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Identification of the receptor tyrosine kinase *AXL* in breast cancer as a target for the human *miR-34a* microRNA

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Abstract

The identification of molecular features that contribute to the progression of breast cancer can provide valuable insight into the pathogenesis of this disease. Deregulated microRNA expression represents one type of molecular event that has been associated with many different human cancers. In order to identify a miRNA/mRNA regulatory interaction that is biologically relevant to the triple-negative breast cancer genotype/phenotype, we initially conducted a miRNA profiling experiment to detect differentially expressed miRNAs in cell line models representing triple-negative (MDA-MB-231), ER⁺ (MCF7), and HER-2 over expressed (SK-BR-3) histotypes. We identified human *miR-34a* expression as being >3-fold down (from its median expression value across all cell lines) in MDA-MB-231 cells, and identified *AXL* as a putative mRNA target using multiple miRNA/target prediction algorithms. The *miR-34a/AXL* interaction was functionally characterized through ectopic over expression experiments with a *miR-34a* mimic in two independent triple-negative breast cancer cell lines. In reporter assays, *miR-34a* binds to its putative target site within the *AXL* 3' UTR to inhibit luciferase expression. We also observed

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degradation of *AXL* mRNA and decreased AXL protein levels, as well as cell signaling effects on AKT phosphorylation and phenotypic effects on cell migration. Finally, we present an inverse correlative trend in *miR-34a* and *AXL* expression for both cell line and patient tumor samples.

Keywords

MicroRNAs; AXL; *miR-34a*; Triple negative; Breast cancer

Introduction

Triple-negative tumors of the breast represent a unique subgroup of an otherwise heterogeneous disease that accounts for 10–25% of all the reported breast cancer cases [1, 2]. These tumors lack the expression of the steroid hormone receptors estrogen and progesterone, and have decreased expression of ErbB-2/human epidermal growth factor receptor 2 (HER-2). In molecular studies that classified breast cancer tumors and cell lines based on global gene expression profiles, most triple-negative samples were representative of the basal B subtype as they expressed markers characteristic of the underlying basal (myoepithelial) layer of cells in breast ducts [3–9]. Triple-negative tumors generally display aggressive metastatic behavior, have a high propensity for recurrence, and are associated with poor patient survival [10]. Premenopausal women of African, African-American, and Hispanic ethnicity have a higher rate of risk for this disease [11]. Although systemic, neoadjuvant chemotherapy has been a viable therapeutic option in some metastatic cases, molecular-targeted therapies having any degree of measured success are currently unavailable.

MicroRNAs represent a novel, conserved class of endogenous non-coding RNAs that negatively regulate gene expression via the RNA interference (RNAi) pathway [12]. A variety of biological processes have been implicated as being regulated by miRNAs including cell development, differentiation, proliferation, metabolism, and the stress response (for reviews see [13–16]). It is now recognized that many miRNAs reside in regions of genomic instability associated with cancer, and that genetic rearrangements, gain or loss in DNA copy number, errors in miRNA processing, epigenetic regulation of miRNA expression, and activation by oncogenic transcription factors may all lead to aberrant miRNA expression [17]. MicroRNAs can function as either oncogenes or tumor suppressors depending on their level of expression and the biological function of their targeted transcript. For instance, oncogenic miRNAs promote tumorigenesis when they are over expressed and negatively regulate genes whose products are involved in cell-cycle arrest and apoptosis [18]. Conversely, miRNAs with tumor suppressor roles are often down regulated resulting in an inability to regulate cell growth and proliferation as a result of their absence [19]. To date, there are >1,000 mature human miRNAs that have been identified, yet the vast majority of mRNA targets (each miRNA is estimated to regulate multiple if not hundreds of mRNA transcripts) remain uncharacterized [12].

The aim of this study was to identify and characterize a novel miRNA/mRNA target interaction that is relevant to the molecular biology and/or pathophysiology of triple-negative breast cancer. We find the down regulated expression of human *miR-34a* to be reciprocally linked to increased *AXL* mRNA and protein expression in triplenegative breast cancer cell lines with an invasive phenotype. AXL is a member of the TAM (Tyro3, AXL, and MER) receptor tyrosine kinase family and its signaling plays a diverse role in cellular processes including survival, proliferation, migration, invasion, angiogenesis, autophagy, platelet aggregation, and natural killer-cell differentiation [20]. High AXL expression was recently shown to be a negative prognostic indicator for survival in breast cancer patients

[21]. Using two independent triple-negative breast cancer cell lines, we show that the ectopic over expression of *miR-34a* results in decreased *AXL* mRNA and protein levels, decreased cell migration potential, and decreased phospho-AKT protein expression levels (MDA-MB-231 cells), without having any significant concomitant effect on the viability or induction of apoptosis in these cell lines. *AXL* is the second receptor tyrosine kinase (c-MET being the first [22-27]) identified as being regulated by *miR-34a*.

Materials and methods

Cell lines

Breast cancer cell lines MCF7 (ER⁺/PR⁺/HER-2⁻), SK-BR-3 (ER⁻/PR⁻/HER-2⁺), MDA-MB-231 (ER⁻/PR⁻/HER-2⁻), BT549 (ER⁻/PR⁻/HER-2⁻), T47D (ER⁺/PR⁺/HER-2⁻), and Hs578T (ER⁻/PR⁻/HER-2⁻); colorectal cell lines SW480 and SW620; and the renal cell line SN12C were maintained in RPMI 1640 basal medium supplemented with 10% v/v heat-inactivated fetal bovine serum (Invitrogen; Carlsbad, CA). The immortal non-tumorigenic breast cell line MCF10A (ER⁻/PR⁻/HER-2⁻) was maintained in DMEM/F12 basal medium supplemented with 5% v/v horse serum (Invitrogen; Carlsbad, CA), 20 ng/ml final epidermal growth factor, 0.5 µg/ml final hydrocortisone, 100 ng/ml final cholera toxin, and 10 µg/ml final insulin (all from Sigma-Aldrich, St. Louis, MO). Antibiotics were excluded from all the media. Cells were grown at 37°C with 5% CO₂ and passaged every 3–4 days for up to 10 generations. MCF10A and MDA-MB-231 cells were obtained from ATCC (Manassas, VA; 2008). MCF7, SK-BR-3, SN12C, and HeLa cells were obtained from the NCI Developmental Therapeutics Program, NCI, and NIH (Bethesda, MD; 2008). Hs578T, BT549, and T47D cells were gifted by Dr. Stan Lipkowitz (NCI, NIH, Bethesda, MD; 2008). SW480 and SW620 cells were gifted by Dr. Thomas Reid (NCI, NIH, Bethesda, MD; 2009). All cell lines tested negative for mycoplasma contamination using the MycoAlert® Mycoplasma Detection Kit with a Lucetta™ Luminometer (Lonza Rockland, Inc.; Rockland, ME).

Tissue collection

Fresh-frozen tumor specimens were obtained at time of surgery from breast cancer patients, as described previously [28]. Patients were recruited, who signed letters of consent at hospitals in Baltimore, Maryland, USA, between 1993 and 2003. Clinical and pathological information was obtained from medical records and pathology reports. Disease staging was performed according to the tumor-node-metastasis (TNM) system of the American Joint Committee on Cancer/the Union Internationale Contre le Cancer (AJCC/UICC). The Nottingham system was used for determining the tumor grade. The protocol for collection of tumor specimens, survey data, and clinical as well as pathological information was reviewed and approved by the University of Maryland Institutional Review Board (UMD protocol #0298229). IRB approval of this protocol was then obtained for all participating institutions (Veterans Affairs Medical Center, Union Memorial Hospital, Mercy Medical Center, and Sinai Hospital). The research was also reviewed and approved by the NIH Office of Human Subjects Research.

Immunohistochemical characterization of basal-like breast cancer

Basal-like breast tumors were identified by immunohisto-chemistry for estrogen receptor (ER), HER-2 receptor, EGFR receptor, and cytokeratin 5/6 using the following antibodies: ready-to-use monoclonal (Clone 6F11) antibody (Ventana Medical Systems; Tucson, AZ) for estrogen receptor; 1:100 diluted rabbit polyclonal antibody (Dako North America, Inc.; Carpinteria, CA) for HER-2; ready-to-use mouse antibody for EGFR; and 1:250 diluted mouse monoclonal antibody for cytokeratin 5 (Lab Vision Corporation; Fremont, CA). Basal-like breast tumors were ER negative, HER-2 negative, and either EGFR positive or

cytokeratin 5/6 positive, as described previously [29]. Aberrant nuclear p53 expression was determined using a 1:100 diluted monoclonal DO-7 antibody (Dako North America, Inc.; Carpinteria, CA). A tumor was scored p53-positive if more than 10% of the cancer cells showed nuclear positivity for p53 protein expression. Tumors were screened for p53 mutations as previously described [30].

MicroRNA expression profiling

The small RNA (sRNA) fractions from four separate passages (biological replicates) of MCF7, SK-BR-3, MDA-MB-231, and MCF10A cells were extracted using Qiagen's miRNeasy kit following the manufacturer's instructions (Qiagen; Germantown, MD). Five micrograms of sRNA was labeled with either Cy3 (MCF10A) or Cy5 (breast cancer cell) fluorescent label (GE Healthcare; Piscataway, NJ) using the *mirVana* miRNA labeling kit (Ambion; Austin, TX). The labeled products were hybridized to *mirVana* miRNA Bioarrays V2 (Ambion, Austin, TX) and washed following the manufacturer's protocol. Each array was composed of 328 human miRNA probes in addition to 114 mouse and 46 rat miRNAs. The comparative hybridization for each breast cancer cell line to the reference MCF10A cell line control was conducted in quadruplicate ($n = 12$ total arrays). Processed arrays were scanned for dual channel hybridization using a GenePix 4000B scanner (Molecular Devices; Sunnyvale, CA). Analyses were performed using BRB-Array Tools Version 3.8.1 (<http://linus.nci.nih.gov/BRB-ArrayTools.html>). Replicate spots for each miRNA were averaged within the array, background adjusted, and $\log_2$ normalized. Normalization was performed using a per chip median normalization method. Only those miRNAs with at least a threefold change in expression from its median value in 4 of 12 total arrays were retained. Samples and genes were subjected to a hierarchical cluster analysis with average linkage under the Euclidian distance similarity metric using the Cluster 3.0 (<http://rana.stanford.edu/software>) and Java Tree View 1.1.4r3 [31] applications. MiRNA probes of mouse, rat, and the manufacturer's (Ambion) origins that passed the gene filter were excluded from future analyses. Microarray expression data are available at the Gene Expression Omnibus (GEO) (accession GSE21834) and are in accordance with MIAME guidelines.

MicroRNA-target predictions

In a PAM analysis of global gene expression array data conducted on 51 breast cancer cell lines, 305 genes were identified as classifiers for the luminal, basal A, and basal B subtypes based on a threshold scaling of 2.8 [6]. From this list, 20 genes (the top 10 highest and lowest scores for the basal B subtype, excluding multiple probes for the same gene) were used in three separate miRNA target prediction searches (TargetScanHuman 5.1, <http://www.targetscan.org/>; Microcosm Targets 5, <http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>; and MiRanda 2008, <http://www.microrna.org/microrna/home.do>) to identify putative miRNA regulators from the list of 34 differentially expressed miRNAs observed in our profiling experiment. For MDA-MB-231 cells, only those predicted miRNAs that matched with the 20 genes from the classifier list and exhibited an inverse relationship in expression to the classifier genes were considered.

Northern hybridization

Total RNA (10 μ g) was resolved on a 15% TBE-Urea gel (1.0-mm thickness) (Invitrogen; Carlsbad, CA), electro blotted onto a positively charged nylon membrane (Roche Diagnostics, Indianapolis, IN), and UV cross linked at 120,000 μ Joules using a UV Stratalinker 1800 (Agilent Technologies; Santa Clara, CA). Following prehybridization in DIG Easy Hyb Buffer (Roche Diagnostics; Indianapolis, IN) for 15 min at 56°C, human *miR-34a* was probed (56°C for 16 h) with a complementary 3'-DIG labeled miRCURY

LNATM detection probe ACAACCA GCTAAGACACTGCCA (Exiqon; Woburn, MA) (2nM final concentration of probe in 20 ml DIG Easy Hyb Buffer). The membrane was washed using a DIG Wash and Block Buffer (Roche Diagnostics; Indianapolis, IN) and RNA was detected by CSPD chemiluminescence on film following the manufacturer's protocol for the DIG Luminescent Detection Kit (Roche Diagnostics; Indianapolis, IN). The blot was stripped at 37°C in 0.2 M NaOH solution containing 0.1% SDS, washed with 10X SSC, and reprobed (2nM final probe concentration, 50°C hybridization for 16 h) with a complementary 3'-DIG labeled miRCURY LNATM detection probe for human U6 (CACGAA TTTGCGTGTCATCCTT) (Exiqon; Woburn, MA).

Quantitative real-time PCR

Total RNA was prepared from cell lines using the miRN-easy extraction kit (Qiagen; Germantown, MD). Single-stranded cDNA was synthesized from 100 ng total RNA using the TaqMan[®] MicroRNA Reverse Transcription kit (Applied Biosystems; Foster City, CA). QRT-PCR was performed on an ABI 7900HT Sequence Detection System using a human *miR-34a* microRNA TaqMan[®] Expression Assay and TaqMan[®] Universal PCR Master Mix (Applied Biosystems; Foster City, CA). The small nucleolar RNA molecule RNU48 was used as an endogenous control for normalization, as it displayed the lowest variance in threshold cycle times (C_t) compared to seven other controls tested across all 10 cell lines. Relative *miR-34a* expression was determined by the comparative C_T method. Three biological replicates for each individual cell line were tested and PCR amplifications were performed in triplicate for each sample. The threshold cycle times were averaged across all technical and biological replicates within a given cell line. Fold difference in *miR-34a* expression for each cell line relative to the MCF10A cell line was calculated using the equation $2^{-\Delta\Delta C_t}$. Error bars for the fold-difference calculations represent the range in $\Delta\Delta C_t$ values based on the incorporation of the standard deviations for this value.

For the analysis of patient tumor samples, total RNA from 26 histologically characterized triple-negative breast cancer samples were used in measuring *miR-34a* and *AXL* expression levels. In brief, total RNA from fresh-frozen breast tissue sample biopsies was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen; Carlsbad, CA). The concentration of each RNA sample was measured using a NanoDrop spectrophotometer (NanoDrop Technologies; Wilmington, DE), and the RNA integrity was confirmed with an Agilent 2100 Bioanalyzer (Agilent Technologies; Palo Alto, CA). *AXL* expression was measured from cDNA synthesized from 1 µg total RNA using the High Capacity RNA-to-cDNA kit, Gene Expression Primer Assays, and TaqMan[®] Gene Expression Master Mix (Applied Biosystems; Foster City, CA). *ACTB* mRNA expression was used for normalization. Both *miR-34a* and RNU48 were measured from each sample as previously described for cell lines.

Construction of luciferase expression vectors

Complementary oligonucleotides (48 bp) spanning the putative *miR-34a*-binding site in the 3'UTR of *AXL* were synthesized with *XhoI* compatible ends. Wild type (WT) and mutant sequences representing changes in the seed sequence-binding region of the *AXL* 3'UTR at positions 2 (M2), 3 (M3), 2 and 4 (M2/4), or 3 and 5 (M3/5) relative to the 5' end of the miRNA are listed in Online Resource Table 1. Following heat denaturation in HEPES buffer, the synthetic forward and reverse oligonucleotides were annealed at 37°C. The annealed inserts were phosphorylated at 37°C using T4 DNA Kinase and ATP. The pMIR-GLO Dual-Luciferase miRNA Target Expression Vector (Promega; Madison, WI) was prepared for ligation by *XhoI* digestion followed by BAP treatment of the ends. A threefold excess of insert to vector was used in the ligation reactions with T4 DNA Ligase at 15°C. Recombinant vector plus inserts were transfected into TOP10 cells (Invitrogen; Carlsbad,

CA) and plated onto ampicillin selective LB agar plates. Positive clones containing inserts were identified as PCR products using pMIR-GLO vector-specific primers (Forward: CGCAAGATCCGCGAGA TTCT and Reverse: TAGTTATTGCTCAGCGGTGG). PCR positive clones were then subjected to sequencing to verify the integrity of the insert. Before the luciferase assays, clones were grown and plasmid DNA was purified using a Qiagen Plasmid Mini Kit (Qiagen; Germantown, MD) followed by endotoxin removal with MiraCLEAN® (Mirus Bio LLC; Madison, WI).

The miTarget™ microRNA 3' UTR target sequence expression clone vector containing the full-length 3' UTR of *AXL* insert (GeneCopoeia; Rockville, MD) and a control vector minus insert were transfected into One Shot® Stbl3™ cells (Invitrogen; Carlsbad, CA) and plated onto kanamycin selective LB agar plates. Plasmid DNA from positive clones was purified using a QIAfilter™ Plasmid Midi Kit with endotoxin removal (Qiagen; Germantown, MD).

Luciferase reporter assay

For assays involving the full-length *AXL* 3' UTR, MDA-MB-231 cells (10,000 cells per well) were seeded in a white 96-well CulturPlate™ (PerkinElmer; Waltham, MA) and incubated for 24 h before transfection. The expression vectors (1 µg) were co-transfected with AllStar Negative Control siRNA (siNEG), *hsa-miR-34a* miScript mimic (UGGCAGUGUCUUAGCUGGUUGU) (34a-mimic), or *hsa-miR-200c* miScript mimic (UAAUACUGCCGGGU AAUGAUGGA) (200c-mimic) (Qiagen; Germantown, MD) at a 10 nM final concentration using Lipofectamine™ 2000 (Invitrogen; Carlsbad, CA). Both firefly and *Renilla* luciferase were measured 24-h post-transfection on a FLUOstar OPTIMA microplate reader (BMG Labtech; Cary, NC) using the Luc-Pair™ miR Luciferase Assay system (GeneCopoeia; Rockville, MD). For the wild-type and mutant seed sequence expression constructs (100 ng), both fast kinetic firefly and *Renilla* luciferase expression was measured 48-h post-transfection in MDA-MB-231 cells (25,000 cells per well) using the Dual-Luciferase Reporter Assay System (Promega; Madison, WI). The mean ± SEM is reported for each transfection condition ($n = 3$). Significance was determined by a two-tailed unpaired t test for means (siNEG vs. mimic or mimic for WT vs. mimic for mutant construct).

Branched DNA capture assay

MDA-MB-231 or Hs578T cells (20,000 cells per well; 96-well plate) were reverse transfected with siNEG, siRNA *AXL* (siAXL) (TCCAAGATTCTAATGATTAA) (Qiagen; Germantown, MD), or 34a-mimic at a 10 nM final concentration using Lipofectamine™ 2000. Relative *AXL* mRNA levels in cell lysates were determined 48-h post-transfection using an *AXL*-specific probeset with the Quantigene® branched DNA reagent (1.0) and capture assay system (Panomics; Fremont, CA) (see Martin et al. for assay details [32]). All transfection experiments were performed in triplicate with a total of 36 measurements per transfection condition. *AXL* mRNA levels were normalized to corresponding *PPIB* mRNA levels measured from the same lysate followed by averaging of replicates across experiments. Significance was determined by a two-tailed unpaired t test for means (siNEG treated samples were used as the control for all comparisons).

Western blots

MDA-MB-231 or Hs578T cells (600,000 cells per well; 6-well plate) were reverse transfected with siNEG, siAXL, or 34a-mimic at a 10 nM final concentration using Lipofectamine™ 2000. Transfected cells were washed in PBS and directly lysed within the well by the addition of Cell Extraction Buffer (Invitrogen; Carlsbad, CA) containing 1X Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific; Rockford, IL)

followed by gentle scraping. After a 30-min incubation on ice, protein lysates were centrifuged at 13,000 rpm for 10 min at 4°C to remove cellular debris. All the protein lysates were harvested 48-h post-transfection. Total protein concentration was measured by the BCA™ protein assay (Thermo Scientific; Rockford, IL). Total cell extracts (5 µg for AXL blots or 10 µg for all other protein blots) were reduced and resolved on a 4–12% Tris–glycine gradient gel (Invitrogen; Carlsbad, CA). Proteins were electroblotted (Tris–glycine transfer buffer + 10% v/v methanol; 3 h, 25 volts constant) to an Invitrolon™ PVDF membrane (Invitrogen; Carlsbad, CA) and blocked with 5% w/v milk in 1 × TBS + 0.1% v/v Tween-20. For immunodetection, membranes were incubated at 4°C overnight with primary antibodies specific for AXL, phospho-p44/42 ERK1/2 (Thr202/Tyr204), p44/42 ERK1/2, phospho-AKT (Ser473), phospho-AKT (Thr308), AKT (pan), and β-actin (Cell Signaling Technology; Danvers, MA). Beta-actin expression was examined as a loading control. Following washes with 1 × TBS + 0.1% v/v Tween-20, membranes were incubated 1 h with either HRP-linked anti-rabbit or anti-mouse secondary antibody (Cell Signaling Technology; Danvers, MA). Chemiluminescence was detected on film using SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific; Rockford, IL). Membranes were stripped with Restore™ Plus Western Blot Stripping Buffer (Thermo Scientific; Rockford, IL) in a 30-min incubation at room temperature. The blots were re-blocked in 5% w/v milk, 1 × TBS + 0.1% v/v Tween-20 for 1 h and re-probed. The signal density for each protein was normalized to the respective β-actin band in that sample and expressed as a fraction of the siNEG transfected sample on the blot using ImageJ 1.43 s (<http://rsb.info.nih.gov/ij/>) software [33].

For rescue experiments, MDA-MB-231 cells (150,000 cells per well) were seeded in six-well plates and incubated for 24 h before transfection. Using Lipofectamine™ 2000, cells were cotransfected with siNEG, siAXL, or 34a-mimic at a 10 nM final concentration along with either an OmicsLink™ Expression-Ready ORF-cloning vector containing an AXL cDNA (NM_021913) insert that lacks the 3′ UTR or a control vector with an EGFP insert (2 µg) (GeneCopoeia; Rockville, MD). Twenty-four hours post-transfection, cells were harvested and lysed as previously described.

Cell migration assay

MDA-MB-231 or Hs578T cells (20,000 cells per well) were reverse transfected on the surface of a microporous (8.0-µm pore size) PET membrane (BD Falcon™ HTS Insert System, BD Biosciences; San Jose, CA) with siNEG, siAXL, siRNA MET (siMET) (ACCGAGGGAATCAT CATGAAA) (Qiagen; Germantown, MD), or 34a-mimic at a 10 nM final concentration using Lipofectamine™ 2000. Cells were starved 48-h post-transfection by aspirating the growth media and replacing it with serum-free media. After a 24-h starvation, 10% serum-containing media was added to the lower chamber (underside of membrane). Cells were allowed to migrate for 16 h before processing. In brief, cells on the transfection side of the membrane were swabbed and cells on the underside of the membrane were fixed in methanol. Following washes in PBS, cells on the underside of the membrane (migrated cells) were DAPI stained, imaged, and counted using a Discovery-1 Screening System (Molecular Devices; Sunnyvale, CA). All migration assays were performed in triplicate with a total of 10 measurements per transfection condition. Significance was determined by a one-way ANOVA with a Dunnett multiple comparison post-test using siNEG treated samples as the control.

Cell viability/proliferation assay

In parallel with the migration assay, MDA-MB-231 or Hs578T cells (20,000 cells per well; 96-well plate) were reverse transfected with the same siRNA/miRNA mimic molecules and either fluorescence (579_{Ex}/584_{Em} nm, CellTiter-Blue®) or absorbance (490 nm, CellTiter

96[®] Aqueous) (Promega; Madison, WI) was determined after the addition of the reagents 76-h post-transfection. All viability assays were performed in triplicate with a total of 30 measurements per transfection condition for each assay. Significance was determined by a two-tailed unpaired *t* test for means (siNEG treated samples were used as the control for all comparisons).

Cell-cycle analysis and apoptosis

MDA-MB-231 or Hs578T cells (600,000 cells per well; 6-well plate) were reverse transfected with siNEG, siAXL, siMET, or 34a-mimic at a 10 nM final concentration using Lipofectamine™ 2000. After 72 h, cells were washed in DPBS and fixed in 70% ethanol overnight at 4°C. DNA staining was performed with 50 µg/ml propidium iodide and 100U RNase A for 30 min at 4°C. Data collection was performed on a FACScalibur flow cytometer (Becton-Dickinson; Franklin Lakes, NJ) with CellQuest Pro software. For each sample, 25,000 events were collected and cell-cycle modeling was performed using FlowJo 9.3 (Tree Star, Inc.; Ashland, OR) software. Aggregated cells were gated out, and the percentages of cells within the different phases of the cell cycle were determined. Flow cytometric measurements were made on transfections performed in triplicate for each condition. Significance was determined by a two-tailed unpaired *t* test for means (siNEG treated samples were used as the control for all comparisons).

For assessing the effects on cell viability and the possible induction of apoptosis following transfection with *miR-34a*, Annexin V-PE and PI flow cytometry was conducted. MDA-MB-231 or Hs578T cells were harvested 72 h post-transfection and stained following the manufacturer's instructions for the Annexin V-APC Apoptosis Kit (eBioscience, Inc.; San Diego, CA). Data collection was performed on a FACScalibur flow cytometer (Becton-Dickinson; Franklin Lakes, NJ) using CellQuest Pro software. For each sample, 10,000 events were collected, and apoptotic fractions were determined using FlowJo 9.3 (Tree Star, Inc.; Ashland, OR). Flow cytometric measurements were made on transfections performed in triplicate for each condition. Significance was determined by a two-tailed unpaired *t* test for means (siNEG treated samples were used as the control for all comparisons).

Statistical analyses

All statistical analyses were performed using GraphPad Prism v5.0c software (GraphPad Software, Inc.; La Jolla, CA).

Results

Differentially expressed miRNAs among three breast cancer cell lines with different histotypes

To identify differentially expressed miRNAs among the breast cancer cell lines that differed in their hormone receptor histotypes, we profiled miRNA expression in cell line models representative of the luminal (MCF7 and SK-BR-3; ER⁺/PR⁺/HER-2⁻ and ER⁻/PR⁻/HER-2⁺, respectively) and basal B (MDA-MB-231; ER⁻/PR⁻/HER-2⁻) molecular subtypes, and normalized each profile to that of a common reference sample (MCF10A; normal breast, non-tumorigenic control). Thirty-four miRNAs from a total of 328 human miRNAs (10.4%) spotted on the array passed the threshold of having at least a threefold change in expression in either direction from its median value across all samples/arrays for at least 33% of all arrays tested (Fig. 1a). Of these 34 miRNAs, 21 (61.8%) were observed to meet this threshold in the MDA-MB-231 cell line alone (with either up or down expression relative to MCF10A cells consistent in at least two of 4 arrays (replicates) that were tested). Interestingly, across the three cell lines, only two of these 21 miRNAs (*miR-200b* and *miR-375*) had the same directionality in expression (up in both MDA-MB-231 and SK-BR-3

cell lines relative to MCF10A); indicating that the majority of these miRNAs in MDA-MB-231 cells were inversely expressed in the luminal cell lines. Some of our results are consistent with a previously reported miRNA microarray expression profiling study in breast cancer cell lines [34]. For example, the down regulated expression of *miR-141* and *200c* (clustered on human chromosome 12) and the up regulated expression of *miR-221* and *miR-222* (clustered on human chromosome X) in the MDA-MB-231 cell line relative to MCF10A were also observations shared in this study. The correlated up regulation of *miR-221* and *miR-222* has also recently been confirmed in triple-negative breast cancer patient samples [35].

Bioinformatic prediction of a miR-34a/AXL interaction is relevant to the basal B molecular subtype in breast cancer cell lines

Previous studies have shown that breast cancer cell lines, as tumor samples, can be grouped into distinct molecular subtypes based on their global gene expression profiles [4, 6]. In a study by Neve et al. [6], a Prediction Analysis of Microarrays (PAM) that was applied to gene expression data from 51 breast cancer cell lines identified 305 classifiers that would distinguish these cell lines as belonging to either the basal A, B, or luminal subtypes. From this list of classifiers, we focused on those genes specific to the basal B subtype that had the top ten positive and negative PAM scores when applying our target prediction analysis. We chose the basal B subtype because cell lines classified within this group (including MDA-MB-231) are often of the triple-negative histotype, with a characteristic mesenchymal-like appearance, high proliferation rates, and high invasiveness [4, 6]. We used three separate miRNA target prediction algorithms (each of which with weight different parameters in binding and having different levels of predictive power, yet all are confined to the 3'UTR of the mRNA) to identify putative interactions between our 34 differentially expressed miRNAs with genes in the basal B gene classifier subset. Only those interactions in which there was an inverse correlation in expression between the miRNA and the mRNA target were considered (Online Resource Table 2) for possible follow-up studies. There were six miRNA/mRNA interactions that were identified by all the three-prediction programs: *miR-34a/AXL*, *miR-34a/FOSL1*, *miR-191/TSPAN13*, *miR-200b/TSPAN13*, *miR-222/TSPAN13*, and *miR-200a/FOXA1*. We excluded the *miR-200a/FOXA1* prediction because the *FOXA1* gene was more of a distinguishing classifier for the luminal subtype (both basal A and B subtypes exhibited low expression of *FOXA1*) and the free energy of binding between *miR-200a*, and its target site was not as strong as some of the other predicted interactions. Likewise, with regard to the miRNAs targeting *TSPAN13*, there were only poorly conserved binding sites for these miRNAs across several species, and the free energy of binding for each miRNA was comparatively weak. The putative interactions predicted for *miR-34a* with either *AXL* or *FOSL1* were much more appealing, as both had highly conserved (across 11 species in the case for *AXL*) seed sequence matches of eight consecutive nucleotides beginning at the 5' end of the miRNA, and strong binding energies ($\Delta G = -28.5$ and $\Delta G = -22.8$ or -24.3 kcal/mol for *AXL* and *FOSL1*, respectively). Although there are two putative binding sites for *miR-34a* in the 3'UTR of *FOSL1* (one site is considered poorly conserved; Online Resource Fig. 1), there appears to be an additional stretch of base complementarity (7 nt) following the seed sequence region of *miR-34a* that is unique to the targeting site within *AXL*; making this binding interaction stronger with the possibility of a higher probability of occurrence (Fig. 1b). We focused our next series of experiments on characterizing the possible interaction between *miR-34a* and the receptor tyrosine kinase *AXL*, as *AXL* has been directly associated with a variety of different cancers including those of the breast [21, 36–38]. Moreover, *AXL* was shown to play an important role in mediating cell motility and invasiveness in breast cancer cells [38].

***miR-34a* expression is significantly downregulated in triple-negative breast cancer cell lines and is inversely related to AXL protein expression**

To confirm the observed expression levels of *miR-34a* from our array results, we performed a Northern blot hybridization and qRT-PCR analysis with probes and primers specific for the mature form of *miR-34a*, respectively, using the same cell lines representative of the array as well as six additional cancer cell lines (Fig. 1c, d). Mature *miR-34a* expression was significantly down regulated in triple-negative breast cancer cell lines representative of the basal B subtype (MDA-MB-231, Hs578T, and BT549), but was expressed in the luminal cell lines MCF7, T47D, and HER-2 expressing SK-BR-3 cell line. MDA-MB-231 cells and other triple-negative metastatic breast cancer cell lines express higher than normal levels of the receptor tyrosine kinase AXL [38–40] (Online Resource Fig. 2); high AXL expression being a positive correlate of cell aggressiveness. Interestingly, AXL expression was also high in the renal cancer cell line SN12C and detectable in the colorectal cell lines SW480 and SW620, whereas *miR-34a* expression in these cell lines was either absent or weakly expressed, respectively. We examined whether decreased mature *miR-34a* expression in these cell lines could be the result of an error in the cellular processing of the precursor molecule to the mature form of the miRNA. Our qRT-PCR results indicate that the primary transcript for *miR-34a* is poorly expressed and that this would explain the low levels of both precursor and mature forms of *miR-34a* in these cells (Online Resource Fig. 3). Since *miR-34a* is known to be under the direct regulatory control of p53 [41–48], and mutations in this gene were previously shown to be present in the cell lines we used, *miR-34a* expression might be influenced by the inability of p53 to bind or activate transcription. Alternatively, a recent study has shown that the inactivation of *miR-34a* transcription could be the direct result of CpG methylation [49]. Given the correlation between low levels of *miR-34a* expression in cell lines exhibiting high levels of AXL, and the putative target site for *miR-34a* in the *AXL* 3' UTR, we hypothesize that *AXL* is a direct molecular target of *miR-34a*.

***miR-34a* targets the 3'UTR of AXL to negatively affect luciferase reporter expression**

To test whether *AXL* contains a putative target site for *miR-34a*, we used the full-length *AXL* 3' UTR as well as a more defined region of the UTR (48 bp representing nucleotides 18–65 from the start site of the 3' UTR; denoted as wild-type or WT) in a luciferase reporter expression system. We also created expression vectors containing base pair mutations within the putative seed sequence of this defined region to examine the specificity of this targeting interaction. In MDA-MB-231 cells, co-transfection of the full-length 3' UTR of *AXL* with a siRNA (target site is within the *AXL* 3' UTR) results in a 97% loss of luciferase reporter expression (Fig. 2a). On average, there was a 38% loss in luciferase expression with the 34a-mimic molecule ($P < 0.0001$) but no significant change in luciferase expression with a *miR-200c* mimic. The *miR-200c* microRNA does not have a predicted target site within the *AXL* 3' UTR and therefore served as a negative control.

In assays involving a portion of the *AXL* 3' UTR that contains the putative target site for *miR-34a*, co-transfection of the WT vector with the 34a-mimic also resulted in an approximate 40% decrease in luciferase expression (Fig. 2b), but this effect was nearly abolished when mutations in the putative seed sequence-binding regions were introduced. A single nucleotide alteration (M2 or M3; corresponding to nucleotide positions 2 and 3, respectively) in the seed sequence region was enough to produce the inhibitory effect.

***miR-34a* represses AXL at the transcriptional and translational level and has a downstream affect on AKT signaling in MDA-MB-231 cells**

Using MDA-MB-231 cells that were previously shown to be deficient in *miR-34a*, we examined the effects of ectopic *miR-34a* expression (in the form of a mimic molecule) on

AXL mRNA and protein levels. Normalized *AXL* mRNA levels (relative to *PP1B*) were determined for each transfection condition using a branched DNA capture assay. As expected, when compared with negative control (siNEG) transfected cells, a loss of ~80% in relative *AXL* mRNA levels was observed with a siRNA targeting *AXL* (Fig. 3a). Surprisingly, transfections with the mimic molecule resulted in an approximate 60% decrease in relative *AXL* mRNA levels that was highly significant ($P < 0.0001$). Similar effects were observed in the triple-negative breast cancer cell line Hs578T, in which there was an 86% loss in *AXL* mRNA levels with the siRNA and a 77% loss in *AXL* mRNA levels with the *miR-34a* mimic (Fig. 3a). This decrease in *AXL* mRNA upon *miR-34a* over expression, however, is consistent with recent reports suggesting mRNA degradation as the major component of mammalian miRNA-mediated gene regulation [50, 51]. For Hs578T cells, the effects of *miR-34a* on *AXL* mRNA levels may have been more pronounced as a result of lower endogenous *AXL* mRNA levels on average in these cells when compared to MDA-MB-231 cells.

At the protein level, the *miR-34a* mimic was effective in decreasing *AXL* protein by 90% compared to siNEG treated MDA-MB-231 cells (Fig. 3b), whereas the receptor tyrosine kinase c-MET (a previously published target of *miR-34a*; see Refs. [22–27]) was decreased by only 30%. In comparison to negative control treated Hs578T cells, a 60% reduction in *AXL* protein levels was seen following transfection with the *miR-34a* mimic, but just a 30% decrease in c-MET levels (Fig. 3b). These data suggest that in both MDA-MB-231 and Hs578T breast cancer cell lines, the affinity of *AXL* as a target for *miR-34a* appears to be greater than that of c-MET.

We also examined the downstream effects on *AXL* signaling when this gene is targeted for regulation by *miR-34a*. One signaling network known to involve *AXL* is the PI3K/AKT effector pathway. Although there was not a significant change in PI3K phosphorylation (data not shown), there was an appreciable decrease in phospho AKT (both Thr308 and Ser473 residues) but not total AKT levels in cells transfected with either siAXL or the 34a-mimic (Fig. 3c). We did not detect any noticeable changes in phospho-ERK1/2 (p42/p44 MAP kinase) and total ERK1/2 levels under these same transfections (data not shown).

More importantly, we conducted an *AXL* cDNA rescue experiment and observed the changes in phospho-AKT signaling (at the Thr308 residue). MDA-MB-231 cells co-transfected with an *AXL* cDNA expression vector lacking the 3'UTR sequence fully recovered *AXL* protein expression as the mRNA transcripts were not targeted by either the siRNA or the 34a-mimic (Fig. 3d). This resulted in a recovery of phospho-AKT (Thr308) levels and supports the targeting influence *miR-34a* has on *AXL*.

AXL targeting by miR-34a results in impaired cell migration but has no significant effect on proliferation or cell cycle regulation

In previous studies, *AXL* was shown to have an active role in cellular migration for a variety of cell types including triple-negative breast cancer [20, 36, 37, 52–55]. We examined the effects of *miR-34a* mediated knockdown of *AXL* protein levels on cell migration in both the MDA-MB-231 and Hs578T cell lines. MDA-MB-231 cells transfected with a *miR-34a* mimic resulted in a 37% decrease in migratory cells ($P < 0.001$), whereas no effect on cell migration was observed in cells treated with an siRNA targeting c-MET (Fig. 4a). The effect on cell migration was even more pronounced in Hs578T cells, as transfections with the *miR-34a* mimic resulted in a 53% decrease in migratory cells (Fig. 4b). Interestingly, the si-MET transfection resulted in an overall increase in the number of migratory Hs578T cells that was statistically significant ($P < 0.001$).

The reduced numbers in migratory cells upon transfection with the *miR-34a* mimic may be the result of impaired growth, proliferation, or even activation of cell death programming; and it may not necessarily reflect a single direct effect on decreased *AXL* levels within these cells. To determine whether the regulation of *AXL* by *miR-34a* principally alters the migration phenotype of MDA-MB-231 and Hs578T cells rather than alterations in cell growth and survival, we measured cell proliferation/viability and examined the cell cycle for growth arrest or induction of apoptosis in our transfected cells parallel to our migration assays. In MDA-MB-231 cells, we observed a modest decrease in cell viability (13%, $P < 0.05$) for the *miR-34a* mimic transfection when using the CellTiter-Blue® Assay (Fig. 4c). The observed results for the Hs578T cell line were very similar to that of MDA-MB-231. Transfection with the *miR-34a* mimic resulted in a 12% decrease in proliferative ability ($P < 0.0001$) whereas transfection with an siRNA targeting *c-MET* increased the viability of the cells by ~ 15% (Fig. 4d).

When comparing the various transfections in regard to cell-cycle analysis, the *miR-34a* mimic molecule did result on average in a 2.9% increase in the number of MDA-MB-231 cells in the G0/G1 phase with a concomitant decrease in cells entering the S-phase of the cell cycle when compared to siNEG transfected cells (Fig. 5a). The Hs578T cell line also exhibited an increase in the number of cells that remained in the G0/G1 cell cycle (~5%) with a decrease in cells at the S-phase (Fig. 5b). In a flow cytometric analysis with Annexin V staining as an indicator of apoptosis, there was a slight increase on average in early or pre-apoptotic MDA-MB-231 cells (compare lower right quadrants) for the siAXL, 34a-mimic, and siMET transfections (Fig. 6a). We did not, however, observe a significant increase in the number of apoptotic cells (upper right quadrant) for each transfection compared to siNEG. Similarly, no significant change in the number of pre-apoptotic or apoptotic Hs578T cells (Fig. 6b) was observed with the 34a-mimic transfection compared to siNEG.

As *AXL* may not be the only *miR-34a* target with influence on cell migration, we conducted a global transcriptome profiling experiment on MDA-MB-231 cells transfected with either siNEG or the 34a-mimic to determine other contributing transcripts that might be affected. Other than *MET* which also exhibited a $-0.8 \log_2$ fold change, we did not observe downregulation of other computationally predicted targets of *miR-34a* with relevance to cell motility (e.g., *RhoA*, *VCL*, *TBCA*, *TBCE*, *VASP*, *ANK2*, and isoforms of *MYO*). Moreover, other than *CCNE2*, known *miR-34a*-targeted transcripts involved in cell-cycle regulation (e.g., *CCND1*, *CDK6*, and *E2F3*) were also not significantly affected.

Overall, our results suggest that transfections with a *miR-34a* mimic did not cause a gross inhibitory effect on the viability or proliferative ability of these breast cancer cell lines, and that the observed negative effect of *miR-34a* expression on cell motility is most likely related to a decreased *AXL* protein expression in these cells.

***miR-34a* expression is inversely correlated with *AXL* mRNA expression in basal-like breast cancer tumor patient samples**

We examined whether the expression of both *miR-34a* and *AXL* mRNA were correlated with one another in a clinically relevant setting by profiling the expression of these RNAs in 26 tumor samples from patients with basal-like breast cancer (Online Resource Table 4). Application of a nonparametric (Spearman) correlation analysis to the expression data (*AXL* mRNA normalized to β -actin and *miR-34a* normalized to RNU48) generated for all the 26 samples shows that *AXL* and *miR-34a* expression are inversely correlated with one another ($r = -0.52$; two-tailed $P < 0.01$) with significance (Fig. 7).

Discussion

The association of *AXL* expression with basal-like breast cancer was initially reported in a gene expression profiling study involving 51 independent breast cancer cell lines [6]; but the factors contributing to this observation have not been thoroughly investigated. *AXL* expression as a result of discrete gene amplification or of amplification of its corresponding genomic region (19q13.1) resulting in copy number variations is not a common phenomenon identified in breast cancer. Furthermore, an actively mutated form of *AXL* is not hypothesized as there have been no reported somatic mutations within this gene in all primary breast carcinomas that have been examined (COSMIC Database) [56]. We hypothesized that *AXL* expression in triple-negative breast cancer is attributed to deregulation at the post-transcriptional level, possibly through the aberrant expression of a miRNA that targets *AXL* mRNA transcripts for sequestration or degradation.

In the present study, we demonstrate that the human *miR-34a* microRNA is significantly downregulated in expression in those highly invasive cell lines of the triple-negative histotype (MDA-MB-231, BT549, and Hs578T) that concomitantly exhibit high *AXL* protein expression. We showed that the 3' UTR of *AXL* contains a target site for *miR-34a* as ectopic expression of this miRNA resulted in loss of luciferase reporter expression as well as decreased endogenous *AXL* mRNA and protein levels. Phenotypically, targeting of *AXL* by *miR-34a* reduced phospho-AKT expression, and impaired the motility of triple-negative breast cancer cells toward a growth substrate, without having a significant affect on the viability of the cells. Furthermore, the *miR-34a/AXL* interaction appears clinically relevant, as there is an inverse correlation in expression for these molecules in basal-like breast cancer patient tumor samples.

The *miR-34* family of microRNAs has garnered significant interest in tumor biology because its transcription is directly regulated by p53, and it participates in a functional role within this tumor suppressor pathway [41–44, 47, 48]. Several studies have shown that ectopic expression of *miR-34a* can cause cell-cycle arrest in the G₁ phase [41, 44, 48], cellular senescence [44], and apoptosis [42, 47, 48, 57]. Several mRNA targets for *miR-34a* have been previously identified, and not surprisingly, a significant portion of them are known to be involved in cell-cycle control (for review see [58]). Of the known mRNA targets for *miR-34a*, the proto-oncogenic hepatocyte growth factor receptor MET has been the most extensively studied to date because of its association in a wide variety of cancers [22–27]. In our study, we compared the effects of *miR-34a* expression on both *AXL* and MET protein levels in two triple-negative breast cancer cell lines and we observed that the loss in protein translation was more pronounced for the receptor tyrosine kinase *AXL* than with MET. These results suggest that the functional consequence of *miR-34a* expression in tumor cells is highly dependent on the cell type and its cellular context. *AXL* mRNA and protein levels in triple-negative breast cancer cell lines are much more abundant than those of MET, and thus, the probability of targeting *AXL* over MET is greatly increased. In addition, the free energy of binding of *miR-34a* to *AXL* ($\Delta G = -28.5$ kcal/ mol) is much lower than with MET ($\Delta G = -16.5$ kcal/ mol), indicating that *miR-34a* has a greater likelihood of binding with *AXL* transcripts. We observed a similar phenomenon in regard to the effect of ectopic *miR-34a* expression on cell-cycle progression and apoptosis. Unlike the induction of apoptosis observed in neuroblastoma cell lines [57], HCT116 p53^{WT} cells [42], and H1299 and HEK293 cells [47, 48]; *miR-34a* expression did not result in the induction of apoptosis in triple-negative breast cancer cell lines. Similarly, we did not observe a drastic increase in G₁ cell-cycle arrest unlike what has been previously reported for other cell lines [41, 44, 48]. We hypothesize that this observed difference reflects the presence of more abundant targets (e.g., *AXL*) within triple-negative breast cancer cell lines such that they are

preferentially targeted minimizing the *miR-34a* effect on other targets involved in cell regulatory and survival pathways.

Recently, Mudduluru et al. have shown that *miR-34a* can regulate *AXL* expression and impair its functions that contribute to the migration, invasion, and formation of distant metastases of cancer cells [49]. *AXL* targeting by *miR-34a* was also confirmed through the application of in vitro assays using a modified method of mRNA pulldown and isolation with biotinylated *miR-34a* (Ashish Lal and Judy Lieberman, personal communication). In this article, we provide additional evidence for the targeting of *AXL* by *miR-34a*, and that this interaction (or lack thereof) is relevant to triple-negative breast cancer. The results of this study corroborates with the former and further substantiates *AXL* as second receptor tyrosine kinase to be identified as a target of *miR-34a* having similar pro-oncogenic properties to that of c-MET. As we continue to unravel the extent of the tumor suppressor role *miR-34a* plays in cells, it is reasonable to assume that one productive mechanism in shutting down the proliferative ability of cells would be to target the most upstream elements that are involved in growth factor binding and cellular signaling. Whether other receptor tyrosine kinases fall under the regulatory control of *miR-34a* awaits further experimentation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. *miR-34a* expression and targeting in triple-negative breast cancer cell lines. **a** Heat map of the most differentially expressed miRNAs in MDA-MB-231, MCF7, and SK-BR-3 breast cancer cell lines compared to MCF10A cells. Each cell line comparison is represented by four arrays (biological replicates). Hierarchical clustering of samples and genes are shown. Only those human miRNAs with at least a threefold change in expression from its median value across all arrays in at least 33% of the arrays are shown. *Red* miRNAs that are overexpressed relative to the reference, *green* miRNAs that are underexpressed relative to the reference. **b** Gene structure showing the location of the predicted binding site for *miR-34a* within the *AXL* 3'UTR, and the structure of the miRNA-target interaction as predicted by the MiRanda program. Seed sequence-binding region is *boxed in gray*. *CDS* coding sequence. **c** Northern blot showing the expression of *miR-34a* precursor and mature RNAs across 10 different cell lines. Breast cell lines: MCF10A, MCF7, MDA-MB-231, SK-

BR-3, Hs578T, T47D, and BT549; renal cell line: SN12C; and colorectal cell lines: SW480 and SW620. U6RNA was probed as a loading control. *Asterisks* denote triple-negative breast cancer cell lines. High AXL protein expression (see Suppl. Fig. S2) is inversely correlated with low *miR-34a* expression in these cell lines. **d** qRT-PCR analysis of mature *miR-34a* expression in cancer cell lines relative to the reference MCF10A. Error bars represent the range in fold difference. *Asterisks* denote triple-negative breast cancer cell lines. The results in **c** and **d** corroborate with one another, as well as to the relative expression levels observed for *miR-34a* in those cell lines that were tested on the microarray

Fig. 2. *miR-34a* targets the 3'UTR of *AXL* and affects expression of a luciferase reporter construct. Full-length *AXL* 3'UTR (a) or a 48 bp fragment containing the putative *miR-34a* target site (b) were cloned into luciferase reporter expression vectors and co-transfected into MDA-MB-231 cells with siNEG siRNA (negative control) siAXL, or mimics for *miR-34a* and *miR-200c*. For each construct, normalized firefly luciferase signal for each transfection is presented relative to the siNEG transfected sample. For the full-length 3'UTR of *AXL*, *miR-34a*, but not *miR-200c*, affects luciferase expression; mutations in the seed sequence-binding region for *miR-34a* nearly abolishes the effect (b). Bars represent the average signal $\pm$ SEM

Fig. 3.

Ectopic *miR-34a* expression decreases *AXL* mRNA and protein levels with a downstream effect on AKT phosphorylation. **a** MDA-MB-231 or Hs578T cells transfected with either siAXL or a *miR-34a* mimic results in a significant decrease in *AXL* mRNA levels within these cells. *AXL* mRNA levels were normalized to *PP1B* for each transfected sample (data point). Horizontal bar represents the average signal $\pm$ SEM. **b** MDA-MB-231 or Hs578T cells transfected with either siAXL or a *miR-34a* mimic also results in a significant decrease in *AXL* protein levels. The receptor tyrosine kinase MET, which was previously shown to be a target for *miR-34a*, was also affected at the protein level but the results are not as dramatic as those seen with *AXL*. Values under each band represent the β -Actin normalized intensity of the band relative to that of the siNEG transfected sample. **c** Decreases in *AXL* protein levels by either siAXL or the *miR-34a* mimic affects AKT phosphorylation (at Thr308 and Ser473) in MDA-MB-231 cells with no decrease in total AKT levels. **d** The effects of siAXL and *miR-34a* on phospho-AKT (Thr308) are rescued with the co-

transfection of an AXL expression vector that lacks the 3'UTR for targeting by these molecules. Western blots are representative of at least duplicate independent experiments

Fig. 4.

Downregulation of *AXL* expression by *miR-34a* results in decreased cell motility but has no major effect on the cell viability of MDA-MB-231 cells. *Box-Whiskers plots* showing the decrease in migratory MDA-MB-231 (a) or Hs578T (b) cells as a result of transfection with siAXL or a *miR-34a* mimic. Knockdown of MET had no negative effect on cell migration. *Bar graph* showing the effects of various transfections on two different measurements/assays of cell viability in MDA-MB-231 (c) or Hs578T (d) cells. *Each bar* represents the mean signal $\pm$ SEM

Fig. 5.

Representative flow cytometric analysis of the cell cycle from the three independent siNEG and 34a-mimic transfections in MDA-MB-231 (a) or Hs578T (b) cells using propidium iodide staining. Percentage of cells within each phase of the cell cycle is indicated. A slight increase in G₀/G₁ cell-cycle arrest is observed in cells transfected with the *miR-34a* mimic. The column graph shows the average of all the independent transfections $\pm$ SEM performed in triplicate

Fig. 6. Representative flow cytometric analysis of apoptosis from the three independent siNEG and 34a-mimic transfections in MDA-MB-231 (a) or Hs578T (b) cells using Annexin V and propidium iodide staining. *Numbers* within the graph represent the percentage of cells detected within a quadrant. No significant change in the percentage of pre-apoptotic (*lower right quadrant*) or apoptotic (*upper right quadrant*) cells is observed between the 34a-mimic and siNEG transfections. *Split column graph* shows the average of all the independent transfections $\pm$ SEM performed in triplicate

Fig. 7.

miR-34a and *AXL* mRNA inversely correlated in basal-like breast cancer patient tumor samples. Scatterplot depicting normalized *AXL* and *miR-34a* expression for the 26 basal-like breast cancer patient samples. The inverse relation in expression was determined through a Spearman nonparametric correlation analysis. *Open squares* indicate those samples that were identified as having p53 mutations