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Expression of microRNAs and their gene targets are dysregulated in pre-invasive breast cancer

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Abstract

Introduction: microRNAs (miRNAs) are short non-coding RNAs that negatively regulate gene expression and may play a causal role in invasive breast cancer. Since many genetic aberrations of invasive disease are detectable in earlier stages, we hypothesized that miRNA expression dysregulation and the predicted changes in gene expression would also be found in early breast neoplasias.

Methods: Expression profiling of 365 miRNAs by RT-qPCR was combined with laser-capture microdissection to obtain an epithelial specific miRNA expression signature of normal breast epithelium from reduction mammoplasty (RM; n=9) and of paired samples of histologically normal epithelium (HN) and ductal carcinoma *in situ* (DCIS) (n=16). To determine how miRNAs may control the expression of co-dysregulated mRNAs we also performed gene expression microarray analysis in the same-paired HN and DCIS samples and integrated this with miRNA-target prediction. We further validated several target pairs by modulating the expression levels of miRNAs in MCF7 cells and measured the expression of target mRNAs and proteins.

Results: Thirty-five miRNAs were aberrantly expressed between RM, HN and DCIS. Twenty-nine miRNAs and 420 mRNAs were aberrantly expressed between HN and DCIS. Combining these two datasets with miRNA-target prediction we identified two established target pairs (miR-195:CCND1 and miR-21:NFIB) and tested several novel miRNA:mRNA target pairs. Over-expression of the putative tumor-suppressor miR-125b, under-expressed in DCIS, repressed the expression of MEMO1, which is required for ErbB2-driven cell motility (also a target of miR-125b); and NRIP1/RIP140, which modulates the transcriptional activity of the estrogen receptor. Knockdown of the putative oncogenic miRNAs miR-182 and miR-183, both highly over-expressed in DCIS, increased the expression of CBX7 (which regulates

E-cadherin expression), DOK4, NMT2, and EGR1. Augmentation of CBX7 by knockdown of miR-182 expression, in turn, positively regulated the expression of E-cadherin, a key protein involved in maintaining normal epithelial cell morphology which is commonly lost during neoplastic progression.

Conclusions: These data provide the first miRNA expression profile of normal breast epithelium and of pre-invasive breast carcinoma. Further, we demonstrate that altered miRNA expression can modulate gene expression changes that characterize these early cancers. We conclude that miRNA dysregulation likely plays a substantial role in early breast cancer development.

Introduction

Considerable molecular pathology research has focused on invasive breast cancer (IBC), however, less attention has been given to the pre-invasive non-obligate precursor, ductal carcinoma *in situ* (DCIS). DCIS is the fourth most common cancer diagnosis among women, and is present in the vast majority of IBC cases [1]. Women diagnosed with DCIS are at an increased risk of subsequently developing IBC and when examined, DCIS and IBC also share many of the same genetic features. However, there is an increased need to better understand the early genetic events and identify biomarkers that are present prior to IBC. miRNAs have emerged as a new class of gene regulators that may serve as both molecular biomarkers and novel therapeutic targets. In this study we sought to investigate miRNA expression changes and their consequences in pre-invasive breast cancer.

miRNAs are short, non-protein-coding RNAs that exert post-transcriptional control over their mRNA targets through the mechanism of RNA interference. By complementary binding to the 3' untranslated region of target mRNAs miRNAs promote mRNA

destabilization, thereby inducing translational repression [2]. It has been demonstrated that miRNAs control major cellular processes including metabolism, developmental timing, stem cell division, cell growth and differentiation, and apoptosis [3-5]. Given this expansive role it is unsurprising their effect on mRNA expression contributes to the pathogenesis of many diseases, including cancer [6, 7]. To date, >900 miRNAs have been identified in humans, constituting >1% of the total coding genome. It is predicted that >60% of mRNAs may be targeted and that a single miRNA may target as many as 200 mRNAs, thus making them the largest class of gene regulators [8-10].

Several studies have established the role of miRNAs in the pathogenesis of IBC. For example, abnormal miRNA expression has been described in breast cancer cell lines, and in bulk primary normal and cancerous breast tissues [11-13]. In this setting, miRNA expression has correlated with specific breast cancer biopathologic features, such as estrogen receptor (ER) and progesterone receptor (PR) expression, tumor stage, vascular invasion, or proliferation index. In addition, many miRNAs that are consistently down-regulated may act as tumor suppressors, *e.g.* miR-206, miR-17-5p, miR-125a, miR125b, and the let-7 family; and many that are consistently up-regulated may acts as oncogenes, *e.g.* miR-21, miR-10b, and miR-27a. Other studies have shown that miRNAs exhibit a specific spatial distribution of expression within breast epithelium [14].

Almost all human breast cancers arise in the epithelial compartment, likely due to the transformation of the epithelial cells, although the surrounding stroma and microenvironment play a crucial role in tumor progression. Therefore, the present work is focused on the genetic changes that occur within the epithelial cell population.

We hypothesized that miRNA expression is dysregulated prior to IBC, that these changes will be associated with mRNA expression changes, and that together these will help to elucidate important steps in early breast tumorigenesis. Therefore, to first obtain a profile of normal miRNA expression we profiled miRNAs in normal epithelium from healthy controls

undergoing reduction mammoplasty (RM). Next, to obtain a profile of miRNAs dysregulated prior to invasion we examined miRNA expression in histologically normal (HN) epithelium and compared this to paired samples of adjacent DCIS. We then integrated the HN-DCIS miRNA expression profile with the gene expression profile from the same samples, and used miRNA-target prediction programs to identify putative miRNA:mRNA functional interactions. We then selected three candidate miRNAs (miR-125b, miR-182 and miR-183) and six of their putative target genes (MEMO1, NRIP1, CBX7, DOK4, NMT2, and EGR1) for validation. This study represents the first report of a miRNA expression profile in normal breast epithelium and the first integrated analysis of dysregulated miRNA and mRNA expression in paired HN and DCIS samples. Many of the dysregulated miRNAs identified in DCIS have previously been identified in IBC. Our data suggest an important role for miRNAs in determining the parallel gene expression changes that characterize the earliest stage of breast disease.

Materials and methods

Tissue sample acquisition and preparation

Primary breast tissues not needed for diagnosis were obtained at Boston Medical Center, from patients undergoing RM and breast cancer surgeries (prior to any chemo or radiation therapy). All samples were de-identified and assigned a number at the time of collection, therefore informed consent was not required according to our specimen collection protocol pre-approved by the Boston University Medical Center Institutional Review Board. Samples were processed as described [15]. Epithelium from 3 groups was examined: normal breast tissue (n=9) from RM (mean age=52.2; range=44-75 years), and paired samples of HN and DCIS (n=16), from 8 individuals undergoing cancer surgeries. H&E stained sections were reviewed by a pathologist (AdlM) to verify normal epithelium and pre-invasive lesions.

Laser-capture micro-dissection and RNA isolation

Laser capture micro-dissection (LCM) was performed as described [15-17], to collect breast epithelial cells of normal appearing ductal tissue (RM and HN) and epithelial cells of identified regions of DCIS. Total RNA was isolated with the RNAqueous miRNA Isolation Kit (Ambion, Austin, TX) and treated with DNaseI, according to the manufacturer. RNA to be utilized for gene expression analysis was processed as described [15, 16]. The pooled RM sample was prepared by combining 400ng of total RNA from each of the 9 RM samples.

miRNA expression profiling and statistical analysis

cDNA was synthesized from 800ng (100ng/per multiplex pool) with the TaqMan miRNA Reverse Transcription (RT) Kit, according to manufacturer. miRNA expression was measured by real-time quantitative PCR (RT-qPCR) utilizing the TaqMan Human miRNA Array Panel (v1.0; based on miRBase version 9.2) and assayed on the 7900 Real-Time PCR System, according to manufacturer. All reagents and equipment from Applied Biosystems, (Foster City, CA). miRNA expression data is available from the NCBI Gene Expression Omnibus (GEO) [18] under accession [GEO:GSE24509].

All probes with cycle threshold (C_T)=40 in >2/3 of PRM replicates or >6/8 of HN and >6/8 DCIS samples were considered 'non-expressed' and removed. Remaining C_T values were global-median normalized by transforming all expression values by re-scaling to a target value of 12. A variance correction was applied to account for the pooled samples as suggested by Churchill, GA [19] and a t-test was performed. A $p<0.005$ in at least one of the comparisons was considered significant. To address the issue of multiple comparisons, we highlight the results that remain significant using two valid procedures: the more restrictive Bonferroni correction (p-value<0.00025) and the less restrictive False Discovery Rate <0.05

(p-value<0.017) which typically results in a greater number of significant results. The fold change values in each comparison were calculated by 2^-(t-test). Heatmaps were generated with the Heatplus package in Bioconductor [20].

Gene expression profiling and statistical analysis

Gene expression analysis was measured on the U133A GeneChip (Affymetrix, Santa Clara, CA). All microarray analyses were performed at the Boston University Microarray Facility, as previously described [15]. The paired data were assembled as follows: 12 paired samples (6 HN and 6 DCIS) were pulled from [15]; 2 HN samples were pulled from [21], and the 2 matching pairs of DCIS samples (to equal 16 paired samples) were collected and processed from tissue acquired from the same patient. Array data was analyzed as previously described [15]. Microarray output data were filtered by removing all probesets present in <15% of all samples. Next, data were analyzed by Bayesian analysis of differential gene expression (BADGE), as previously reported in [15] and found at [22]. The gene expression data is available from GEO [18] under accession [GEO:GSE24509].

miRNA target prediction

SigTerms [23] was utilized to extract predictions from: PicTar [24], TargetScan (4.1 and 5.1) [25] and miRanda (Jan 08 and Sep 08) [26]. miRNA target predictions were extracted two separate times, the first using TargetScan release 4.1 and miRanda release Jan 08 and the second using TargetScan release 5.1 and miRanda release Sep 08. The final prediction results are a combination of the two queries. Pearson correlations and associated p-values were calculated across all 16 HN and DCIS samples for each of the target pairs identified from the intersection of the programs.

Gene ontology and pathway analysis

Gene annotation, ontology, and pathway analysis were conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) [27]. A modified Fisher Exact test (EASE Score) was utilized to calculate the p-values.

miRNA Pre-miR and Anti-miR Transient Transfection

MCF7 cells were kindly provided by G. Sonenshein (Tufts-New England Medical Center, Boston, MA) and were maintained in Dulbecco's modified Eagle's Medium (Invitrogen, Carlsbad, CA) with 4.5g/L glucose and sodium pyruvate, supplemented with 5.8g/L L-glutamine (Cellgro, Manassas, VA), 10% FBS (Sigma-Aldrich, St. Louis, MO), and 1% penicillin-streptomycin (Cellgro). For all experiments, 5 x 10^4 cells/well of a 12-well plate were seeded for 24 hours and then transfected with 100nM of Pre-miR-125b or scrambled negative control sequence (Scramble); or 50nM of Anti-miR-182, Anti-miR-183 or Scramble, using the siPORT NeoFX Transfection Agent (Ambion).

RNA extraction-cell culture

Cells were rinsed with 1X phosphate buffered saline (PBS), lysed with 600ul lysis buffer, total RNA was isolated with the mirVana Isolation Kit (Ambion) and treated with DNase I, according to manufacturer. RNA quantity was determined using Quant-it RiboGreen RNA Quantitation Reagent (Invitrogen), according to manufacturer.

Quantitative RT-PCR for target gene expression

cDNA from 500ng total RNA was synthesized using TaqMan RT Reagents, according to manufacturer. RT-qPCR was performed by diluting RT product in 2X Universal PCR Master Mix and 20x TaqMan Gene Expression Assay for each gene to be measured: CBX7 (Hs00980916_g1); DOK4 (Hs00902919_g1); EGR1 (Hs00152928_m1); GAPDH (4333764F); MEMO1 (Hs00831646_uH); NMT2 (Hs01013924_g1); NRIP1(RIP140)

(Hs00942766_s1). PCR reactions were run on the 7500 Real-Time PCR instrument under the following conditions: HOLD at 95°C for 10 min, and 40 cycles of 95°C for 15 sec, and 60°C for 1 min. All reagents are from Applied Biosystems. Relative gene expression was assessed using the delta delta C_T (ddC_T) method after normalization to GAPDH. Fold-changes were calculated by 2^-(ddC_T).

Immunoblot analysis

Cells were washed with 1X PBS 48 hours post-transfection and collected in RIPA buffer (25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Protein (50ug) was electrophoresed through a 4-15% Tris-HCl Ready Gel (BioRad, Hercules, CA) under reducing conditions, and transferred to a PVDF membrane. Membrane was incubated with primary antibodies against CBX7 (ab21873, Abcam, Cambridge, MA), E-cadherin (610181, BD Biosciences, San Jose, CA), and β-actin (A5441, Sigma-Aldrich). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce Biotechnology, Rockford, IL).

Results

miRNA expression profiles of RM, HN and paired DCIS

Our first goal was to generate a set of miRNA expression profiles in primary human healthy and diseased breast epithelium. Therefore, we micro-dissected 25 samples to enrich for epithelial-RNA from 17 patients, (see Additional file 1 for representative epithelial lesions) separated into 3 groups: the control group of normal epithelium from 9 patients undergoing RM; the paired diseased groups, which consists of 16 samples from 8 patients: 8

samples of histologically normal epithelium (HN) and 8 samples of adjacent ER and PR positive DCIS, Table 1.

Next, to test the reproducibility of the miRNA expression array we combined equal amounts of RNA from 9 RM samples into a pooled RM (PRM). The PRM served as both a heterogeneous biological control and as a technical replicate. The PRM was run in triplicate and showed a high correlation between each replicate with mean Pearson's correlation of 0.95 (Additional file 2).

miRNAs are differentially expressed in normal and pre-invasive breast cancer

By comparing the miRNA expression profiles between PRM, HN and DCIS (see Additional file 3 for list of miRNAs expressed in each group) we found that 35 miRNAs were differentially expressed ($p<0.005$) in at least one comparison (see heatmaps Figure 1A and Additional file 4, and Table 2). As expected, the fewest differences were found in the HN-PRM comparison, where 11 miRNAs were different (7 over-expressed and 4 under-expressed). More than twice that number were different in both the DCIS-PRM and DCIS-HN comparisons, with 29 miRNAs being different, and 23/29 overlapping between these comparisons. In the DCIS-PRM, 17 were over-expressed and 12 were under-expressed and in DCIS-HN, 15 were over-expressed and 14 were under-expressed. It has been noted that miRNAs that are present at the same genetic loci within 50kb are often coordinately expressed [28]. We examined these 35 miRNAs for coordinate expression due to close genomic proximity. The 35 miRNAs are located at 29 different loci and 16/35 miRNAs are clustered at 7 distinct loci (indicated in Additional file 4). As expected, 14/16 (88%) miRNAs, <13 kb apart were positively correlated, with the exception of miR-17-3p and miR-18a.

To validate our miRNA dataset, we queried the literature to determine if any of these miRNAs are implicated in IBC. Twenty of the 35 (57%) miRNAs have previously been implicated (noted in Table 2) [13, 14, 18, 29-41]. Eleven of these 20 miRNAs were over-expressed in DCIS compared to HN and PRM (miR-181b, miR-200b, miR-200c, miR-18a, miR-21, miR-365, miR-7, miR-182, miR-191, miR-193b and miR-93). The directional change in expression in DCIS of 7 of these 11 (64%) miRNAs is consistent with their reported expression in IBC (the exceptions are miR-18a, miR-193b, miR-200b and miR-200c). Nine of these 20 miRNAs were under-expressed in DCIS compared to HN and PRM (let-7c, miR-10b [14], miR-125b, miR-127, miR-145, miR-17-3p, miR-195, miR-204, and miR-383), and the directional change in expression in DCIS of 8 of these 9 (89%) miRNAs is consistent with their expression in IBC (except miR-195) . Overall, 15/20 (75%) of miRNAs that are dysregulated are concordantly dysregulated in IBC. Of the 29 miRNAs dysregulated in DCIS compared to HN, 18/29 (62%) are implicated in IBC and concordant with the reported changes in expression.

Differentially expressed miRNAs are predicted to target differentially expressed genes in HN-DCIS

The identification of miRNA targets is crucial to understanding the biological role of miRNAs. We have recently shown that gene expression is altered in paired HN and DCIS epithelial cells [15]. Because mRNA destabilization is a [39] mechanism of miRNA-mediated gene repression, we sought to determine if any of the 29 miRNAs altered in the HN-DCIS comparison may participate in the regulation of these differentially expressed genes through this mechanism. First, we examined the gene expression profile from the same 16 paired HN and DCIS samples. Data were analyzed by Bayesian Analysis of Differential Gene Expression (BADGE). BADGE uses a model-averaging approach to calculate the posterior

probability of a fold-change >1 for each probeset and ranks the genes, so that probesets with a very small probability (<0.025) or very large (>0.975) are considered differentially expressed. From the BADGE analysis we selected the set of probesets in which the probability of a fold-change >1.5 was either >0.975 or <0.025. These probesets were further analyzed using a linear mixed model with log-normal errors and random effects to account for patient-matching across probesets. From this analysis we obtained a set of 497 probesets (420 genes) that were differentially expressed ($p<0.05$, fold-change >1.5) between HN and DCIS, 208 probesets (181 genes) were over-expressed, and 289 probesets (239 genes) were under-expressed, Figure 1B. The list of probesets and mean expression values is provided in Additional file 5.

We then combined the two expression profiles to identify putative miRNA:mRNA functional pairs linking these 420 mRNAs and 29 miRNAs. In order to increase specificity (at the cost of lower sensitivity), we integrated results of 3 target prediction programs and examined only the intersection. We found that 113 unique miRNA:mRNA target pairs were predicted by all 3 programs, composed of 74 genes and 13 miRNAs, Figure 1C.

Returning to the expression data, we found that 59/113 miRNA:mRNA pairs (45 mRNAs and 12 miRNAs) were inversely expressed and 54/113 miRNA:mRNA pairs (46 mRNAs and 13 miRNAs) were coordinately expressed. The inverse pairs are the canonical understanding of miRNA:mRNA interactions, meaning as the expression of one changes it is expected that the expression of the other would change in the opposite direction. The coordinately expressed pairs can represent either false-positive predictions or positive target-regulation. The degree of anti-correlation for the inverse pairs was calculated and listed in Table 3. Similarly, the degree of correlation for the coordinate pairs was calculated and is provided in Additional file 6.

Gene ontology and pathway analysis of gene targets reveals enriched involvement in transcription

To determine if a particular molecular or biological function or pathway was over-represented among the 74 predicted target genes we conducted gene ontology and pathway analysis. According to these results: 23/74 (31%) of the genes regulate transcription. Of the 23, 15 have transcription factor activity, 4 are transcriptional repressors, and 9 are sequence specific DNA-binding factors (see Additional file 7). For example, EGR1 and HOXA9 are predicted targets (miR-183:EGR1 and let-7c/miR-182:HOXA9) and are both transcription factors that regulate many genes including: EIF4E, COL2A1, NAB1, and SNAIL (by EGR1) and EIF4E and MEIS2 (by HOXA9). Interestingly, these genes were also differentially expressed in the HN to DCIS comparison, and several of these are also predicted targets (miR-7:COL2A1, let-7c:NAB1, and let-7c:MEIS2). Due to the use of such a small gene list, no pathways reached a level of significance (see Additional file 8), however we noted that several genes associated with cancer-related pathways, such as cell cycle regulation (CCND1 and YWHAZ), MAPK signalling (FGF2 and DOK4), nucleotide excision repair (RAD23B), and p53 interactions (CCND1, CBX7, EZH2, WWP1, and PTB4A1).

miR-125b, miR-182 and miR-183 target validation

Two of the 59 inverse miRNA:mRNA target pairs we identified have been validated: miR-21:NFIB (an oncogenic interaction) in leukemia cells [42] and miR-195:CCND1 (a tumor suppressive interaction) in hepatocellular carcinoma [43]. Our data suggest a new role for these proven interactions in DCIS.

We wished to validate additional miRNA:mRNA inverse target pairs. Based on a high degree of differential expression in DCIS, multiple predicted targets and potential relevance to cancer, we selected 6/59 inverse target pairs, consisting of 3 miRNAs (miR-125b, miR-182, and miR-183) for experimental manipulation in a breast cancer cell line.

miR-125b expression is greatly reduced in DCIS compared to HN (0.05-fold) and PRM (0.08-fold). Target prediction analysis identified 3 inverse putative miR-125b targets, see Table 3. We selected MEMO1 and NRIP1 for validation. MEMO1 (mediator of ErbB2-driven cell motility 1) is a non-heme iron-dependent dioxygenase that binds to the C-terminus of ErbB2/Her2 (also a known target of miR-125b) and is required for ErbB2-driven cell motility [29, 44]. NRIP1/RIP140 (nuclear receptor interacting protein 1), is a nuclear protein that modulates the transcriptional activity of the estrogen receptor [45].

To determine if MEMO1 and NRIP1 are authentic miR-125b targets, we transiently expressed the precursor of miR-125b in MCF7 cells. This reduced the endogenous expression of MEMO1 as early as 24 hrs (0.75-fold), expression was significantly further decreased at 48 hrs (0.62-fold) and remained below baseline at 72 hrs (0.84-fold). Similarly, the expression of NRIP1 was reduced at 24 hrs (0.63-fold), significantly further decreased at 48 hrs (0.53-fold) and remained below baseline at 72 hrs (0.68-fold), see Figure 2A. These results suggest that both MEMO1 and NRIP1 are negatively regulated by miR-125b.

miR-182 and miR-183 expression is greatly increased in DCIS compared to HN (72.35 and 51.88-fold), and to PRM (106.24 and 19.06-fold), and slightly increased in HN compared to PRM (2.0 and 2.52-fold), respectively. Target prediction analysis identified 13 inverse targets of miR-182 and 4 inverse targets of miR-183, see Table 3. We selected CBX7, DOK4, NMT2 for miR-182, and EGR1 for miR-183 validation. CBX7 (chromobox homolog 7) is a chromobox family protein and a member of the polycomb-repressive-complex 1 that positively regulates E-cadherin expression through interaction with HDAC2 [46]. DOK4 (docking protein 4) acts as an anchor for c-Src kinase, inhibits tyrosine kinase signalling and can activate mitogen-activated protein kinase (MAPK) [47, 48]. NMT2 is a N-myristoyltransferase; myristoylated proteins have diverse biological functions in signal transduction and oncogenesis [49]. EGR1 (early growth response 1) is a C2H2-type zinc-

finger protein that functions as a transcriptional regulator of target genes required for differentiation and mitogenesis [50].

To determine if these genes are authentic targets of miR-182 and miR-183, we introduced an antisense-RNA specifically designed to knockdown the expression of mature miR-182 and miR-183 into MCF7 cells. We observed that from 24 through 72 hrs, there was an upward trend in expression for each of the target genes, which was significant for 3 out of the 4 targets at 72 hrs. By 72 hrs the endogenous expression of CBX7 increased by 1.23-fold, 1.13-fold for DOK4, 1.34-fold for NMT2. EGR1 was unexpectedly decreased at 24 hrs (0.46-fold) expression returned to baseline at 48 hrs and was increased at 72 hrs (1.2-fold), see Figure 2B.

Knockdown of miR-182 induces the expression of E-cadherin through up-regulation of CBX7

It has been suggested that the loss of CBX7 expression may influence the invasiveness of epithelial cancers by promoting epithelial-to-mesenchymal transition [46]. This effect is believed to be due to CBX7's ability to promote the expression of E-cadherin, a cell-adhesion molecule that plays a role in maintaining normal epithelial cell morphology, by associating with and inhibiting the repressive action of HDAC2 within the E-cadherin promoter region. Loss of E-cadherin expression during neoplastic progression is associated with several cancers, including breast cancer. In this study, the expression of CBX7 was reduced by 0.44-fold in the DCIS-HN comparison, however we did not observe significant decrease of E-cadherin in our pre-invasive clinical samples. It may be that the loss of E-cadherin expression is more characteristic of the invasive transition, or of lobular histology. However, we asked if up-regulation of CBX7 due to the reduction of its targeting miRNA (miR-182) would also lead to an up-regulation of E-cadherin expression *in vitro*. We found

that by 48 hrs post-knockdown of miR-182, the protein levels of both CBX7 and E-cadherin were up-regulated by approximately 35-40%, relative to control, see Figure 3A and B.

Discussion

In this study, we identified a set of miRNAs that are expressed in normal breast epithelium and found that major miRNA expression changes occur at the transition of normal to DCIS, thereby defining a set of putative oncogenic and tumor suppressor miRNAs that are dysregulated at the pre-invasive stage of breast cancer. A greater number of miRNAs were expressed in PRM compared to HN and DCIS, this is contrary to what we have observed in our gene expression studies and recognize that the high Ct cut-off values employed could influence these results. [15, 16]. However, this observation fits with the current understanding of miRNA regulation of mRNA expression, given that a greater number of expressed miRNAs would correspond to fewer expressed mRNAs.

Twenty of these miRNAs have been previously implicated in IBC and 62% of the miRNAs dysregulated in the HN and DCIS comparison are directionally concordant with miRNAs dysregulated in IBC. This work identifies a role for these previously implicated miRNAs at an early stage of breast cancer development. For example, we found that miR-145 expression was under-expressed in the DCIS-HN comparison. Using an *in situ* hybridization approach, Sempere, L. *et al* [14] found that miR-145 was restricted to the myoepithelial/basal cell compartment of normal mammary ducts and lobules, and was reduced or absent in matching tumor specimens. This finding lends support to our discovery of decreased miR-145 in DCIS, because we know our epithelial samples include myoepithelial cells.

Although we observed a high concordance rate with previous reports in IBC, in contrast to the seminal study by Iorio, M. *et al* [13], which examined miRNA expression in bulk tumor tissue versus normal, miR-155 was identified as highly over-expressed in breast tumor tissues. However, miR-155 was not differentially expressed in any of our comparisons.

This suggests that either miR-155 is an invasive specific miRNA, or its expression is not epithelial specific, and was detected due to the heterogeneous cell population presenting bulk tumor tissue. miR-155 has since been described in immune cell function, which supports the latter scenario [51]. Comparison of our dataset to others may shed light on other miRNAs whose expression is specific to either cancer stage or a particular cell-type.

The identification of miRNA targets is crucial to the understanding of their biological role. We hypothesized that there is a coordinate mechanism of dysregulation between the abnormal expression of miRNAs and target mRNAs in very early breast tumorigenesis. By combining miRNA and gene expression data and integrating miRNA-target prediction, we obtained a set of candidate miRNA:mRNA target pairs. Approximately half of these were coordinately expressed and are either false-positive predictions or may in fact positively regulate the target mRNA, albeit a less well understood phenomenon. However, several instances of miRNA positive regulation of a target gene have been described [52-54]. In addition, it has been noted that two classes of miRNA network motifs, corresponding to positive and negative regulation of a miRNA and its target, may co-exist, and in neuronal cells miRNAs tend to be co-expressed in the same direction with their target genes [55]. This may in part explain our observations, although further studies are needed.

Our approach has identified many potentially important early-acting cancer-promoting mRNA targets and miRNA dysregulation is a potential mechanism causing these early mRNA changes. Many of the identified target genes have known cancer or anti-cancer activity. For example, TXNIP, EGR1, CBX7, HOXA9, and FOXN3 have tumor suppressor functions and are targeted by the potentially oncogenic miRNAs: miR-93, miR-183, miR-181b, miR-182 and miR-7. Similarly, WWP1, SDC1, EZH2, CCND1, ADAM9, and MEMO1, have oncogenic activities and are targeted by the potentially tumor suppressor miRNAs: miR-195, miR-10b, let-7c, miR-17, and miR-125b.

Many of these target pairs are likely to be relevant to cancer in general, and breast cancer in particular, however we could only validate a subset of these. We found that with modulation of miR-125b, miR-182 and miR-183 expression we obtained results that suggest these miRNAs do regulate the expression of their predicted target genes. The expression of miR-125b is reduced in many cancers including breast cancer [13, 56] and serous ovarian carcinoma [57]. In addition, it has been established that miR-125b targets ErbB2/Her2, by also targeting MEMO1, which interacts with ErbB2/Her2, miR-125b is regulating two functionally related genes. miR-182 and miR-183 are clustered at 7q31.2, a region that is frequently amplified in melanoma [58] and both miRNAs are commonly co-dysregulated in many cancers including prostate, colon, and breast [33, 59, 60]. In this study we found that by suppressing the expression of miR-182 and miR-183 *in vitro*, the expression of their 4 predicted targets CBX7, DOK4, NMT2 and EGR1 were up-regulated. Two of these CBX7 and EGR1, have well described tumor suppressor functions, and recently DOK4 family members (DOK1-3) were identified as lung tumor suppressors [61]. In addition, the secondary effect of miR-182 repression resulting in up-regulation of E-cadherin through CBX7, that we have shown, may have important implications in reversing epithelial neoplasias to a more normal state. Furthermore, in future studies, combined modulation of miR-125b, miR-182, and/or miR-183, as well as other miRNAs altered in DCIS, may be effective in reversing the forward progression to IBC.

Admittedly, our study has several limitations, most notably the small sample size and the inclusion only of ER and PR positive DCIS. With the use of micro-dissected paired breast tissue samples and robust statistical analysis, we sought to minimize potential biases elicited by small sample size. In fact, the many similarities between our miRNA expression profile of DCIS and others' miRNA expression profiling of IBC suggest that our results are reliable. However, an expansion of this study to include other histological categories could identify subtype specific dysregulated miRNAs.

Conclusions

The present study provides the first report of a miRNA expression profile in normal breast epithelium and the first integrated analysis of miRNA and mRNA expression in paired samples of histologically normal and preinvasive breast cancer. We further demonstrate, by modulating the expression of several miRNAs, the expression of their predicted target genes is affected. Taken together these findings support our hypothesis that changes in miRNA expression in early breast cancer may control many of the parallel changes in gene expression at this stage. This work also implicates the loss of the tumor-suppressor miR-125b and the gain of the oncogenic miRNAs, miR-182 and miR-183 as major contributors to early breast cancer development. Additionally, this study has revealed novel candidate markers of pre-invasive breast cancer, which could contribute to identification of new diagnostic and therapeutic targets. The miRNAs and miRNA:mRNA target pairs identified in this study are natural candidates for future investigations.

Abbreviations

miRNA: microRNAs; RT-qPCR: real time-quantitative polymerase chain reaction; RM: reduction mammoplasty; HN: histologically normal; DCIS: ductal carcinoma *in situ*; PRM: pooled reduction mammoplasty; mRNA: messenger RNA; MEMO1: mediator of ErbB2-driven cell motility; NRIP1/RIP140: nuclear receptor interacting protein 1; CBX7:chromobox homolog 7; DOK4: docking protein 4; NMT2: N-myristoyltransferase 2; EGR1: early growth response 1.

Competing interests

The authors declare that they have competing interests.

Authors' contributions

BNH conceived of and designed the study, executed miRNA expression profiling, subset of gene expression profiling, target prediction, target validation, and drafted the manuscript. PS participated in the design of the study and performed gene and miRNA expression statistical analysis. AdlM reviewed and identified lesions from all histological slides. JL provided expert technical advice and helped to design validation experiments. CLR conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1. Expression profiling and target prediction of miRNAs and genes in HN and DCIS. Hierarchical clustering heatmap of **(A)** miRNAs ($p<0.005$, fold-change >3) and **(B)** mRNAs ($p<0.05$, fold-change >1.5) over-expressed (red) and under-expressed (green) in DCIS *vs.* paired HN. Bracketing indicates the most significant miRNAs and probesets. *Rows*, transcripts/miRNAs; *columns,* profiled patient samples; *shaded-boxes*, lesion type. **(C)** Venn diagram depicting number of predicted functional pairs for each algorithm and the intersection of algorithms, 1st and 2nd queries are separated by a slash.

Figure 2. Effect of miR-125b over-expression and miR-182 and miR-183 knockdown on predicted target gene expression. (A) Reduced average relative fold-change expression of predicted miR-125b targets: MEMO1 (*$p<0.08$) and NRIP1 (*$p<0.05$) **(B)** Increased average relative fold-change expression of predicted miR-182 targets: CBX7, DOK4, and NMT2; and miR-183 target, EGR1 (*$p<0.05$ and ** $p<0.02$). Target mRNA expression measured at 24, 48 and 72 hrs post-transfection (n=4, all conditions). Significant differences determined by Student's t-test. Error bars indicate standard error of the mean.

Figure 3. Effect of miR-182 on CBX7 and E-cadherin expression. (A) Protein levels of CBX7 and E-cadherin after transfection with anti-miR-182 or scramble. β-actin served as a loading control. **(B)** The percent change in levels of CBX7 and E-cadherin were quantitated and normalized for β-actin.

Additional files

Additional file 1. LCM series of representative breast epithelial lesions. Lesions were obtained from healthy normal (RM), histologically normal (HN) and paired adjacent ductal carcinoma *in situ (*DCIS). Lesions were micro-dissected from 10-μm thick consecutive tissue sections; *left-right:* standard hematoxylin and eosin (H&E) stained 'guide slide', dilute H&E stained pre-capture, post-capture stromal compartment, and captured epithelial compartment. 40X magnification.

Additional file 2. X-Y scatter correlation plot of triplicate PRM samples. The cycle threshold (C_T) values for each of the 385 assays from the 3 replicate PRM samples, are plotted against one another. R^2 =0.88, 0.91, 0.90 (mean=0.90) and Pearson's correlation coefficient of 0.94, 0.96, and 0.95 (mean=0.95)

Additional file 3. List of miRNAs considered present/absent in each histological group.

Additional file 4. Expression profiling heatmap of 35 miRNAs differentially expressed between PRM, HN and DCIS. Hierarchical clustering heat map representation of 35 miRNAs over-expressed (red) and under-expressed (green) between PRM, HN and DCIS ($p<0.005$, fold-change >3), sorted by physical (chromosomal) position from top to bottom, black indicates no change in expression. Bracketing indicates the 16 clustered miRNAs. *Rows*, miRNAs; *columns*, profiled patient samples; *shaded-boxes*, indicate lesion type or replicate sample.

Additional file 5. Table. All genes significantly differentially expressed in paired HN and DCIS.

Additional file 6. Table. Expression correlation of miRNAs and coordinately expressed predicted targets.

Additional file 7. Table. Enriched gene ontology terms for predicted target genes.

Additional file 8. Table. Cancer specific functional annotation and pathway analysis of predicted target genes.

Table 1. Paired HN and DCIS sample summary

Sample	Age (years)	Grade of DCIS	Histology of DCIS	ER/PR/HER2 of IDC	IDC Grade
379	43	2 and 3	Cribiform	+/+/+	2
444	48	2	Cribiform	+/+/NA	Not present
248	49	1	Micropapillary	+/+/NA	2
274	49	2	Solid	+/+/-	1
380	53	2 and 3	Solid/Cribiform	+/+/-	2
446B	54	3	Comedo/Solid	+/+/-	2
258	65	1 and 2	Cribiform/Micropapillary	+/+/-	2
405	67	2	Micropapillary/Cribiform	+/+/-	2
Mean Age	53.5				
Median Age	51.0				

Abbreviations: NA=not applicable; HN=histologically normal; DCIS=ductal carcinoma *in situ;* ER=estrogen receptor; PR=progesterone receptor; HER2=human epidermal growth factor receptor-2;IDC=invasive ductal carcinoma.

Table 2. Differentially expressed microRNAs among pooled RM, HN and DCIS.

microRNA	HN *vs.* PRM		DCIS *vs.* PRM		DCIS *vs.* HN	
	Fold change	p-value[a]	Fold change	p-value[a]	Fold change	p-value[a]
let-7c [62]	15.23	3.46E-03#	0.31	1.25E-01	0.03	1.19E-03#
miR-7 [38]	3.12	1.35E-01	22.35	1.53E-03#	5.19	4.92E-02
miR-10b [13, 39]	2.90	1.59E-01	0.82	7.82E-01	0.04	2.58E-03#
miR-17-3p [32, 40]	19.04	2.14E-03#	2.20	2.85E-01	0.12	1.73E-02
miR-18a [34]	1.91	3.76E-01	20.88	1.76E-03#	3.53	1.12E-01
miR-21 [13, 31, 36, 40, 41]	6.74	2.23E-02	55.20	2.64E-04#	20.92	3.21E-03#
miR-93[34]	7.53	1.72E-02	193.86	3.33E-05*	160.47	1.59E-04*
miR-99a	1.90	3.79E-01	0.53	3.79E-01	0.02	9.19E-04#
miR-99b	3.08	1.39E-01	14.84	3.66E-03#	6.24	3.33E-02
miR-125b [13, 29]	0.93	9.24E-01	0.08	5.39E-03#	0.05	4.03E-03#
miR-127 [30, 31]	1.77	4.30E-01	0.25	7.81E-02	0.02	5.30E-04#
miR-130a	0.29	1.06E-01	0.01	1.38E-04*	0.09	1.01E-02
miR-145 [13, 14]	3.00	1.47E-01	0.80	7.52E-01	0.03	1.20E-03#
miR-181b [31, 40]	3.40	1.11E-01	14.90	3.64E-03#	5.46	4.42E-02
miR-182 [33]	2.00	3.45E-01	106.24	8.54E-05*	72.35	4.55E-04#
miR-183	2.52	2.15E-01	19.06	2.14E-03#	51.88	7.38E-04#
miR-191 [13]	6.75	2.23E-02	78.21	1.43E-04*	31.59	1.60E-03#
miR-193b [18]	2.20	2.84E-01	15.67	3.25E-03#	6.87	2.73E-02
miR-195 [35]	1.37	6.57E-01	0.06	2.78E-03#	0.13	2.15E-02
miR-200b [37]	1.70	4.63E-01	6.07	2.86E-02	51.31	7.50E-04#
miR-200c [37]	1.90	3.80E-01	26.41	1.08E-03#	19.98	3.48E-03#
miR-204 [13]	0.77	7.12E-01	0.04	1.32E-03#	0.08	8.12E-03#
miR-324-5p	2.86	1.64E-01	24.38	1.28E-03#	92.53	3.24E-04#
miR-365 [31]	4.05	7.45E-02	17.65	2.51E-03#	25.05	2.35E-03#
miR-376a	0.45	2.73E-01	0.06	3.14E-03#	0.24	7.91E-02#
miR-382	2.03	3.32E-01	0.11	1.20E-02#	0.06	4.71E-03#
miR-383 [34]	0.05	2.15E-03#	0.01	1.58E-04*	0.62	5.18E-01
miR-410	0.96	9.56E-01	0.15	2.33E-02#	0.00	1.11E-04*
miR-425-5p	1.38	6.57E-01	9.47	1.01E-02#	74.46	4.37E-04#
miR-449a	10.65	7.71E-03#	15.12	3.52E-03#	2.53	2.23E-01
miR-449b	0.79	7.37E-01	11.57	6.39E-03#	21.20	3.13E-03#
miR-486	0.02	2.06E-04*	0.01	9.73E-05*	0.34	1.65E-01
miR-489	0.06	2.73E-03#	0.10	8.61E-03#	0.25	8.62E-02
miR-511	0.40	2.20E-01	0.07	3.72E-03#	0.18	4.11E-02
miR-517c	0.07	3.48E-03#	0.01	1.29E-04*	0.36	1.82E-01

Abbreviations: RM=reduction mammoplasty; PRM=pooled reduction mammoplasty; HN=histologically normal; DCIS=ductal carcinoma *in situ*. Fold change in expression between comparisons was considered significant if p<5E-02. A p<1.7E-02 implies significance with an FDR of 0.05 (denoted by #), and a p<2.5E-04 (denoted by *) implies significance after the Bonferroni correction. [a]p-value determined by Student's t-test;* p<2.5E-04; miRNAs in **bold** have been previously implicated in invasive breast cancer.

Table 3. Expression correlation of microRNAs and inversely expressed predicted targets.

microRNA expression		Gene expression			miRNA:mRNA	
miRNA	**Fold Change**	**Probe ID**	**Gene Symbol-Gene Name**	**Fold Change**	**Expression correlation**	**p-value[a]**
let-7c	0.03	203481_at	C10ORF6-chromosome 10 open reading frame 6	1.58	-0.58	1.88E-02
		218567_x_at	DPP3-dipeptidyl-peptidase 3	2.31	-0.49	5.4E-02
		203358_s_at	EZH2-enhancer of zeste homolog 2 (*Drosophila*)	2.36	-0.64	7.69E-03
		209283_at	SLC20A1-solute carrier family 20 (phosphate transporter), member 1	2.06	-0.30	2.53E-01
		203358_s_at	TRIB1-tribbles homolog 1 (Drosophila)	1.77	-0.48	5.84E-02
miR-10b	0.04	202357_s_at	SDC1-syndecan 1	2.06	-0.28	2.96E-01
miR-125b	0.05	203744_at	HMGB3-high-mobility group box 3	2.13	-0.61	1.27E-02
		209613_s_at	**NRIP1-nuclear receptor interacting protein 1**	**2.25**	**-0.39**	**1.36E-01**
		213004_at	**MEMO1-mediator of cell motility 1**	**1.73**	**-0.59**	**1.54E-02**
miR-17-3p	0.12	203744_at	HMGB3-high-mobility group box 3	2.13	-0.49	5.66E-02
		212446_s_at	LASS6-LAG1 longevity assurance homolog 6	1.78	-0.31	2.38E-01
		211653_x_at	MAP7-microtubule-associated protein 7	1.73	-0.49	5.19E-02
		213492_at	SERP1-stress-associated endoplasmic reticulum protein 1	1.80	-0.23	3.84E-01
		203213_at	SAR1B-SAR1 gene homolog B (*S. cerevisiae*)	1.95	-0.07	8.03E-01
		202381_at	ADAM9-ADAM metallopeptidase domaine 9	2.04	-0.45	7.89E-02
miR-181b	5.46	221234_s_at	BACH2-BTB and CNC homology 1 basic leucine zipper transcription factor 2	0.47	-0.30	2.62E-01
		212914_at	CBX7-chromobox homolog 7	0.44	-0.30	2.67E-01
		204753_s_at		0.24	-0.19	4.84E-01
		204755_x_at	HLF-hepatic leukemia factor	0.28	-0.52	3.82E-02
		204754_at		0.31	-0.45	7.70E-02
		204567_s_at	NMT2-N-myristoyltransferase 2	0.58	-0.35	1.81E-01
		202274_at	NR3C1-nuclear receptor subfamily 3, group C, member 1	0.46	-0.44	8.86E-02
		200974_at	(glucocorticoid receptor)	0.57	-0.57	2.21E-02
miR-182	72.35	221234_s_at	BACH2-BTB and CNC homology 1 basic leucine zipper transcription factor 2	0.47	-0.73	1.41E-03
		210347_s_at		0.27	-0.65	6.31E-03
		219497_s_at	BCL11A-B-cell CLL/lymphoma 11A	0.42	-0.69	2.86E-03
		219498_s_at		0.43	-0.80	2.19E-04
		221530_s_at	BHLHB3-basic helix-loop-helix domain containing, class B, 3	0.47	-0.55	2.71E-02
		204851_s_at	DCX-doublecortex; lissencephaly, X-linked (doublecortin	0.13	-0.83	7.48E-05
		204850_s_at		0.30	-0.67	4.63E-03
		209691_s_at	**DOK4-docking protein 4**	**0.56**	**-0.53**	**3.65E-02**
		209905_at	HOXA9-homeobox A9	0.10	-0.64	8.18E-03
		214651_s_at		0.31	-0.55	2.63E-02
		64900_at	LPHN2-latrophilin 2	0.37	-0.62	9.87E-03
		219497_s_at	PRKD1-protein kinase D1	0.54	-0.59	1.61E-02
		210735_s_at	RIMS3-regulating synaptic membrane exocytosis 3	0.52	-0.73	1.25E-03
		205022_s_at	FOXN3-checkpoint repressor 1	0.50	-0.67	4.40E-03
		221935_s_at	NCAM1-neural cell adhesion molecule 1	0.43	-0.41	1.19E-01
		212914_at	**CBX7-chromobox homolog 7**	**0.44**	**-0.57**	**2.22E-02**
		204567_s_at	**NMT2-N-myristoyltransferase 2**	**0.58**	**-0.26**	**3.26E-01**
miR-183	51.88	221234_s_at	BACH2-BTB and CNC homology 1 basic leucine zipper transcription factor 2	0.47	-0.75	7.83E-04
		204851_s_at	DCX-doublecortex; lissencephaly, X-linked (doublecortin)	0.13	-0.82	1.06E-04
		204850_s_at		0.30	-0.80	2.16E-04
		201693_s_at	**EGR1-early growth response 1**	**0.45**	**-0.61**	**1.12E-02**
		202274_at	NR3C1-nuclear receptor subfamily 3, group C, member 1	0.46	-0.70	2.77E-03
		200974_at	(glucocorticoid receptor)	0.57	-0.45	8.08E-02
miR-195	0.13	217852_s_at	ARL8B-ADP-ribosylation factor-like 8B	1.64	-0.31	2.49E-01
		208712_at	***CCND1-cyclin D1 [43]***	2.30	-0.33	2.19E-01
		208653_s_at	CD164-CD164 molecule, sialomucin	2.40	-0.54	3.16E-02
		202596_at	ENSA-endosulfine alpha	2.03	-0.37	1.54E-01
		211653_x_at	MAP7-microtubule-associated protein 7	1.73	-0.52	4.00E-02
		221935_s_at	RAD23B-RAD23 homolog B	1.79	-0.08	7.61E-01
		201341_at	TMEM33-transmembrane protein 33	1.62	-0.30	2.64E-01
		212464_s_at	WWP1-WW domain containing E3 ubiquitin protein ligase 1	2.79	-0.49	5.57E-02
		216442_x_at		2.83	-0.25	5.57E-02
miR-204	0.08	217852_s_at	ARL8B-ADP-ribosylation factor-like 8B	1.64	-0.27	3.06E-01
		213492_at	SERP1-stress-associated endoplasmic reticulum protein 1	1.80	-0.57	2.22E-02
miR-21	20.92	218992_at	***NFIB-nuclear factor I/B [42]***	0.43	-0.75	9.15E-04
		219060_at		0.52	-0.69	3.28E-03
miR-7	5.19	213492_at	COL2A1-collagen, type II, alpha 1	0.51	-0.12	6.61E-01
		204359_at	FLRT2-Fibronectin leucine rich transmembrane protein 2	0.35	-0.49	5.36E-02
		206765_at	KCNJ2-potassium inwardly-rectifying channel, subfamily J, member 2	0.33	-0.73	1.35E-03
		214112_s_at	SNCA-synuclein, alpha	0.48	-0.54	3.00E-02
		205022_s_at	FOXN3-checkpoint repressor 1	0.50	-0.61	1.22E-02
miR-93	160.47	210347_s_at		0.27	-0.75	7.40E-04
		219497_s_at	BCL11A-B-cell CLL/lymphoma 11A	0.42	-0.72	1.51E-03
		219498_s_at		0.43	-0.71	2.11E-03
		204753_s_at		0.24	-0.68	3.60E-03
		204755_x_at	HLF-hepatic leukemia factor	0.28	-0.81	1.44E-04
		204754_at		0.31	-0.81	1.49E-04
		218992_at	NFIB-nuclear factor I/B	0.43	-0.74	1.14E-03
		219060_at		0.52	-0.65	6.30E-03
		203963_at	RGL1-ral guanine nucleotide dissociation stimulator-like 1	0.53	-0.58	1.79E-02
		204422_s_at	TXNIP-thioredoxin interacting protein	0.36	-0.69	2.96E-03
		205117_at		0.49	-0.57	2.14E-02

Note: miRNA:target mRNA pairs that were tested in this study are indicated in **bold**, previously confirmed targets from the literature are italicized and in **bold,** [a]p-value determined by Student's t-test.

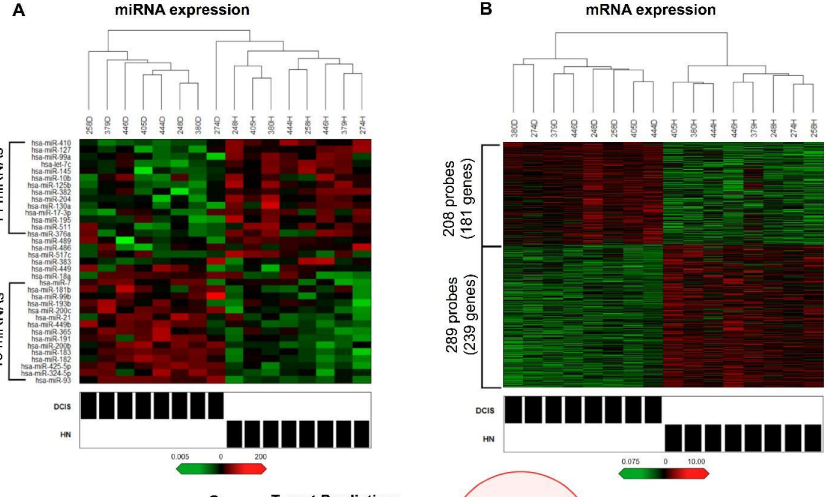
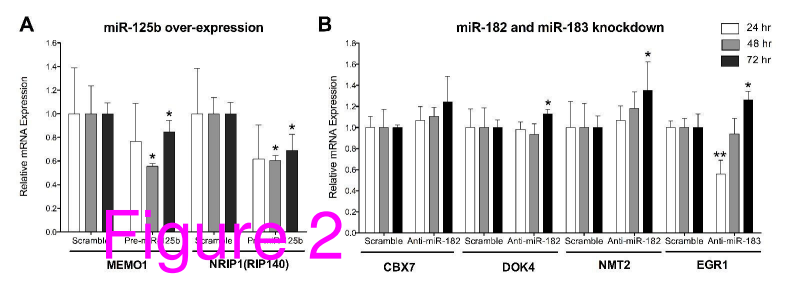

A
miRNA expression
B
mRNA expression
14 miRNAs
15 miRNAs
208 probes
(181 genes)
289 probes
(239 genes)
DCIS
HN
C
Target Prediction:
29 miRNAs + 420 genes
PicTar
244
181/123
130/126
106/91
(113)
TargetScan
411/557
209/266
miRanda
1424/1601

Figure 1


A
miR-125b over-expression
Relative mRNA Expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Scramble
Pre-miR-125b
Scramble
Pre-miR-125b
MEMO1
NRIP1(RIP140)
B
miR-182 and miR-183 knockdown
24 hr
48 hr
72 hr
Relative mRNA Expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Scramble
Anti-miR-182
Scramble
Anti-miR-182
Scramble
Anti-miR-182
Scramble
Anti-miR-183
CBX7
DOK4
NMT2
EGR1
Figure 2

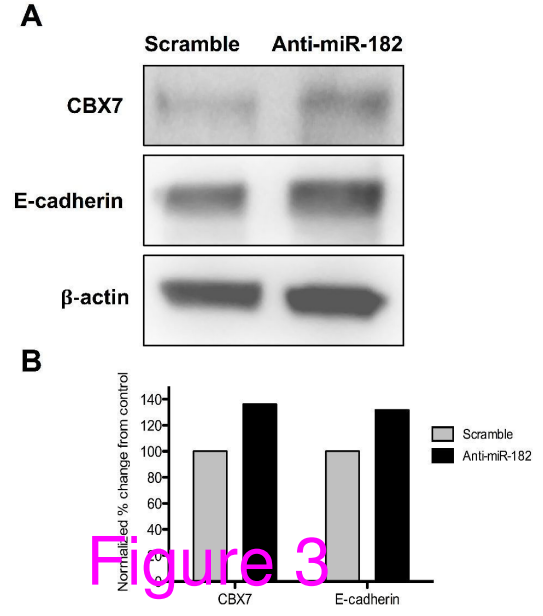

A
Scramble
Anti-miR-182
CBX7
E-cadherin
β-actin
B
Normalized % change from control
140
120
100
80
60
40
CBX7
E-cadherin
Scramble
Anti-miR-182

Figure 3

Additional files provided with this submission:

Additional file 1: S1.pdf, 1606K
http://breast-cancer-research.com/imedia/1340438710452681/supp1.pdf
Additional file 2: S2.pdf, 199K
http://breast-cancer-research.com/imedia/1357468501452681/supp2.pdf
Additional file 3: S3.xls, 209K
http://breast-cancer-research.com/imedia/1148315437452681/supp3.xls
Additional file 4: S4.pdf, 142K
http://breast-cancer-research.com/imedia/1514869980524718/supp4.pdf
Additional file 5: S5.pdf, 221K
http://breast-cancer-research.com/imedia/1200493974526817/supp5.pdf
Additional file 6: S6.pdf, 85K
http://breast-cancer-research.com/imedia/7683270054526817/supp6.pdf
Additional file 7: S7.pdf, 73K
http://breast-cancer-research.com/imedia/5449056104526817/supp7.pdf
Additional file 8: S8.pdf, 155K
http://breast-cancer-research.com/imedia/3283667924526817/supp8.pdf