in Fig. 1C. The differences between these two cell lines may reflect the differential expression of mRNAs and miRNAs, as well as presence of different isoforms of mRNAs 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 Ingenuity pathway analysis system (IPA, 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 panel). 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 cells was targeted by miRNAs in MDA-MB-231 cells (40). 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 bone fide miRNA targets. To validate that the identified AGO2-mRNA interactions were indeed mediated by miRNAs, we examined 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 4 copies of miR-21 target sites in the 3'-UTR (41). 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-IPs 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 mRNAs 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. 2C, 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 wasn’t significantly affected.
miRNA targets in breast cancer cells

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. 2D). The expression of these genes wasn’t 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 (designed as DROSHA-KD) compared to control cells that were transduced with a lentiviral construct expressing scramble RNA (designed 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 miRNA 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. 3B and 3C). These results demonstrated that DROSHA knockdown abolished processing of some, but not all, of pri-miRNAs in MDA-MB-231 cells. The various effects of DROSHA knockdown on different pri-miRNAs are likely due to the presence of multiple miRNA processing pathways (42). 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 examine its impact on the expression of AGO2-IP mRNAs 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 mRNAs (>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 mRNAs 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 expression datasets are publicly available (43-45). The reported expression levels of individual miRNAs appear to vary greatly among these datasets, presumably due differences in RNA sample processing and the platforms used for miRNA profiling. To
miRNA targets in breast cancer cells

compile a reliable list of abundantly expressed miRNAs in these two cell lines, we performed a meta-analysis of a total of seven datasets as indicated in Fig. 5A. The expression levels of individual miRNAs in each dataset were ranked according to Z-scores (46), and the average Z-scores of seven datasets were used to identify the fifty 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), while 28 miRNAs were differentially expressed (Group A and B).

Among the abundantly expressed miRNAs grouped in Fig. 5A, approximately 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-IPs. The relative abundance of the 27 miRNAs detected in AGO2-IPs 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 MDA-MB-231, while higher levels of Group B miRNAs were detected in AGO2-IP from MDA-MB-231 than MCF7. This result suggests that AGO2-binding is well correlated with expression levels of most miRNAs.

These abundantly expressed miRNAs in MCF7 (Group A and C in Fig. 5A) and MDA-MB-231 (Group 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 miRNA Target Filter of IPA system based on 3'-UTR sequences of mRNAs. As summarized in Table 2, over 60% of AGO2-IP mRNAs were predicted targets of the abundantly expressed miRNAs in corresponding cells. Compared to all expressed genes detected in the input RNA samples, the predicted miRNA targets were significantly overrepresented in AGO2-IP mRNAs (p<0.05, Chi-square test with Yates correction). These results suggest that the majority of the AGO2-IP mRNAs are targets of miRNAs abundantly expressed in MCF7 and MDA-MB-231 cells. The absence of miRNA target sites in the 3'-UTRs of ~40% AGO2-IP mRNAs could be attributed to the presence of miRNA target sites outside the 3'-UTRs. AGO2-mRNA interaction mediated by miRNAs expressed at low levels, and undefined miRNA binding sequences.

Alternative polyadenylation contributes to cell-type specific AGO2-interaction of mRNAs. Among the cell-type specific AGO2-IP mRNAs are putative targets of miRNAs commonly expressed in both MCF7 and MDA-MB-231 cells. One possible cause of this cell-type specific AGO2 binding is differential expression of mRNA isoforms with 3'UTRs of varying lengths (47). A previous study reported that ~1/3 of mRNAs in various human tumor cells use alternative polyadenylation (APA) to generate multiple mRNA isoforms differ in their 3'-UTRs (48). The usage of APA appears to be cell context dependent, resulting in expression of cell-type specific mRNA isoforms (48-51).

To investigate whether APA plays a role in cell-type specific mRNA-miRNA interactions in breast cancer cells, we examined the expression ratio of the extended 3'-UTR regions (between proximal and distal polyadenylation site) relative to 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 AREsite and xPAD-Expression & PolyA Database (48,52). 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 RTPRK 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, DGCR8, FXR1, MMD, PRKRA, RUNX1, TARBP2, TFDP1, TRAM2, UBE2N, XPO5 and ZCCHC11. 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 autophagy pathway (e.g., CTSL1, 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 (53). 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 (LC3A-II), which correlates with autophagosome formation (54). Increase of LC3A-II could result from two opposing events, accelerated production of autophagosomes due to autophagy pathway activation or blockage of autophagosome degradation due to lysosome dysfunction. These two events can be distinguished by examining the impact of autophagy activation (e.g., nutrient starvation) and lysosome inhibition (e.g, chloroquine treatment) on LC3A-II levels. Lysosome inhibition will lead to LC3A-II accumulation in cells with baseline autophagy activity. As shown in Fig. 7B, LC3A-II was not detected in untreated control cells (MDA-MB-231/C), but was increased by glucose depletion or chloroquine treatment. In contrast, the DROSHA-KD cells exhibited higher baseline level of LC3A-II, which was not
affected by glucose depletion or chloroquine treatment (Fig. 7B). These results suggest that DROSHA knockdown impaired autophagy flux, supporting a role of miRNAs in regulating autophagy.

Next, we examined the effect of DROSHA knockdown on cell sensitivity to stressors that are known to activate autophagy, including suspension culture (anoikis), glucose depletion, and ER stress induced by thapsigargin (53,55-57). Apoptotic cells were detected by using YO-PRO-1 staining (Invitrogen), which labels cells with compromised membrane permeability. As shown in Fig. 7C, DROSHA knockdown significantly sensitized cells to apoptosis induced by anoikis, ER stress and glucose depletion. Together, these results suggest that miRNAs play a protective role in tumor cells under stress by regulating autophagy flux.

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 (7.5x10^5 cells in PBS) were surgically inoculated into the 4th inguinal mammary gland fat pads of 4-week old female NSG 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, upper panel). The difference in tumor growth was further confirmed by tumor weights at 7 weeks after inoculation (Fig. 8A, lower panel). 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 form 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 (lower panel, left). In contrast, large areas of lung parenchyma were replaced by tumor cells in mice received DROSHA-KD cells (lower panel, left). 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 (Fig. 8C). These results implicates 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
miRNA targets in breast cancer cells

lines that represent luminal and basal-like breast cancer, respectively.

The AGO2-IP mRNAs identified in this study likely represent bona fide miRNA targets based on the following findings: 1) ~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) in silico analysis revealed that putative targets of the fifty most abundantly expressed miRNAs were significantly overrepresented by AGO2-IP mRNAs, implicating a role of these miRNAs in mediating AGO2-mRNA association; 3) ~30% of the AGO2-IP mRNAs 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 experiment 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 deadenylatation and degradation.

Signaling pathway and function mapping of the AGO2-IP mRNAs 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.(58-64) Notably, more anti-proliferation and pro-apoptotic genes were identified as miRNA targets than pro-proliferation and anti-apoptotic 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, while global miRNA inhibition by DROSHA knockdown in human colon adenocarcinoma HT29 cells has been shown to enhance apoptosis induced by 5-FU treatment (65,66).

miRNA deregulation has been frequently described in metastatic tumors, implicating a role of miRNAs in regulating cell properties associated with metastasis (67). We found that genes involved in cell adhesion/migration/invasion were overrepresented in AGO2-IP mRNAs, 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 downregulation rather than upregulation occurs frequently in metastatic tumor cells (67). 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 (64). In addition, DICER1 downregulation has been shown to enhance tumor metastasis (68).

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 damages and facilitate adaptation. Recent studies suggest that autophagy is a common downstream event of various types of cellular stress and plays an important role to promote tumor cell survival and adaptation (53,55-57,69,70). 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 (53). We 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 underline 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 levels 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 experiment evidence supporting 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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provides insight into miRNA sorting and reveals Argonaute association with RNA fragments of diverse origin. RNA Biol 8, 158-177
miRNA targets in breast cancer cells


43. ArrayExpress - functional genomics data: http://www.ebi.ac.uk/arrayexpress/.


miRNA targets in breast cancer cells


chemotherapeutic 5-FU through mediation of an NF kappaB and microRNA network. *Mol Cancer* 9, 98


**FOOTNOTES**

*This work was supported by NIH grants, CA140346 (MF) and CA133322 (LMP).

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2The abbreviations used are: IP, immunoprecipitation; UTR, untranslated region; RISC, RNAi induced silencing complex; KD, knockdown

**FIGURE LEGENDS**

**FIGURE 1.** Identification of mRNAs associated with AGO2 in MCF7 and MDA-MB-231 cells. A. Specificity and efficiency of anti-AGO2 antibody. AGO2 protein from whole cell lysate, AGO2-IP, and IP flow through was detected using immunoblotting. GAPDH was used as loading control. B. Enrichment of miRNA targeted mRNAs by AGO2-IP. Total RNA was prepared from cell lysate or AGO2-IP and subjected to qPCR analysis. The mRNA levels were normalized to GAPDH. The fold enrichment was calculated as: Fold enrichment = mRNA level detected in AGO2-IP / mRNA level in cell lysate, and presented as mean ± SE, n=3. C. Vann diagrams and enriched cell signaling pathways of AGO2-IP mRNAs in MCF7 and MDA-MB-231 cells.

**FIGURE 2.** miR-21 sponge inhibits AGO2 association of miR-21 targets in MDA-MB-231 cells. A. Luciferase reporter that harbor miR-21 target sites in the 3’-UTR was used to monitor the efficiency of miR-21 inhibition by sponge mRNA. B. Enrichment of mRNAs by AGO2-IP in the absence (black bars) and presence (grey bars) of miR-21 sponge. The results were presented as mean ± SE, n=3. *p < 0.05 (Student t-test). C. Increased expression of miR-221 targets in MDA-MB-231 cells transfected with miRCURY LNA miR-221 inhibitor. D. Increased expression of miR-200a targets in MCF7 cells transfected with miRCURY LNA miR-200a
inhibitor. The results were presented as mean fold change (miRNA inhibitor vs. control oligonucleotide) ± SE, n=3.

**FIGURE 3.** DROSHA knockdown in MDA-MB-231 cells leads to pri-miRNA accumulation and mature mRNA reduction. A. DROSHA expression levels in cells stably expressing shRNA (MDA-MB-231/DROSHA-KD) or scramble RNA (MDA-MB-231/C). DROSHA mRNA and protein levels were examined by qPCR and immunoblotting, respectively. B. Fold changes of pri-miRNAs in response to DROSHA knockdown. Pri-miRNA levels were examined by using TaqMan Pri-miRNA Assays. The results were presented as mean fold change (DROSHA–KD vs. control) ± SE, n=3. *p < 0.05 (Student t test). C. Expression levels of mature miRNA in DROSHA knockdown and control cells. The results were presented as mean expression levels of miRNAs (normalized to U6) ± SE, n=3.

**FIGURE 4.** DROSHA knockdown in MDA-MB-231 cells increases expression levels of putative miRNA targets identified by AGO2-IP. A. Fold change of individual mRNAs in response to DROSHA knockdown. Gene expression was examined by array analysis using the Illumina HT-12 expression BeadChips. B. Boxplots of expression levels of putative miRNA targets and non-miRNA targets. The box shows 25th to 75th percentile with a line at the median.

**FIGURE 5.** Identification of the fifty most abundantly expressed miRNA 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 publically available datasets. B. Relative miRNA levels detected in AGO2-IPs from MCF7 and MDA-MB-231 cells. The results were presented as mean ± SE, n=3.

**FIGURE 6.** Alternative polyadenylation contributes to cell-type specific AGO2-interaction of mRNAs. The left panel shows the presence of alternative polyadenylation sites (red arrows) in the 3’-UTRs of mRNAs. The qPCR primers used to detect the expression of the extended 3’-UTR regions (between proximal and distal polyadenylation site) are showed as purple bars. The middle panel shows the cell-type specific interaction with AGO2 of the indicated mRNA. The right panel exhibits the expression ratio of the extended 3’-UTR region relative to coding region of mRNAs.

**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 were presented as: mean number of the cells per field ± SE, n=3. 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 comprised membrane integrity were detected with YO-PRO-1 dye, followed by flow cytometer analysis.
**FIGURE 8.** DROSHA knockdown in MDA-MB-231 cells reduces growth of orthotopic xenografts, but increases lung metastasis. A. Growth rates of xenograft tumors derived from DROSHA knockdown or control cells inoculated in mammary gland fat pads. The results were presented as: average tumor volume ± STDEV, n=12 (up panel); Wet weight of tumors at 7 weeks after inoculation. The results were presented as: average tumor weight ± STDEV, n=8 (low panel). B. H&E staining of tumor sections. Necrosis loci were observed in tumors derived from DROSHA knockdown cells, but not in tumors from control cells. C. Metastatic burden in lungs. H&E staining showed the presence of clusters of human tumor cells in mouse lung sections. Human tumor cells in mouse lung tissues were quantified by qPCR using primers specific for human Alu sequences. The cell number was calculated using standard curve of genomic DNA purified from cultured MDA-MB-231 cells and presented as mean ± STDEV, n=8.
### Table 1. Seed sequences of abundantly expressed miRNAs

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### Table 2. Distribution of miRNA targets

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* Chi-square test with Yates correction, one tailed
** References: 6, 15-21
Fig. 1

Panel A: Western blot analysis showing expression of AGO2 and GAPDH in MCF7 and MDA-MB-231 cells.

Panel B: Bar graph depicting fold enrichment of anti-AGO2 compared to IgG control for various genes.

Panel C: Venn diagram illustrating the overlap of gene expression between MCF7 and MDA-MB-231 cells, with categories of signaling pathways listed.
Fig. 2

A

Luciferase activity

miR-21 sponge, ng/well

0 100 250 500

B

Fold enrichment (anti-AGO2 vs. IgG)

EV
miR-21 sponge

miR-21 sponge

BTG2 COL4A1 DCUN1D3 EIF4EBP2 EPHA4 JAG1 SPRY4 ZCCHC3

C

Fold change of mRNA levels (miR-221AS vs. Sc)

D

Fold change of mRNA levels (miR-200aAS vs. Sc)
Fig. 3

A

DROSHA mRNA levels

C

KD

DROSHA

GAPDH

B

Fold change (DROSHA:KD vs. Control)

Pri-MIR21

Pri-MIR22HG

Pri-MIR100HG

Pri-MIR221

Pri-MIRLET7D

Pri-MIR130A

Pri-MIR17HG

C

hsa-miR-21-5p

hsa-miR-22-3p

hsa-miR-100-5p

hsa-miR-221-3p

hsa-let-7d-5p

hsa-miR-130a-3p

hsa-miR-17-5p

mRNA levels

C

KD

C

KD

C

KD

C

KD

C

KD

C

KD

C

KD
Fig. 4

A

AGO2-IP mRNAs (putative miRNA targets)

Fold change of mRNA express (DROSHA KD vs. control)

mRNAs not associated with AGO2

Fold change of mRNA express (DROSHA KD vs. control)

B

Expression levels (AGO2-IP mRNAs)

Expression levels (non-AGO2-IP mRNAs)

C KD

P<0.0001
Fig. 5

Data sets:
1. E-MEXP-1029
2. E-TABM-23
3. GSE16579
4. GSE18693
5. GSE21834
6. GSE26375
7. Sanger Institute
**Fig. 7**

(A) Bar graphs showing migration (cells/field) and invasion (% of migration) with controls (C) and KD (KD).

(B) Western blot analysis showing LC3A-I, LC3A-II, and GAPDH with controls (Control) and DROSHA KD (KD).

(C) Bar graphs showing apoptosis (%) in suspension culture (48 h), Thapsigargin 50nM (48 h), and Glucose depletion (16 h) with controls (C) and KD (KD).
Comprehensive Analysis of miRNA Targets in Breast Cancer Cells
Meiyun Fan, Raisa Krutilina, Jing Sun, Aarti Sethuraman, Chuan He Yang, Zhaohui Wu, Junming Yue and Lawrence M. Pfeffer

J. Biol. Chem. published online August 6, 2013

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