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Epigenetic Regulation of microRNA Expression: Targeting the
Triple-Negative Breast Cancer Phenotype

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14. ABSTRACT The primary long-term objective of this research is to identify HDAC inhibitor (HDACi) regulated microRNAs which regulate the epithelial-to-mesenchymal transition (EMT) in triple-negative breast cancer (TNBC). Thus far we have assessed the microRNA expression profiles of two TNBC cell lines following treatment with HDACi identifying a number of up- and down-regulated microRNAs. The overall microRNA profile after HDACi treatment was indicative of a less aggressive phenotype. Treatment with HDACi also resulted in increased expression of E-cadherin as well as decreased migration of TNBC indicating a reversal of the EMT phenotype. These results, along with our ongoing research, suggest the ability of HDACi treatment to reverse the EMT phenotype, associated with metastatic and aggressive disease, of TNBC and support future evaluation of HDACi as therapeutic options for the clinical treatment of TNBC.					
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INTRODUCTION

The term „Triple Negative Breast Cancer’ (TNBC) represents a heterogeneous group of diseases and clearly does not comprise a “single entity”. While triple-negative cancer is not a synonym for basal-like cancer, basal-like cancers are preferentially negative for estrogen receptor (ER) and progesterone receptor (PR) and lack human epidermal growth factor receptor 2 (HER2) expression. While it is clear that all TNBC does not fall into the basal-like phenotype and vice versa, there is microarray-based gene expression analysis demonstrating a significant overlap (1). Clinical similarities also exist between triple-negative tumors and basal-like tumors including: a higher prevalence in African-American women, greater frequency in younger patients, and a more aggressive phenotype than other molecular subgroups. EMT (epithelial to mesenchymal transition) is the process by which epithelial cells convert to mesenchymal cells and is essential in embryonic development. It appears that aberrant activation of EMT later in life drives cancer progression, and is involved in highly aggressive, poorly differentiated breast cancers with increased potential for metastasis and recurrence (2). Basal-like breast carcinomas express genes associated with an EMT phenotype and is found in normal basal/myoepithelial cells of the breast, including high-molecular-weight 'basal' cytokeratins, vimentin, and N-cadherin. Interestingly invasive breast cancers with a mesenchymal (basal-like) phenotype have been described to exhibit the loss of expression of certain miRNAs (3). MicroRNA's are a class of short non-coding RNAs found in many plants and animals and often act post-transcriptionally to inhibit gene expression. It has been shown that cells that had undergone EMT in response to TGF- β demonstrated marked downregulation of miR-200 family members, while enforced expression of miR-200 was sufficient to prevent TGF β -induced EMT (4). Epigenetic alterations including modification of histones and others proteins by acetylation and/or phosphorylation play critical roles in the control of gene regulation. Inhibitors of HDACs (HDACi) function to block the deacetylation of histones by HDACs, which in turn blocks the inhibition of gene expression including miRNA expression. Using microRNA array analysis, a rapid alteration of miRNA levels in response to HDACi in the breast cancer cell line SKBr3 has been reported (5). The work described here tests the hypothesis that HDACi-induced increases of miRNA levels regulate the epithelial-to-mesenchymal transition in TNBC. These results indicate the potential therapeutic uses of HDACi to reverse the EMT phenotype and metastatic progression of TNBC.

Task 1. HDAC analysis of miRNA profiles in triple-negative breast cancer cells.

Genome-wide microRNA (miR) microarray analysis on TNBC cell lines (MDA-MB-231, MDA-MB-468) has revealed changes in the miR expression patterns following treatment with an HDAC inhibitor (HDACi). Cells were treated with HDACi (1uM) for 18hrs. Following treatment, cells were harvested, total RNA isolated and samples sent to LC Sciences, LLC for miR microarray analysis by cross-array normalization, ANOVA, and SAM (Significance Analysis of Microarrays). Venn diagrams illustrate miR expression similarities and differences across the two TNBC cell lines. Table 1 summarizes significant increases in miR expression in both cell lines, while Table 2 summarizes significant decreases in miR expression in both cell lines following treatment with an HDACi.

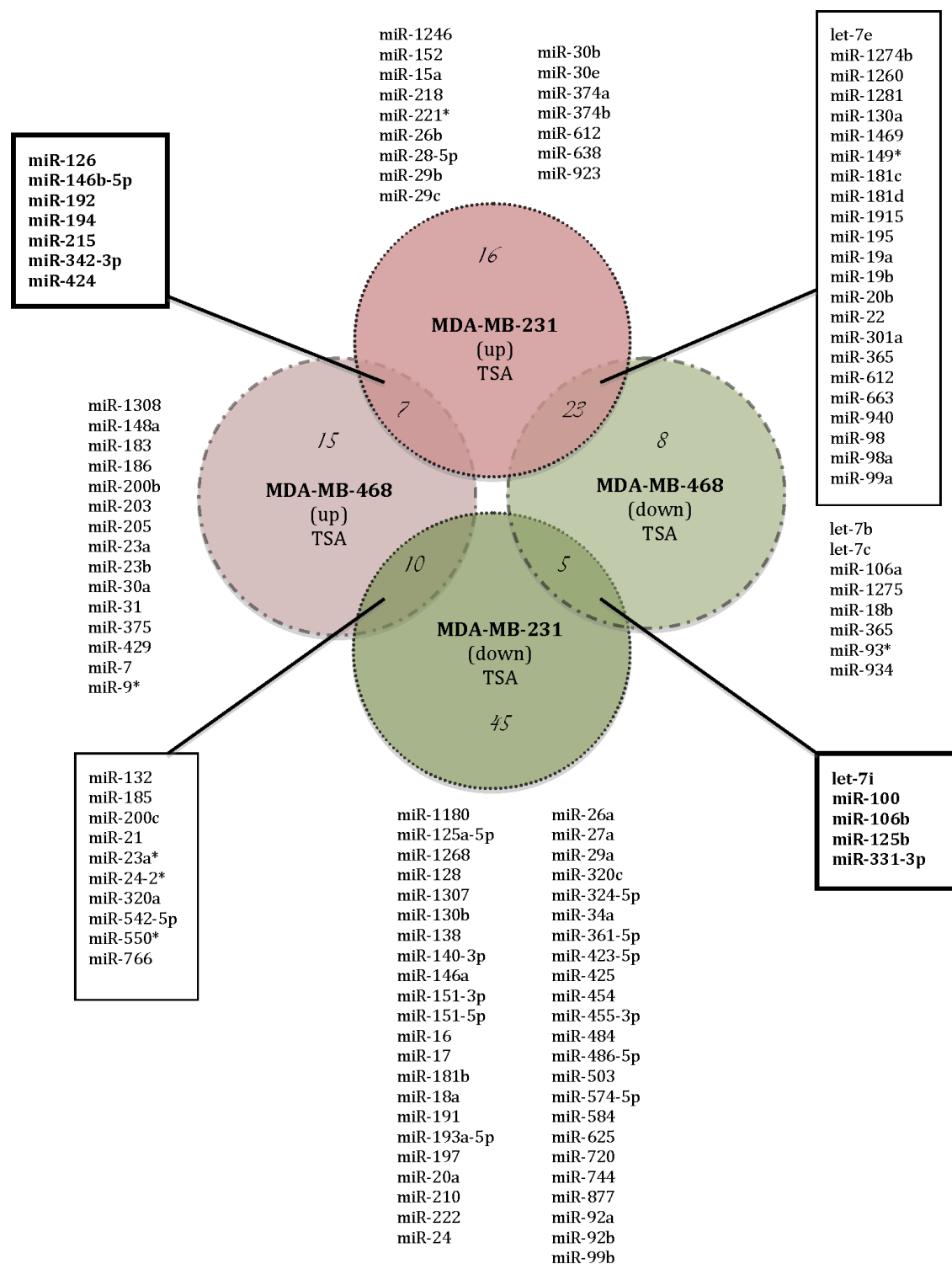
Table 1. HDACi induces increased expression of microRNAs.

	MDA-MB-231		MDA-MB-468		Description
	Fold change	p-value	Fold change	p-value	
miR-126	2.66	0.001340	2.19	0.006560	Metastasis suppressor, Anti-proliferative
miR-146b-5p	6.50	0.000019	23.75	0.000008	Migration/Invasion suppressor
miR-192	4.14	0.000752	2.85	0.000087	Anti-proliferative
miR-194	5.90	0.001020	2.93	0.000042	Anti-proliferative
miR-215	--	ns	5.94	0.004970	Anti-proliferative
miR-342-3p	1.66	0.005280	1.72	0.003620	--
miR-424	1.35	0.005230	2.23	0.000081	--

Table 2. HDACi induces decreased expression of microRNAs.

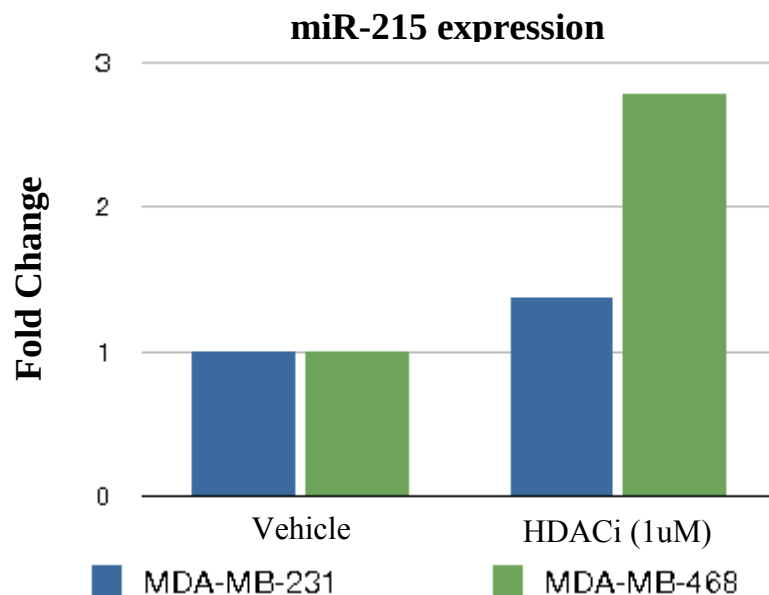
	MDA-MB-231		MDA-MB-468		Description
	Fold change	p-value	Fold change	p-value	
let-7i	-1.39	0.000016	-1.17	0.001980	Chemo-resistance
miR-100	-1.45	0.000004	-2.27	0.006250	Metastasis associated
miR-106b	-1.17	0.007900	-1.09	0.002370	OncomiR, metastasis
miR-125b	-1.27	0.001580	-1.52	0.001380	OncomiR
miR-331-3p	-1.48	0.000058	-1.44	0.004120	Growth factor receptor regulation

Venn diagram of microRNA expression changes in TNBC cell lines.

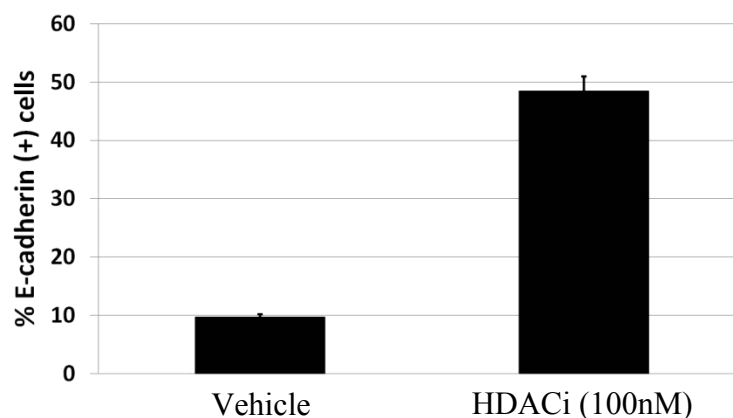


Task 2. qPCR validation of HDAC regulated miRNAs.**Task 2a. qPCR validation of HDAC regulated miRNAs.**

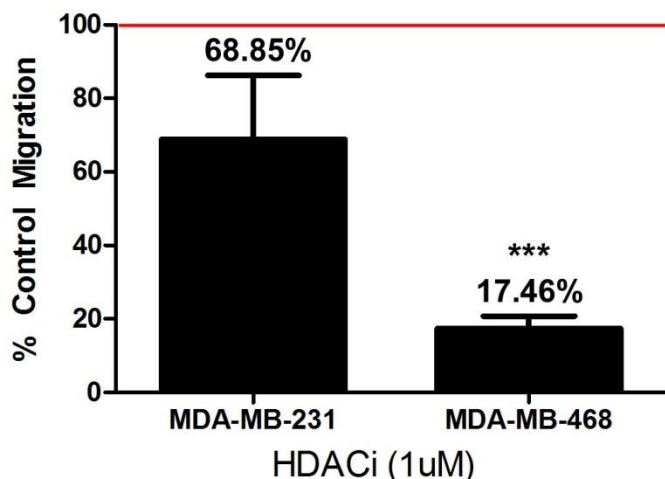
This task has been initiated but not yet completed. Preliminary results for miR-215 expression indicate that changes in the miR microarray induced by HDACi are consistent with qPCR results. Other miRs are currently being validated as well.

**Task 2b. Determine effects of HDACi on EMT phenotype of TNBC cells.**

This task has been initiated but not yet completed. MDA-MB-231 cells treated with HDACi (100nM) for 18hrs demonstrate a significant increase in E-cadherin expression, an epithelial cell marker, by flow cytometry. Studies examining vimentin expression, a mesenchymal cell marker, following HDACi treatment are also being conducted, but we do not have the results at this time.



Additionally, to confirm that HDAC treatment has an effect on TNBC cell biology, we performed migration assays. TNBC cells are characterized as highly aggressive and metastatic cells. Our results demonstrate that treatment with an HDACi (1uM) for 24hrs results in decreased migration of TNBC cell lines.



Task 3. Anti-miR strategies to reverse effects of HDACi and miR on EMT.

These experiments have not yet been conducted. Once we have completed confirmation of miR expression by qPCR, TNBC cells will be transfected with anti-miRs (targeting miRs validated by task 2). Cells will then be treated with HDACi or vehicle and analyzed for effects of on EMT phenotype (flow cytometry for E-cadherin, vimentin).

Task 4. miR lentiviral expression library analysis of effects on EMT-MET in triple-negative breast cancer cells.

These experiments have not yet been conducted. We will transduce TNBC cells with a miRNA lentiviral library (miR tRFP tagged) and analyze cells for E-cadherin expression. Cells positive for E-cadherin (GFP+) which express a miR construct (tRFP +) will be sorted via FACS analysis. The resulting cell population will contain cells expressing miRs which regulate the EMT-MET phenotypes.

Task 5. Follow up analyses for miR library screen.

The experiments outlined below have not yet been conducted.

Task 5a. qPCR confirmation of miRNA expression in the library transduced TNBC cells.

Cells from task 4 will be diluted to a single cell suspension and expanded to generate clonal miR expressing cell lines. Samples will be taken for sequencing to identify the miRs which reverse EMT.

Task 5b. Determine effects of miR expression on TNBC EMT phenotype.

Cell lines generated in task 5a will be analyzed for effects of miR expression on proliferation, migration/invasion, and expression of epithelial and mesenchymal cell markers.

Task 5c. Validation of miR effects on target gene expression.

TargetScan algorithms will be used to predict targets for miR identified in the above tasks. The effects of miR expression on predicted targets will be tested by qPCR analysis.

KEY RESEARCH ACCOMPLISHMENTS

- Identified HDACi-induced miR changes in TNBC cell lines, many of which have been implicated in EMT and metastasis regulation.
- Demonstrated increased expression of E-cadherin in TNBC cells following treatment with HDACi, indicating the reversal of the EMT phenotype.
- Demonstrated inhibition of cell migration of TNBC cell lines treated with HDACi.

REPORTABLE OUTCOMES

None at this time.

CONCLUSION

As triple-negative breast cancer has not benefited from the advances seen in the realm of endocrine and targeted therapy thus far, it is imperative to develop novel treatment strategies for this disease. Our results reported here have identified putative microRNAs, regulated by HDAC inhibitors, whose altered expression may play a role in the initiation of an invasive phenotype. Validation of the biological roles of these microRNAs in the regulation of EMT holds promise as therapeutic targets for the reversal of the invasive and metastatic phenotype associated with the lethality of triple-negative breast carcinoma.

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