MicroRNA-301 Mediates Proliferation and Invasion in Human Breast Cancer

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Abbreviations: IR (ionizing radiation); qRT-PCR (quantitative real time polymerase chain reaction); MTS (soluble tetrazolium salt assay); siRNA (small interfering RNA); CT (chemotherapy); SCID (severe combined immunodeficient)

None of the authors have any conflict of interest.
ABSTRACT
Several microRNAs have been implicated in human breast cancer but none to date have been validated or utilized consistently in clinical management. miR-301 over expression has been implicated as a negative prognostic indicator in lymph node negative (LNN) invasive ductal breast cancer, but its potential functional impact has not been determined. Here we report that in breast cancer cells, miR-301 attenuation decreased cell proliferation, clonogenicity, migration, invasion, tamoxifen resistance, tumor growth and microvessel density, establishing an important oncogenic role for this gene. Algorithm-based and experimental strategies identified FOXF2, BBC3, PTEN and COL2A1 as candidate miR-301 targets, all of which were verified as direct targets through luciferase reporter assays. We noted that miR-301 is located in an intron of the SKA2 gene which is responsible for kinetochore assembly, and both genes were found to be co-expressed in primary breast cancer samples. In summary, our findings define miR-301 as a crucial oncogene in human breast cancer that acts through multiple pathways and mechanisms to promote nodal or distant relapses.

PRECIS
This study establishes a novel oncomiR in breast cancer that acts through several pathways to promote metastatic tumor progression.
INTRODUCTION

Since the initial discovery of micro-RNAs (miRNAs) in 1993 (1), they have been shown to influence multiple cellular processes (2), and in particular, have been demonstrated to play important roles in cancer development and progression (3, 4). The majority of miRNAs map to fragile genomic sites, and aberrant patterns of miRNA expression have been observed in multiple cancer types, including leukemias and lymphomas (5), colon (6), hepatocellular (7), head & neck (8), and breast cancers (2, 9-11). Functionally, aberrant miRNA expression can affect cell proliferation (12), apoptosis (4), chemotherapeutic and radiation-sensitivity (13), development of metastases (14), epithelial-mesenchymal transition (5), and could even potentially define the cancer stem cell phenotype (5).

Breast cancer is unfortunately the most commonly diagnosed cancer in women, and the second leading cause of cancer deaths in the developed world (15). The majority of women presenting with lymph node-negative (LNN) disease fare well (16); however, the development of metastatic relapse is almost always incurable (17). Several studies have described putative breast cancer-specific miRNA signatures (2, 9, 10, 18); others have correlated miRNA expression with bio-pathologic features, including estrogen and progesterone receptor status (10). None to date however, has been consistently validated, or utilized in clinical management.

In an effort to contribute to the understanding of miRNAs in human breast cancer, we had previously conducted a global miRNA profiling (11), wherein four miRNAs were identified to dichotomize relapse risk; one of which was hsa-miR-301, associated in particular with nodal and distant relapses. MiR-301 has been previously noted to be deregulated in pancreatic (19), hepatocellular (20), and small cell lung cancers (21). It is located in the intron of SKA2 (spindle and kinetochore associated complex subunit 2), and was shown to regulate the expression of miR-301 in a lung cancer cell line (22), but little else is known about miR-301. In this study, we
report that miR-301 promotes breast cancer proliferation, invasion, and tumour growth, mediated at least by FoxF2, BBC3, and PTEN. Furthermore, miR-301 likely co-operates with its host gene SKA2, contributing to the aggressive breast cancer phenotype with promotion of distant disease.

MATERIALS AND METHODS

Patients

Seventy-one formalin-fixed paraffin-embedded (FFPE) blocks of the 769 participants in a Phase III clinical trial comparing Tamoxifen vs. Tamoxifen plus breast radiotherapy (RT) for LNN breast cancer (16) served as the cohort for micro-RNA profiling evaluation. The current study utilized a case control design. Case patients (n=33) were identified as any individual who experienced local, lymph node, or distant metastasis within 10 years. Controls (n=38) were identified as patients who had never experienced any relapse or death from cancer with >10 years’ follow-up. The clinical characteristics of these 71 patients are described in Table 1. Five reduction mammoplasty specimens served as normal comparators. All studies have received Research Ethics Board approval from the University Health Network.

Cell lines and transfections

Primary human breast cancer cell lines MCF-7, MDA-MB-231, MDA-MB-468, and T47D were obtained from ATCC (2005-2007), and freshly recovered from liquid nitrogen (< 6 months). They were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. MCF-10A cells were obtained from Dr. Linda Penn (Ontario Cancer Institute), and grown in DMEM/HAM F12 supplemented with 5% horse serum, insulin, hEGF, hydrocortisone (Clonetics) and cholera toxin (Sigma). All cells have been authenticated using Short Tandem Repeat analyses, and are regularly examined using light microscopy. All cells are tested and determined to be free from Mycoplasma contamination every month.
The cells were transfected using the LipofectAMINE 2000 (Invitrogen, Canada) reverse transfection protocol, according to the manufacturer’s instructions. AntagomiR-301, anti-miR negative control #1 (NC) (Ambion), siRNA-SKA2 and scrambled siRNA (siC) were all transfected at a final concentration of 40 nM.

**Quantitative real-time PCR analysis of miRNAs and mRNAs**

The expression of hsa-miR-301 was measured using the standard Taqman MicroRNA assay (Applied Biosystems) as previously reported (11). The primers used for mRNA expression are listed in Supplementary Table 1. RNA was isolated using the Norgen kit (Norgen Biotek Corporation, Canada).

**Cell proliferation and colony-forming assays**

Cytopathic effects of depleting miR-301 were evaluated in MCF-7 and T47D cells using the MTS assay (Promega, Canada), which is a colorimetric method for determining the viable cells in proliferation and cytotoxicity. For colony formation assays, MCF-7 and T47D cells were seeded onto plates 48 hours after transfection, incubated for two weeks, then fixed and stained, followed by colony counting.

**Cell migration and invasion assays**

For scratch migration assay, antagomiR-301 or NC-transfected cells were scratched using a standard 200 AL tip. Serial photographs were obtained at different time point using an Axiovert 100 M phase contrast microscope (Carl Zeiss, Inc). Invasion of MDA-MB-231 cells was assessed using the Matrigel Invasion Chamber (BD Biosciences), with conditions detailed in the Supplemental Materials and Methods.

**Target identification by mRNA profiling**

MCF-7 and MB-231 cells were transfected with either antagomiR-301 or NC. RNA was isolated 24 and 72 hours later, and gene expression was analyzed using the Whole Human
Genome 4x44K Array (Agilent, Santa Clara, CA). Genes with greater than two-fold expression change relative to NC were considered as potential targets of miR-301, hence selected for further investigation.

**Western blotting**

MDA-MB-231 cells were transfected with either antagomiR-301 or NC, then harvested on ice after 72 hours. Proteins were probed with mouse anti-FoxF2 (Abnova, Taiwan), rabbit anti-PTEN, p-Akt (S473), total Akt (Cell Signaling Technology, USA), rabbit anti-PUMA BBC3 (Novus Biologicals LLC, USA), or anti-GAPDH (Abcam, USA). SKA2 protein was detected using a rabbit anti-SKA2 antibody (ProSci Inc, USA).

**Luciferase assay**

The 3’-UTRs of FoxF2, PTEN, BBC3 and Col2A1 were amplified by PCR and cloned downstream of the *luciferase* gene in a pMIRREPORT luciferase vector (Ambion). A mutant sequence was also cloned as a validation plasmid. Either pMir-luciferase or pMir-luciferase–gene specific vectors were co-transfected with antagomiR-301 or NC in MDA-MB-231 or MCF-7 cell lines. pRL-SV vector (Promega) containing *Renilla luciferase* was also transfected with each condition as a reference control. Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay (Promega).

**In vivo experiments**

Six to eight-week-old SCID CB.17 female mice were utilized for the xenograft experiments. MDA-MB-231 cells were transfected with antagomiR-301, NC or LipofectAMINE 2000. At 48 hours post-transfection, cells were harvested, 5x10^5 viable cells were diluted in 100 μl culture medium, then mixed with 100μl of Matrigel (BD Biosciences, USA). Cells were then injected subcutaneously into the flank of SCID mice. Tumour volume was measured twice weekly using a caliper, calculated as: (tumor length x width^2)/2. MCF-7 xenograft studies were
Conducted as described, but with the addition of implanting a 17β-estradiol pellet subcutaneously one day before cell injection.

**Detection of miR-301 using In-Situ Hybridization (ISH)**

*In-situ* hybridization was performed using a catalyzed signal amplification method (GenPoint signal amplification system; DakoCytomation, USA) using the 5’ biotin-labeled miR-301 miRCURYTM LNA detection probe, or a scrambled negative control probe (50 nM) (Exiqon, USA). Positive hybridization signals were visualized by adding the chromogenic substrate diaminobenzidine.

**Immunohistochemistry for SKA2, CD31 and Ki-67**

Expression of SKA2, CD31 and Ki-67 were evaluated on tumor sections using microwave antigen retrieval in combination with the Level-2 Ultra Streptavidin system. Rabbit polyclonal anti-human SKA2 antibody (1:200 dilution; PorSci Inc), rat anti-mouse CD31 antibody (1:50 dilution; BD Pharmingen), and mouse anti-human Ki-67 antigen (1:100 dilution; DakoCytomation) were utilized.

**Statistical analysis**

Box plots were utilized to visually explore the relationship between miR-301 or SKA2 expression using a Mann-Whitney-Wilcoxon test. All other data were expressed as the mean ± SE; a p-value of <0.05 was considered to be statistically significant.

**RESULTS**

*miR-301 was over-expressed in LNN breast cancer, strongly associated with tumor recurrence*

Global miRNA profiling was conducted on 71 archival FFPE LNN invasive ductal breast cancer samples using the TaqMan Low Density Array (Panel v1.0) (Applied Biosystems, USA). In comparison with 5 normal mammary epithelial tissues, 57 miRs were significantly
deregulated with adjusted p-values <0.05, amongst which 54 were up-regulated and 3 were down-regulated (data not shown). Amongst the top deregulated miRNAs was miR-301, which was ~6.7-fold up-regulated, compared to normal tissues (Suppl Fig 1A). Furthermore, patients with higher miR-301 expression level experienced a worse DFS survival compared to those with lower miR-301 expression (Suppl Fig 1B). Most interestingly, the relapses associated with miR-301 expression were nodal or distant metastases, not local recurrences (Fig 1A and Suppl Fig 1C).

AntagomiR-301 caused significant reduction in miR-301 expression, leading to reduced viability and decreased clonogenicity

The expression of miR-301 was evaluated in four human breast cancer cell lines: MCF-7, T47D, MDA-MB-231, and MDA-MB-468, compared to that of the normal human mammary epithelial cell line MCF-10A. As shown in Figure 1B, miR-301 was over-expressed in all four cell lines, indicating that these models are reasonable models for the primary human cancer scenario. To assess the biological role of miR-301, antagomiR-301 was utilized in MCF-7 and T47D cells to knock-down its expression, demonstrating a >9-fold reduction as early as 24 hours post-transfection, persisting for 72 hrs (Fig 1C).

This reduction in miR-301 led to a decrease in viability in both MCF-7 and T47D cells by 40% and 50% respectively at 72h post-transfection (Fig 1D), which in turn translated to reduced clonogenicity (Fig 1E), more so in the MCF-7 than the T47D cells (50% and 80%, respectively), concordant with the MTS data (Fig 1D).

Reducing miR-301 expression can increase sensitivity to Tamoxifen

Given that all the participants in this clinical trial were treated with Tamoxifen (16), and the association of miR-301 with breast cancer relapses, this raised the question as to whether miR-301 could modulate Tamoxifen sensitivity. A dose response curve was first established,
demonstrating that MCF-7 cells (estrogen-receptor positive) were indeed sensitive to 4-hydroxy-tamoxifen, with an IC$_{50}$ of 300 nM at 48 h (Suppl Fig 2A). When MCF-7 cells were co-treated with antagomiR-301 plus 4-hydroxy-tamoxifen (300 nM), a time-dependent cytotoxicity was observed, down to 38% at 48 hrs, compared to NC, antagomiR alone, or NC with tamoxifen (Fig 1F). However, this observation only applied at the IC$_{50}$ dose of 4-hydroxy-tamoxifen (300 nM). A similar result was also observed in the T47D cells (Suppl Fig 2B), suggesting that miR-301 depletion could modulate Tamoxifen sensitivity, although this appeared to be dependent upon the dose of Tamoxifen.

*AntagomiR-301 regulated cell migration and invasion, in addition to suppressing tumour growth in vivo*

Given the strong association between miR-301 over-expression with nodal or distant relapse, we asked whether miR-301 knock-down could affect cell migration or invasion. Indeed, using the scratch assay, antagomiR-301 did reduce migration for both MCF-7 and MDA-MB-231 cells (Fig 2A). Furthermore, miR-301 depletion reduced the invasive ability of the aggressive MDA-MB-231 cells to 44% vs. 62% for NC-cells (p<0.05) (Fig 2B).

The reduced clonogenicity *in vitro* translated to the *in vivo* model wherein antagomiR-301-treated MDA-MB-231 or MCF-7 cells demonstrated significantly delayed tumour growth compared to mice injected with either NC- or Lipofectamine-treated cells (p=0.04 and p= 0.05, respectively) (Fig 2C). To acquire further insights into the mediators of miR-301 suppression leading to reduced tumour growth, tumors from the NC- and antagomiR-301-treated mice were collected 45 days after implantation. Figure 2D demonstrated reduced tumor microvessel density, observed *via* CD31 immuno-expression in the antagomiR-301-treated MDA-MB-231 tumour compared to a control tumour. Ki-67 expression was only slightly reduced (data not shown), indicating that miR-301 affected tumour angiogenesis.
**miR-301 directly targets FoxF2, PTEN, BBC3iso-2 and Col2A1 in breast cancer cells**

These phenotypic data thus far indicate that miR-301 over-expression causes a myriad of effects including breast cancer cell proliferation, migration, invasion, along with tumour growth and angiogenesis. To identify its potential mRNA targets which could mediate these changes, a tri-pronged approach was undertaken (23, 24); details provided in Supplementary Fig 2C. Fourteen overlapped candidate targets were thus identified (FoxF2, BBC3, PTEN, CoL2A1, ITGB8, IL18, MMP13, Bcl2L14, ARRP1, E2F2, RAG1, IGFBP2, IGFBP5, and SMOC2). Individual qRT-PCR assays demonstrated that the basal expression of 7 of these candidate transcripts were under-expressed in the majority of the 4 human breast cancer cell lines (FoxF2, PTEN, BBC3, Col2A1, Il18, ITGB8 and MMP13), which were thus selected for further evaluation (Suppl Fig 3). Knocking down miR-301 induced the expression modestly in 5 of 7 mRNA transcript levels for both MCF-7 and MDA-MB-231 cells, by only 1.1-2-fold, at either 48 or 72h post-transfection (Fig 3A). Western blotting confirmed increased protein expression for FoxF2, PTEN, BBC3 with reduced expression of p-Akt, compared to NC (Fig 3B).

The direct interaction of miR-301 with the top four candidate mRNA transcripts: FoxF2, PTEN, BBC3, Col2A1 was clearly demonstrated by the induction of their respective luciferase expression by 1.2- to 2-fold when MDA-MB-231 cells were co-transfected with antagomiR-301 and the luciferase reporter plasmid carrying the relevant wild-type 3’ UTR sequences, which was completely abrogated by the mutant 3’-UTR sequences (Fig 3C). Almost identical data were observed for the MCF-7 cells (Suppl Fig 4A), thereby corroborating that these four mRNA transcripts were indeed *bona fide* targets for miR-301.

In order to identify some of the further downstream effectors of the observed cellular phenotypic changes, Wnt5a, a target of FoxF2 was evaluated post-miR-301 depletion. The over-expression of Wnt5a has been correlated with increased cell motility and invasion in melanoma.
and breast cancer cells (25). In three of four tested breast cancer cell lines, the basal expression of Wnt-5a was slightly increased (Suppl Fig 4B). Depletion of miR-301 in turn reduced Wnt5a expression (Fig 3D), suggesting that miR-301 could be regulating Wnt5a via FoxF2, at least in the MCF-7 and MDA-MB-231 cells.

Given the reduced micro-vessel density observed in the MDA-MB-231 tumors when miR-301 was depleted, and VEGF being a known downstream effector of PTEN-Akt, we asked whether VEGF levels would be differentially expressed in our primary human breast cancer samples. Indeed, the median VEGF transcript expression level analyzed in the remaining samples with sufficient RNA was 7.7-fold higher than normal tissues, compared to 15.4-fold between the non-relapsed vs. relapsed breast cancer patient samples (Fig 3E), suggesting that VEGF could indeed be a downstream effector of breast cancer relapse.

**SKA2 is the host gene of miR-301**

It is well known that more than 50% of mammalian miRNA genes are located within introns of protein-coding or non-coding units (26), wherein miRNAs could be transcribed in parallel with their host genes. Interrogation of the Sanger microRNA database revealed that miR-301 overlaps with the first intron of the SKA2 gene, located on 17q22 (Fig 4A). SKA2 is a recently-described member of the SKA complex, which is essential for proper chromosomal segregation (27). SKA2 has been shown to be over-expressed in human lung and breast cancer tissues, perhaps mediating cancer cell proliferation (28). Hence, the expression of SKA2 was investigated in the same LNN breast cancer specimens using qRT-PCR. Amongst the 64 tumors examined (with sufficient remaining RNA), 44 demonstrated up-regulation of SKA2, with 37 (84%) exhibiting over-expression of both SKA2 and miR-301 (Suppl Fig 5A). Interestingly, SKA2 over-expression was also significantly associated with only a higher risk of nodal or distant relapse (p=0.03; Fig 4B). Similarly, SKA2 over-expression was also observed in all four
human breast cancer cell lines (Fig 4C), which when knocked down using siRNA, led to a significant reduction in cell viability down to 50% in the MCF-7, or 70% in the MDA-MB-231 cells, in a time-dependent manner up to 72h post-transfection (Fig 4E). Reduced mRNA and protein levels of SKA2 after siSKA2 were corroborated using qRT-PCR and Western blotting respectively (Fig 4D). These data indicated that SKA2 could also independently influence tumour cell proliferation in vitro.

**Co-localization of miR-301 and SKA2 in tumor cells**

Finally, to further ascertain that miR-301 and SKA2 were indeed co-expressed in primary human breast cancer, miR-301 was visualized using in-situ hybridization, and SKA2 via immunohistochemistry. As shown in Fig 5A & B, intense miR-301 signals were observed in the cytoplasm of tumor cells, not observed with a scrambled control probe (Suppl Fig 5B). In a serial section of the same patient’s tumour, SKA2 immuno-expression was also observed in the tumour cytoplasm (Fig 5C & D); again not in the negative control (Suppl Fig 5C). Note that neither miR-301 nor SKA2 were observed in the surrounding normal tissues. This therefore provided the visual corroboration of the previously demonstrated qRT-PCR data for the co-over-expression of both miR-301 and SKA2 in primary human breast cancer tissues (Suppl Fig 5A).

**DISCUSSION**

Current clinical decisions on breast cancer management for LNN patients are based on parameters such as age, menopausal status, tumour size, and histology, which are sub-optimal, and likely result in the over-treatment of many of our patients (29). Several molecular prognostic markers have been proposed (30); the most frequently utilized set in North America is Oncotype DX, a 21-gene assay applicable to LNN ER-positive cancers (31). The true utility of these predictive signatures however, are still under evaluation, underscoring the biological
complexities of LNN disease; hence the importance of determining whether novel insights could be acquired through the lens of miRNAs.

The current study reported miR-301 as a novel oncogene in LNN breast cancer. MiR-301 over-expression was associated with an increased risk of nodal or distant relapse, and mediated proliferation, migration, invasion, resistance to tamoxifen, and tumour formation with increased angiogenesis. These pleiotropic effects of miR-301 were directly mediated by several well-known oncogenic targets and pathways, including PTEN, FoxF2, BBC3, and Col2a1. Furthermore, its co-expression with SKA2 likely co-operatively promotes breast cancer progression (Fig 5E).

Dysregulation of the PTEN-PI3K/Akt signaling pathway is well characterized in its myriad of roles in causing proliferation, increasing angiogenesis, migration, and invasion, leading to metastases (32). In fact, we had previously reported dysregulation of the PTEN-PKB axis in primary human breast cancer samples (33). Although the mechanism of dysregulation was unclear, further examination of the 29 overlapping samples (from the previous and the current cohorts), demonstrated that 14 samples over-expressed both miR-301 and PTEN; 5 over-expressed miR-301 with concomitantly low PTEN; and 10 were reversed with low miR-301 and high PTEN expression (p=0.07), suggesting that in this small cohort, there might indeed be a trend of a reciprocal relationship between miR-301 with PTEN. In the current study, PTEN appears to be a central target of miR-301 in mediating many of the observed phenotypic changes in the breast cancer cells consequent to its knock down. We observed increased proliferation, migration, and invasion in vitro (Fig 1D, 1E, 2A & 2B), which can certainly all be attributed by the mitogenic signals of a down-regulated PTEN cascade, leading to activated PI(3)K-Akt, with multiple further downstream signals involving matrix metalloproteinases, and integrins (34). Furthermore, we observed enhanced tumour formation, associated with increased micro-vessel
density in vivo, corroborated by increased VEGF mRNA expression in the same primary breast cancer tissues, which in turn was associated with relapse (Fig 3E); again, these phenotypes would be consistent with an activated Akt cascade (34).

FoxF2 is a member of the forkhead family of transcription factors (35), which regulate diverse cellular processes (36). FoxF2 has been recently described to be under-expressed in prostate cancer (37), and can down-regulate Wnt5a (38). Our data demonstrate that FoxF2 is directly targeted by miR-301, and miR-301 depletion reduced Wnt5a expression (Fig 3). Wnt5a activates the non-canonical Wnt pathways, with distinct signaling and biological phenotypes depending on context, and availability of partner receptors (39). There is controversy regarding the role of Wnt5a in human malignancies, with reports of both tumour suppressive (40), and oncogenic effects (41). Certainly Wnt5a can mediate cell polarity, migration and invasion through its interactions with the Frizzled receptors (39), which would be concordant with our observations via the miR-301/FoxF2/Wnt5a axis (Fig 5E), although clearly, the roles of FoxF2 and Wnt5a in human malignancies are just beginning to be understood, definitely warranting further examination.

In addition to elucidating the cancer relevant pathways downstream of miR-301 such as the aforementioned PTEN and FoxF2, we also noted that miR-301 was located within the first intron of SKA2 (42). Not only were both miR-301 and SKA2 co-over-expressed in our breast cancer samples (Fig 5, Suppl Fig 5A), but SKA2 with miR-301 also associated with clinical outcome in that the worst disease-free survival was observed for the tumours which over-expressed both genes vs. the best outcome when neither was over-expressed (Suppl Fig 6A). SKA2 forms at the kinetochore-microtubule interface during mitosis (27, 43). Transient depletion of the SKA complex destabilizes the microtubule-kinetochore attachment, resulting in metaphase arrest, while more stringent and prolonged SKA depletion causes chromosome...
congregation defects with subsequent cell death (27). Very little is known about SKA2 in human cancers, but a recent study with the A549 cancer cell line described miR-301 to be indirectly up-regulating SKA2, and its depletion reduced colony formation in agar (22). Their observations would be consistent with our observed phenotype wherein over-expression of SKA2 (with miR-301) appears to promote proliferation and tumour progression, although the precise mechanisms and pathways remain to be elucidated.

The two other direct targets of miR-301, BBC3, and Col2A1 were demonstrated at the transcript and protein levels along with luciferase reporter data (Fig 3A-C, Suppl Fig 3 & 4). We were unable to demonstrate a significant anti-apoptotic process after increasing BBC3 levels in any of our tested breast cancer cells. Col2A1 encodes the alpha chain of type II collagen (44), which is an important component of the extracellular matrix (45). It is certainly conceivable that the migration and invasion observed after miR-301 depletion (Fig 2A & B) could be mediated through the down-regulation of Col2A1.

Finally, given that all the participants in this randomized clinical trial received Tamoxifen, and miR-301 over-expression was associated with relapse, we asked whether miR-301 depletion could affect Tamoxifen sensitivity, and indeed demonstrated a modest effect in both MCF-7 and T47D cells (Fig 1F, Suppl Fig 2B). Despite decades of clinical experience with Tamoxifen, the precise mechanisms of Tamoxifen resistance remain largely unknown. Our data do not clearly demonstrate the pathways of resistance, but activation of Akt has certainly been implicated (46); hence it is plausible that the miR-301/PTEN/Akt pathway could be influencing Tamoxifen sensitivity, based on our cell line and clinical observations.

In conclusion, our experiments have demonstrated a novel pleiotropic oncogenic role for miR-301 through regulation of key signaling pathways involving at least PTEN, FOXF2, and Col2A1, and working in co-operation with SKA2. The resulting phenotype of an up-regulated
miR-301 includes increased proliferation, migration, invasion, and tumour formation. Furthermore, miR-301 over-expression is a candidate prognostic marker for metastases in patients with LNN breast cancer, which should be validated in a larger patient cohort.

ACKNOWLEDGEMENTS

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REFERENCES

### TABLE I—CLINICAL CHARACTERISTICS OF PATIENTS

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NHS*: Nottingham histologic score  
(*)-Uniform, intense complete cytoplasmic membrane staining of >30% invasive tumour cells.  
(**)-No cytoplasmic membrane staining or weak incomplete membrane staining in any proportion of invasive tumour cells.  
(***)-Complete circumferential membrane staining that is either non uniform or weak in 10% or more of invasive tumour cells or intense complete cytoplasmic membrane staining in 30% or less of invasive tumour cells.
FIGURE LEGEND

Figure 1. miR-301 over-expression in LNN breast cancer was strongly associated with tumor relapse. AntagomiR-301 caused significant reduction in miR-301 expression, leading to reduced viability, decreased clonogenicity, and increased sensitivity to Tamoxifen. A) Over-expression of miR-301 was strongly associated with distant/nodal (*p=0.03) or multiple (*p=0.02) relapses. The Mann-Whitney-Wilcoxon test was used to explore the relationship between miR-301 expressions with relapse type. B) miR-301 was over-expressed in all four human breast cancer cell lines. C)-E) AntagomiR-301 significantly reduced cell viability and decreased clonogenicity in both MCF-7 and T47D cells. F) Depleting miR-301 increased Tamoxifen sensitivity (300 nM) in MCF-7 cells. Each datum represents the mean fold change ± SE from triplicates. AntagomiR vs. NC, *p<0.05, **p<0.001.

Figure 2

Knockdown of miR-301 significantly reduced cell migration and invasion, in addition to suppressing tumour growth, and micro-vessel density. A) The scratch migration assay. Serial photographs were obtained at indicated times post-transfection in MCF-7 and MDA-MB-231 cells. B) The migration and invasion rates were determined by counting the number of cells which have migrated onto collagen IV-coated plates (upper), or invaded through Matrigel (bottom). C) Tumour formation in SCID mice after subcutaneous injection of transfected-MDA-MB-231 or MCF-7 cells. The indicated tumor volumes represent the mean ± SE. D) Representative photomicrograph of CD31 immunostaining in MDA-MB-231 xenograft tumours; quantitation of the number of micro-vessels in 10 representative hpf’s; the datum represents the mean ± SE from 2 independently treated tumours (*p<0.05).
Figure 3

MiR-301 target identification and validation. A tri-pronged approach identified seven down-regulated genes as potential targets for miR-301. A) qRT-PCR demonstrated up-regulation in 6 and 5 of the tested 7 genes after miR-301 knockdown in the MCF-7 and MDA-MB-231 cells, respectively. B) Western blotting for FoxF2, PTEN, BBC3, p-Akt and total Akt at 72 hrs post-transfection of antagomiR-301 in MDA-MB-231 cells. C) Luciferase reporter assays confirmed that FoxF2, PTEN, BBC3 and Col2A1 were direct targets of miR-301 in the MDA-MB-231 cells, with luciferase activity ranging from 1.2 to 2-fold induction. D) Wnt5a expression was reduced after miR-301 knockdown for both MCF-7 and MDA-MB-231 cells. Each datum represents the mean fold change in expression from three independent experiments ± SE. E) Pre-treatment expression VEGF in non-recurrent (11) vs. recurrent (11) primary human breast cancer samples compared with 4 normal mammoplasty specimens (Nor). *p<0.05.

Figure 4

SKA2 is the host gene for miR-301. A) A schema demonstrating the location of miR-301 on chromosome 17q22, within the intron of SKA2. B) Box-plot demonstrating a correlation between SKA2 expression with distant or nodal relapses. *p<0.04. C) qRT-PCR measurements of SKA2 over-expression in all four human breast cancer cells, relative to that of MCF-10A. D). Reduced mRNA and protein levels of SKA2 after siSKA2 in MDA-MB-231 cells demonstrated using qRT-PCR and Western blotting, respectively. E) SKA2 knockdown significantly reduced cell viability in both cell lines. Each datum represents the mean fold change from three independent experiments ± SE. *p<0.05, **p<0.001.
Figure 5

Co-localization of miR-301 and SKA2 in a primary breast cancer specimen. In-situ hybridization (ISH) demonstrating granular brown cytoplasmic expression of miR-301 in tumour (T), but not in the normal tissues (N). A) 100x; B) 200x magnifications. In an adjacent serial 4 uM section, immunohistochemistry demonstrated significant cytoplasmic expression of SKA2 also in tumour (T), but not in normal tissues. C) 100x; D) 200x magnifications. E) A proposed model wherein miR-301 over-expression in human breast cancer can down-regulate several mRNA targets, including Col2A1, PTEN, FoxF2, and BBC3. In turn, down-regulation of Col2A1 can lead to increased migration and invasion. Similarly, PTEN down-regulation will lead to Akt activation, which can promote VEGF expression, with increased tumour angiogenesis. Down-regulation of FoxF2 can increase expression of Wnt5a, which can promote cell proliferation. In parallel, SKA2 is also co-expressed with miR-301, which can also potentially increase proliferation.
**Figure 1**

**A**
miR-301 expression (fold change) for different types of relapse:
- No relapse (n=38)
- Local Only (n=9)
- Distant or LN (n=15)
- Multiple (n=9)
(p = 0.0071)

**B**
Relative fold change for different cell types:
- MCF-7
- T47D
- MDA-231
- MDA-468

**C**
Relative fold change over time (24, 48, 72 hours) for different treatments:
- MCF-7
- T47D

**D**
Viable cell fraction over time (24, 48, 72 hours) for different treatments:
- MCF-7
- T47D

**E**
Survival fraction for different treatments:
- Lipo
- NC
- anta-301

**F**
Cell viability over time (24, 48, 72 hours) for different treatments:
- NC
- A tam5
- NC+Tam1
- A tam5+Tam1
- NC+Tam3
- A tam5+Tam3
- NC+Tam5
- A tam5+Tam5

Note: All data points are represented with error bars indicating standard deviation.
Figure 3

A

Relative fold change of genes in MCF-7 and MDA-MB-231 cells treated with either NC or AntamiR301.

B

Western blot analysis of FoxF2, PTEN, BBC3, pAKT, AKT, and GAPDH in MDA-MB-231 cells treated with NC or AntamiR301.

C

 Luciferase activity of Luc_FoxF2_3'UTR and Luc_PTEN_3'UTR in MDA-MB-231 cells treated with NC or AntamiR301.

D

Wnt5a expression after depletion of miR-301 in MCF-7 and MDA-MB-231 cells.

E

VEGF expression in Breast cancer samples in MCF-7 and MDA-MB-231 cells.
**Figure 4**

A. Diagram showing the location of miR-301a and SKA2 on chromosome 17.

B. Box plot showing the expression fold changes of SKA2 across different types of relapse.

C. Bar chart comparing the relative fold changes of miR-301 and SKA2 in MCF-10A, MCF-7, T47D, MDA-231, and MDA-468 cell lines.

D. Bar chart showing the relative fold changes and cell viability of MDA-MB-231 cells after treatment with SiC or siSKA2.

E. Bar chart showing the cell viability of MCF-7 and MDA-MB-231 cells after treatment with SiC or siSKA2.

SiC siSKA2

SKA2

GAPDH
Figure 5

**A** miR-301 ISH

**B** (X200)

**C** SKA2 IHC

**D** (X100)

**E** Schema of miR-301 regulated pathways
MicroRNA-301 Mediates Proliferation and Invasion in Human Breast Cancer

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