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Award Number: W81XWH-08-1-0458

TITLE: Altered MicroRNA Activity Promotes Resistance to Endocrine Therapy

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REPORT DATE: July 2009

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 31-07-2009		2. REPORT TYPE Final		3. DATES COVERED (From - To) 1 July 2008-30 June 2009	
4. TITLE AND SUBTITLE Altered MicroRNA Activity Promotes Resistance to Endocrine Therapy				5a. CONTRACT NUMBER W81XWH-08-1-0458	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Diana M. Cittelly Ph.D. Email: Diana.Cittelly@ucdenver.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Colorado Health and sciences. Mail Stop F428 PO BOX 6508 Aurora, CO 80045-0508				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT  Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT MicroRNAs (miRNAs) have tumor suppressive and oncogenic potential in human cancer, but little is known about the extent at which miRNA expression is modified after anti-estrogen treatment and the contribution of specific miRNAs to the acquisition of anti-estrogen resistance. To answer this question, in <b>Aim 1</b> , we performed miRNA profiling of tamoxifen-resistant and sensitive breast cancer cells treated with Estradiol or Tam. Several miRNAs were intrinsically downregulated in tam-resistant cells or were deregulated after treatments, suggesting that endocrine signaling can modulate miRNA expression. One miRNA sensitized resistant cells to tam-induced apoptosis and may emerge as a potential therapeutic target to sensitize endocrine refractory breast tumors. In <b>Aim 2</b> , we tested the hypothesis that regulation of miRNAs involved in modulating anti-apoptotic BCL-2 plays a role in tamoxifen resistance in breast cancer cell lines. Equivalent levels of BCL-2 mRNA were observed in tam-sensitive and resistant cells; however, tam-resistant MCF-7Δ16 cells upregulated BCL-2 protein and expressed reduced levels of BCL-2 targeting miRNAs miR15a and miR16. Reintroduction of miR15a/16 reduced tam-induced BCL-2 expression and sensitized MCF-7Δ16 to tam. Conversely, suppression of miR15a/16 activated BCL-2 expression and rendered cells tam-resistant. In conclusion, miRNAs constitute a novel mechanism for endocrine-therapy evasion and provides a template for unique therapeutic interventions involving modulation of miRNAs in combination with tamoxifen.					
15. SUBJECT TERMS none provided.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT  UU	18. NUMBER OF PAGES  42	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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## INTRODUCTION

MicroRNAs (miRNAs) are small non-coding ssRNA regulatory molecules of approximately 22 bp that suppress gene expression by binding the 3' UTR of target gene mRNAs. Once bound to their target mRNA, miRNAs may repress gene expression through enhanced degradation of the mRNA or more commonly by inhibiting target gene translation. Microarray studies on miRNA levels in various human breast cancer tissues have shown that some miRNAs are up-regulated in breast cancer vs. normal tissue, and a smaller cohort of miRNAs are upregulated in ER(-) vs ER(+) tumors (1,2). miRNAs have tumor suppressive and oncogenic potential in human cancer, but little is known about the extent at which miRNA expression is modified after anti-estrogen treatment and the contribution of specific microRNAs to the acquisition of anti-estrogen resistance.

Nearly, 70% of breast cancer patients develop tumors expressing the estrogen receptor (ER $\alpha$ ) and are candidates for endocrine therapy. The selective ER $\alpha$  modulator, tamoxifen is the most commonly prescribed endocrine therapy and has recently been recommended as a preventative (3). Nevertheless, 30-40% of patients fail adjuvant tamoxifen therapy and nearly all patients with metastatic disease develop tamoxifen resistance (4-6). Unfortunately, *de novo* and acquired tumor resistance to tamoxifen therapy remains a poorly understood and serious clinical problem.

Several clinical studies implicate tumor expression of the HER2 receptor tyrosine kinase as a significant risk for tamoxifen failure. Patients with HER2 expressing tumors account for approximately half of the ER $\alpha$  positive population and over 70% of these patients may exhibit *de novo* tamoxifen resistance (6). Furthermore, a large percentage of HER2/ER $\alpha$  positive tumors are estrogen-independent and therefore continue to grow when patients are estrogen depleted (7). Preclinical models of HER2 overexpression have provided insights into possible mechanisms underlying tamoxifen resistance; however, only the occasional HER2 overexpressing ER $\alpha$ (+) cell line exhibits at best partial tamoxifen resistance and all remain estrogen dependent (8-12).

In Aim 1, we study the role of microRNA dysregulation to the acquisition of anti-estrogen resistance, by analyzing the miRNA expression profiles of tamoxifen resistant and sensitive breast cancer cells lines alone and in response to Tamoxifen. Consistent with our hypothesis, several miRNAs were intrinsically downregulated in tamoxifen-resistant cells when compared to sensitive cells. Mir-342 and other miRNAs are further downregulated in response to tamoxifen in resistant cell lines, suggesting that endocrine signaling can modulate miRNA expression. Consistent with a role of miR-342 downregulation in the acquisition of tamoxifen resistance, reintroduction of miR342 using synthetic miRNAs sensitized resistant cells to tamoxifen-induced cell growth arrest and apoptosis. Microarray analysis of gene expression in tam-resistant cells stably overexpressing mir-342 showed differential expression of 257 genes, 13 of which are predicted to be direct targets of mir-342. Experiments to validate miR-342 targets and their impact on tamoxifen-response are needed. Our findings suggest that mir-342 may play a significant role in tamoxifen response and may emerge as a potential therapeutic target to sensitize endocrine refractory breast tumors.

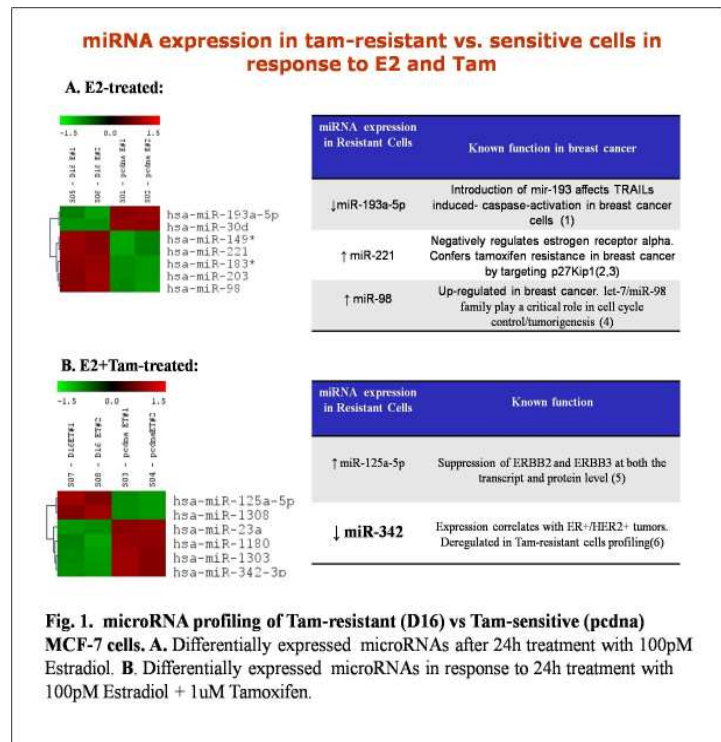
In Aim 2, we tested the hypothesis that antiestrogen resistance is mediated by altered post-transcriptional regulation (ie. miRNA deregulation) of pro-survival genes such as Bcl-2 in tumors overexpressing an oncogenic variant of HER2. We showed that an oncogenic isoform of HER2, HER2 $\Delta$ 16, promotes tamoxifen resistance and estrogen independence of ER $\alpha$  positive MCF-7 xenografts. Equivalent levels of BCL-2 mRNA are observed in tamoxifen sensitive and resistant cells lines; however, tamoxifen resistant MCF-7/HER2 $\Delta$ 16 cells upregulate BCL-2 protein and also express reduced levels of BCL-2 targeting microRNAs miR-15a and miR-16. Reintroduction of miR-15a/16 reduced tamoxifen induced BCL-2 expression and sensitized MCF-7/HER2 $\Delta$ 16 to tamoxifen. Conversely, suppression of miR-15a/16 activated BCL-2 expression and converted cells to a tamoxifen resistant phenotype. Our results suggest that miRNA modulation constitute an additional and novel mechanism for endocrine-therapy evasion, and provides a template for unique therapeutic interventions involving modulation of microRNAs and/or derepressed apoptosis in combination with tamoxifen.

## BODY

### **Aim 1. To test the hypothesis that miRNAs deregulation is involved in the response to anti-estrogens in breast cancer cell lines (Appendix 1)**

#### **Task 1. Global quantification of relative levels of miRNAs in endocrine- resistant vs. endocrine sensitive MCF-7 cell lines, treated for 24h with E2 alone or in combination with Tam (1uM) or ICI after 48h serum starvation.**

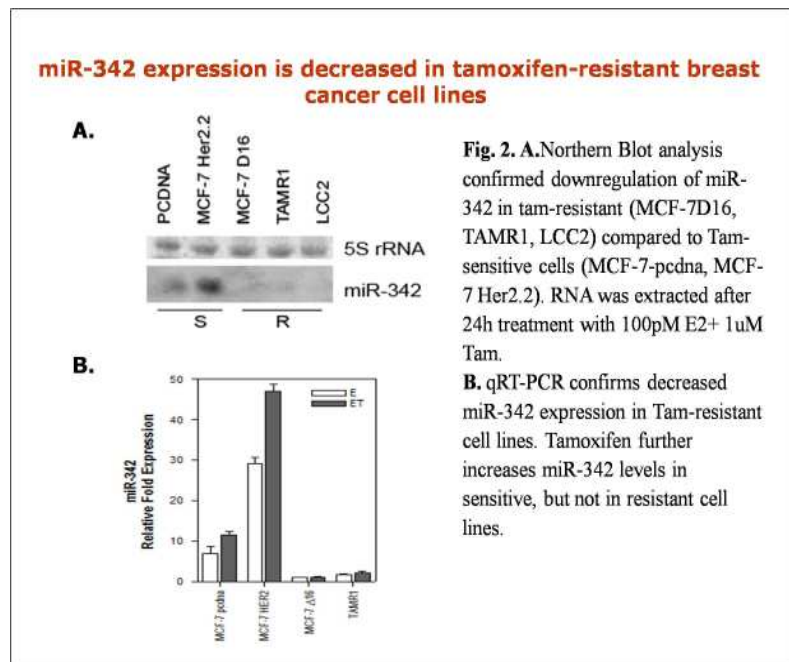
To decipher the contribution of miRNA deregulation to the acquisition of anti-estrogen resistance, we analyzed the miRNA expression profiles of tamoxifen resistant (MCF-7) and sensitive breast cancer cells lines(MCF-7-D16) after 24h treatment with 100pM estradiol or 1uM Tamoxifen (Figure 1). Total RNA was isolated from biological duplicates of each treatment and then analyzed using LC Sciences miRNA detection arrays, containing probes for all miRNAs included in miRBase Release 11.0. Consistent with our hypothesis, several miRNAs were intrinsically downregulated in tamoxifen-resistant cells when compared to sensitive cells. Among them, five miRNAs are further downregulated in response to tamoxifen in resistant cell lines, suggesting that endocrine signaling can modulate miRNA expression. Given the amount of significant data obtained from cells treated with estradiol and tamoxifen, and the high cost of additional miRNA-profiling, we did not performed miRNA profiling of ICI-treated cells as stated in this task, but we focused in testing the ability of specific miRNAs in conferring the tamoxifen-resistant phenotype, as stated in Tasks 3 and 2.



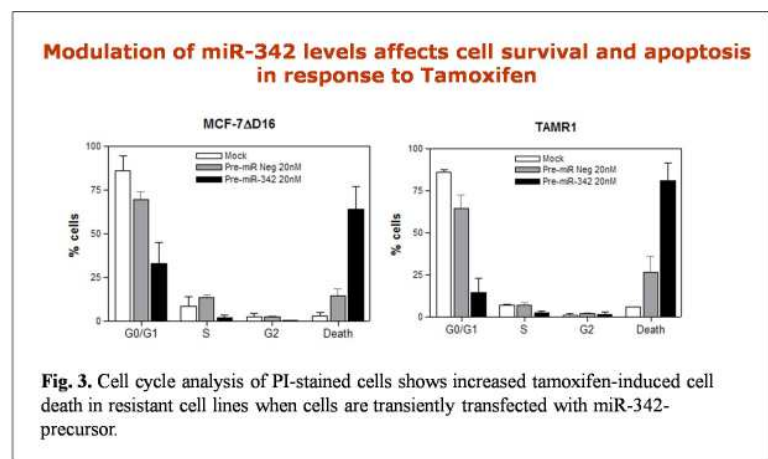
#### **Task 3. Quantification of levels of particular miRNAs by real-time PCR**

Since the profiling analysis resulted in several miRNAs potentially involved in the tamoxifen-resistant phenotype, we first validated miRNA expression levels by northern blot. We used p32-labeled DNA-probes specific for the most deregulated miRNAs in response to Estradiol o tamoxifen (miR-342-3p, miR-203, miR-98) to evaluate whether each miRNA was consistently deregulated in several tamoxifen-

sensitive (pcdna, MCF-HER2) or resistant (MCF-7D16, TAMR1, LCC2) breast cancer cell lines, and therefore represented a mechanism of conferring anti-estrogen resistance in the clinical setting. Only miR-342-3p appears to be consistently down-regulated in several tamoxifen-resistant ER+ breast cancer cells (Figure 2A), thus, we focused our analyses in this particular miRNA. Confirming northern blot results, more sensitive RT-PCR analyses show that miR-342-3p is highly expressed in sensitive compared to resistant-cell lines and is further up-regulated in response to tamoxifen only in sensitive cells (Figure 2B), suggesting that miR-342 mediates tam-induced cell death and that its loss might be either a marker or a mediator of acquisition of tamoxifen-resistance.

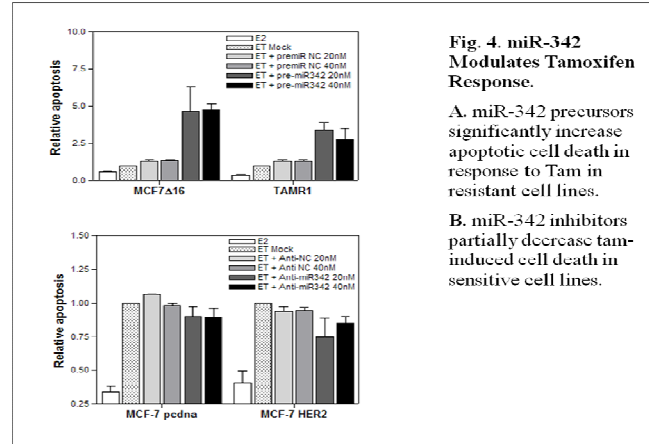


In order to test the direct role of miR-342-3p downregulation in the acquisition of tamoxifen resistance, we modulated the levels of miR-342 in sensitive and resistant cell lines using transient transfections with different concentrations of miR-342 precursors or inhibitors and measured its effect on tamoxifen-induced cell death. As shown in Figure 3, cell cycle analysis using PI-staining 48h after transfection, showed that transient transfection with 20nM pre-miR-342 but not Negative scrambled control sensitized resistant cells (MCF-7D16, TAMR1) to 1uM tamoxifen-induced cell death.



To further decipher whether the increase in tam-induced cell death was a result of increased apoptosis we measured the levels of apoptosis in tam-resistant cells treated with miR-342 precursor using a Cell Death ELISA kit (Roche), a measurement of the amount of Oligonucleosomes that are released to the cytosol during apoptosis (but not in necrosis). As shown in Figure 4, tamoxifen alone induced only marginal increases in apoptosis in resistant cells (D16, TAMR1), but 48h treatment with miR-342 precursor increased apoptosis up to 5 fold compared to negative control and mock-treated

cells (Fig. 4A). As expected, tamoxifen alone induced significant apoptosis in sensitive cells (MCF-7 pcdna, MCF-7-Her2) that express high levels of miR-342. Transient transfection with miR-342 inhibitors partially decreased tamoxifen-induced apoptosis, suggesting that miR-342 is not the only mediator of tam-induced apoptosis, but most likely, that we did not reach a complete inhibition of the endogenous miR-342. In support of this hypothesis, several groups have shown that even marginal levels of miRNA are sufficient to have a functional effect.



## Task 2. Analysis of microRNA arrays results and search for putative targets

For clarity purposes, we changed the order of tasks in this report, since we focused in studying the possible targets of miR-342 (Task 2) only after we confirmed its importance in the tamoxifen-resistant phenotype (Task 3). We utilized two different approaches to identify miR-342 putative targets:

**Approach 1.** In order to identify targets of miR-342 involved in the acquisition of antiestrogen resistance we used several miRNA target prediction algorithms including PicTar, miRANDA and TargetScan. Since each individual algorithm predicts between hundreds to thousands of putative targets, we speculated that the likelihood of targeting real miR-342 targets was higher if a target was predicted by the three algorithms. Using this approach, we found 13 putative target genes, two of which (PDGFRA and ID4) were found to be over expressed in breast cancer when searched in the Oncomine<sup>TM</sup> Research platform (Using this database, one can search among hundreds of microarray profiling databases to evaluate the possible impact of a given gene in different types of cancer).

PDGFRA is a tyrosine kinase receptor (170-185 kDa) involved in actin reorganization, transcription, cell growth, migration and differentiation and a key regulator of MAPK pathway. Interestingly, PDGFRA was identified as one of seven genes causing anti-estrogen resistance (13), suggesting that it might be a real miR-342 target and mediator of miR-342 effect in tamoxifen-resistance.

ID4 is a member of Helix-loop-helix transcription factors (HLH-TF) that lacks of DNA binding-domain and acts as dominant negative regulators of HLH TF. Members of this family are over-expressed in several tumor cells and correlate with dedifferentiated, proliferative and invasive phenotypes. The role of ID4 in breast cancer is not clear. ID4 has been reported to be involved in hormone-dependent down-regulation of BCRA1 (14), and ID4 and BCR1A expression are inversely related in sporadic breast cancer. ID4 is expressed in ER(-) carcinomas (15), so we speculate that ID4 overexpression in ER(+) Tam-



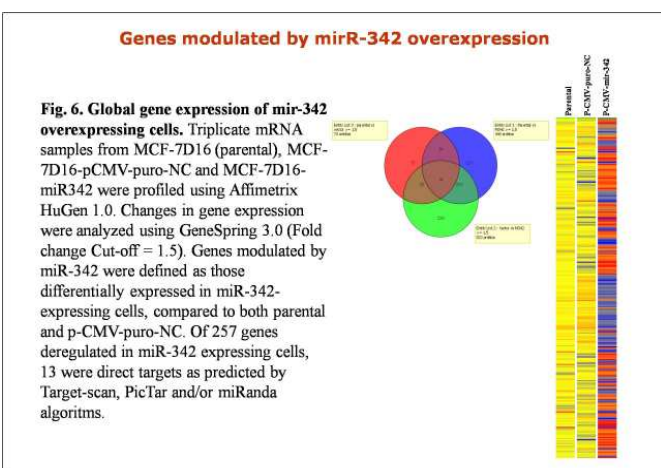
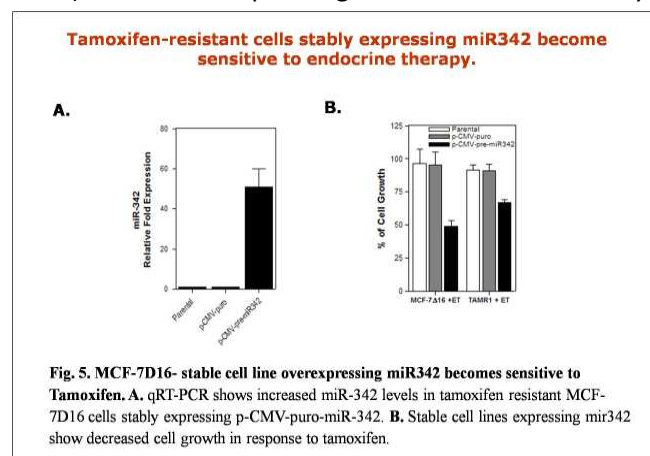
resistant cells might confer cells with the ability to grow when tamoxifen blocks ER-signaling. Therefore, we tested the hypothesis that miR-342 downregulation in tam-resistant cell lines, leads to up-regulation of PDGFRA and/or ID4 and that these genes have a functional role in the acquisition of Tam-resistance mediated by miR-342 (Continues in **Task 4**).

**Approach 2.** Recent studies have shown that identification of individual miRNA targets by computational algorithms can be difficult and that a particular miRNA can have global effects in the the gene expression that result in a given phenotype. In order to identify direct targets of miR-342 that can be responsible for its role in tamoxifen-response, we created stable cell lines over expressing pre-miR342 and then analyzed changes in gene expression profiles compared to parental or vector-expressing cells.

Briefly, a 342bp sequence containing the pre-miR-342 sequence flanked by 100bp up and downstream were cloned in a siRNA expression vector driven by the CMV-promoter (p-CMV-puro-silencer from Ambion) to produce p-CMV-miR342. The same vector expressing a short scrambled sequence (p-CMV-puroNC) was used as a control. Either p-CMV-miR-342 or p-CMV-puroNC were cloned in tamoxifen resistant cell lines (MCF-7D16 and TAMR1) and stable-expressing clones were selected by long-term selection with puromycin. As shown in figure 5A, MCF-7D16-p-CMV-miR342 stable cell lines express miR-342 in levels similar to those of tam-sensitive MCF-7 Her2 cells (see fig 2B) as measured by qRT-PCR.

Consistent with transient transfection results (Fig 3, 4), tamoxifen-resistant cells stably expressing miR-342 but not vector-control, became sensitive to tamoxifen treatment as demonstrated by a ~50% decrease in cell growth measured by MTT assay (Fig. 5B).

We isolated total RNA from triplicate samples of parental (MCF-7D16), NC-vector-expressing (p-CMV-puroNC) and miR-342-expressing (p-CMV-miR-342), and analyzed their gene expression profiles using HuGene 1.0 Affymetrix-microarray (Fig 6.) Changes in gene expression were analyzed using GeneSpring. Genes that changed over 1.5 fold compared to parental-control cells were selected, and miR-342-regulated genes were defined as those differentially regulated in miR-342-expressing cells, but not by the vector-NC control. Tam-



resistant cells stably overexpressing miR-342 showed differential expression of 257 genes, 13 of which are predicted to be direct targets of miR-342. Interestingly, a high percentage of genes is down-regulated as result of miR-342 expression, suggesting that these are regulated as a result of miR-342 action on its direct-targets. Experiments to validate these miR-342 targets and their impact on tamoxifen-response will be performed when additional funding is obtained.

#### Task 4. Quantification of protein expression of putative microRNA targets

As a result of the initial analysis of putative targets in Task 2 (Approach 1), we identified two potential miR-342 targets, PDGFRA and ID4. We then tested the hypothesis that miR-342 downregulation in tam-resistant cell lines, leads to up-regulation of PDGFRA and/or ID4 and that these genes have a functional role in the acquisition of Tam-resistance mediated by miR-342. First, we analyzed the protein levels of PDGFRA and ID4 in tamoxifen-sensitive and resistance breast cancer cells lines (Fig. 7). In agreement with a role of miR-342 in regulating PDGFRA and ID4 expression, tamoxifen-sensitive cell lines (expressing high levels of miR-342) express low levels of both PDGFRA and ID4 proteins, while tamoxifen resistant cell lines (expressing low levels of miR-342) have high expression levels of both proteins.

**PDGFRA and ID4 expression increases after Tamoxifen treatment in resistant but not sensitive MCF-7 cells**

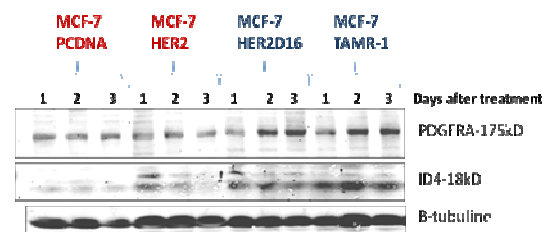


Fig. 7. Western blot analyses showed a time-dependent increase in expression of both PDGFRA and ID4 in response to tamoxifen in resistant breast cancer cells lines compared to sensitive cells. Cells were treated for 24, 48 or 72 h with 100nM tamoxifen and total cell lysates analyzed by western blot. B-tubuline was used as loading control.

To demonstrate the direct regulation of PDGFRA and ID4 by miR-342 we transfected cells with 20 or 40 nM pre-miR342 or Neg-control pre-miR in tamoxifen-resistant MCF7D16 and TAMR1 cells, and measured the protein levels putative targets PDGFRA and ID4. Even though there was a slight downregulation of PDGFRA when cells were treated with 40nM pre-miR342, we were unable to consistently reproduce these results and therefore to confirm the downregulation of PDGFRA by miR-342 transient overexpression. Similarly, we tested different conditions but still were unable to determine the effect of miR-342 in ID-4 expression using this transient-transfection approach. Furthermore, we tested the expression levels of both protein targets in the stable cell-lines described in Task 3 (approach 2). If our hypothesis was correct and PDGFRA and/or ID4 were direct targets of miR342, we expected to see a decrease in one or both proteins in miR-342 stably-over expressing cells. However, western blot analysis showed that despite the significant over-expression of miR-342, there were not changes in PDGFRA or ID4 protein levels in any of the stable cell lines. Together, these results suggested that PDGFRA and ID4 might not be direct targets of miR-342, so we decided to approach the search of putative targets by analyzing the global gene expression changes in stable cells lines as described in approach 2 (Task2).

## **AIM 2. To test the hypothesis that miRNAs deregulate translation of anti-apoptotic Bcl-2 in anti-estrogen resistant breast cancer cell lines**

The results corresponding to this aim have been submitted for publication and are currently under review (Cittelly DM et al, 2009.) A detailed description of these results can be found in the attached manuscript (Appendix 2).

Tumor cell apoptosis plays an important role in both preclinical and clinical responses to tamoxifen and overexpression of anti-apoptotic BCL-2 promotes tamoxifen resistance. We therefore determined the influence of endogenous BCL-2 expression on the response of tamoxifen resistant and sensitive MCF-7 cell lines and tested if Bcl-2 regulation by miRNAs played a role in tam-resistance in breast cancer cell lines. Consistent with previous observations, estrogen stimulated BCL-2 mRNA expression in each MCF-7 cell line tested and tamoxifen suppressed BCL-2 expression to below basal levels (Appendix 2, Fig. 2A). Despite tamoxifen induced suppression of BCL-2 mRNA the MCF-7/HER2 $\Delta$ 16 cell line accumulated BCL-2 protein in response to tamoxifen (Appendix 2, Fig. 2B and Supplementary Fig. S2A). A similar upregulation of BCL-2 protein was observed in MCF-7/HER2 $\Delta$ 16 cells treated with fulvestrant (ICI 182780) and during estrogen withdrawal (Appendix 2, Supplementary Fig. S2B).

### **Task 1. Quantification of miR-15a and miR-16 expression levels in anti-estrogen responsive and antiestrogen resistant cell lines.**

In order to elucidate the molecular mechanisms underlying altered BCL-2 expression in tamoxifen resistant cells, we tested the hypothesis that BCL-2 targeting miRNAs miR15a and miR16 (miR15a/16) which have been shown to alter BCL-2 gene expression, are regulators of Bcl-2 expression in breast cancer cells. Total RNA was extracted from each cell line and analyzed for miR15a and miR16 expression by qRT-PCR. The levels of miR15a/16 were similar in tamoxifen sensitive cells (Appendix 1, Fig. 3A). In contrast, the levels of miR15a/16 in the tamoxifen resistant MCF-7/HER2 $\Delta$ 16 cell line were reduced by 71% and 64%, respectively (Appendix 2, Fig. 3A).

### **Task 2. Evaluation of functional role of miR-15 and miR-16 in up-regulation of Bcl-2 in breast cancer cells**

Increasing the expression of miR15a/16 resulted in suppression of BCL-2 expression and sensitized MCF-7/HER2 $\Delta$ 16 cells to tamoxifen with a nearly 4 fold increase in apoptosis (Appendix 2, Fig. 3B-D). Importantly, miR15a/16 expression was not significantly altered in response to estrogen or tamoxifen (Appendix 2, Supplementary Fig. S3A). In addition, HER2 $\Delta$ 16 expression failed to impact miR15a/16 in the ER $\alpha$  negative MDAMB231 cell line which expressed lower levels of miR15a/16 when compared to MCF-7 cells and lacked tamoxifen induced expression of BCL-2 (Appendix 2, Supplementary Fig. S3B,C).

### **Task 3. Evaluation of functional role of miR-15 and miR-16 in tamoxifen/fulvestrant resistance**

Based upon the ability of enhanced miR15a/16 expression to sensitize MCF-7/HER2 $\Delta$ 16 cells to tamoxifen, we next determined if miR15a/16 suppression promotes tamoxifen resistance. Pretreatment

of anti-estrogen sensitive MCF-7/Vector and MCF-7/HER2 cells with inhibitors of miR15a or miR16 resulted in enhanced BCL-2 expression and a partial but significant tamoxifen resistance with a significant reduction in tamoxifen induced apoptosis (Appendix 2, Fig. 4A-C). These results further implicate the BCL-2 targeting miR15a/miR16 as important regulators of tamoxifen response and suggest that tumor cell suppression of miR15a/16 may represent an important mechanism of tamoxifen resistance.

## KEY RESEARCH ACCOMPLISHMENTS

With this award we were able to demonstrate that:

- miRNAs are intrinsically downregulated in tamoxifen-resistant cells when compared to sensitive cells, suggesting that microRNA(s) may contribute to the acquisition of the tamoxifen resistant phenotype.
- MicroRNA expression varies in response to E2 and Tam, suggesting that endocrine signaling can modulate miRNA expression.
- miR-342 is down-regulated in tamoxifen-resistant breast cancer cells and transient or stable reintroduction of miR-342 restores sensitivity to Tam, thus, it constitutes a potential therapeutic target to sensitize endocrine refractory breast tumors.
- miR-342 regulates the expression of multiple genes in breast tumor cells.
- Upregulation of anti-apoptotic proteins such as Bcl-2, are regulated in part by modulation of miR-15a/16 levels in antiestrogen resistant MCF-7 Her2D16 cells.

## REPORTABLE OUTCOMES

### 1. Manuscripts:

**Cittelly DM, Salvo VA, Fonseca JP, Burrow M, Jones FE.** Oncogenic HER2 Evades Tamoxifen Intervention through Suppression of miR15a and miR16. *Under review. Cancer Research.*

**Cittelly DM, Thor A, Jones FE.** microRNA 342-3p contributes to tamoxifen-resistance in ER+HER2+ breast cancer. *In preparation.*

### 2. Abstracts-Poster presentations:

**Cittelly DM, Jones FE.** Altered microRNA activity promotes resistance to endocrine therapy in breast cancer cells. Poster presentation at “MicroRNA and cancer” Keystone Symposia. Keystone Resort, CO, USA. June 10-15<sup>th</sup>, 2009.

### 3. Cell lines developed:

Stable MCF-7D16 and TAMR-1 cell lines expressing miR-7, miR27b and miR-342 were produced and stored for further studies. Cells lines were included in Dr.Frank Jones lab cell-line bank.

### 4. Research opportunities:

Dr. Cittelly has acquired expertise in the study of microRNAs which will be invaluable asset for the development of her career. The preliminary data obtained in this study will be used for further funding applications.

## CONCLUSION

Using microarrays we identified several miRNAs that are intrinsically downregulated in tamoxifen-resistant cells when compared to sensitive cells, suggesting that miRNA(s) may contribute to the acquisition of the tamoxifen-resistant phenotype. Furthermore, we demonstrated that miRNA expression varies in response to E2 and Tam, indicating that endocrine signaling can modulate miRNA expression. These results can have profound clinical implications since they suggest that differentially expressed miRNAs in resistant cells could serve as potential biomarkers of anti-estrogen resistant tumors and prove to be valuable markers in the personalized clinical management of breast cancer. In particular, our finding that miR-342 is down-regulated in Tam-resistant breast cancer cells and transient or stable reintroduction of miR-342 restores sensitivity to Tam, constitutes a novel and potential therapeutic target to sensitize endocrine refractory breast tumors. We need further studies to validate the use of miR-342 as a clinical marker of endocrine-resistance to clinical samples, for instance, by evaluating the expression of miR-342-3p by *in situ* hybridization in tumor samples from patients that are responders and non-responders to anti-estrogen treatment.

Determination of the target genes/pathways of these dysregulated miRNAs will further enhance our knowledge of antiestrogen resistance and facilitate design of new targeted therapeutic agents that might allow for the prevention or reversal of resistance. For example, therapeutic strategies aimed at blocking expression of these miRNAs in antiestrogen-*sensitive* breast cancer may allow for a greater response to anti-estrogen therapy in a subset of breast cancers. The main obstacle that miRNA analysis poses is the lack of highly accurate prediction methods to select and validate real miRNA targets. As each miRNA can target up to 200 mRNA sequences, and mRNAs can have multiple miRNA target sites, elucidating clinically relevant miRNA targets proved to be challenging. In our study we found that 2 putative targets predicted by 3 different computational algorithms failed to be targeted by miR-342, even though their protein expression levels were consistent with regulation by this miRNA. We used an alternative method to search for miRNA targets by performing microarray analysis of cells stably-expressing miR-342-3p in an attempt to evaluate the global changes in gene-expression due to miR-342-3p. Our results suggest that miR-342-3p overexpression alters expression of around 250 genes, but a high percentage of them are upregulated (instead of downregulated as expected for direct targets), suggesting that miRNA overexpression has a global effect in gene expression. We need further studies to validate the group of genes that are predicted to be direct targets for miR-342-3p that could explain its role in acquisition of tamoxifen resistance.

In conclusion, miRNA modulation constitutes an additional and novel mechanism for endocrine-therapy evasion, and provides a template for unique therapeutic interventions involving modulation of microRNAs and/or derepressed apoptosis in combination with tamoxifen.

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## **APPENDICES**

Appendix 1: Abstract presented at Keystone Symposium Meeting “MicroRNAs and Cancer”

Appendix 2: Manuscript submitted to Cancer Research

Appendix 3: List of personnel paid from the research effort.

## **Altered microRNA activity promotes resistance to endocrine therapy in breast cancer cells**

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Antiestrogens (i.e., Tamoxifen) have been the therapeutic agents of choice for breast cancer patients whose tumors express Estrogen Receptor (ER), but the majority of patients will develop resistance to these drugs. MicroRNAs (miRNAs) have tumor suppressive and oncogenic potential in human cancer, but little is known about the extent at which miRNA expression is modified after anti-estrogen treatment and the contribution of specific microRNAs to the acquisition of anti-estrogen resistance. To address this question, we analyzed the miRNA expression profile of tamoxifen resistant and sensitive breast cancer cells lines alone or in response to Tamoxifen. Several miRNAs, including miR-342-3p, were intrinsically downregulated in tamoxifen-resistant cells when compared to sensitive MCF-7 cells. Mir-342 and three other miRNAs were further downregulated in response to tamoxifen in resistant cell lines, suggesting that endocrine signaling can modulate miRNA expression. Consistent with a role of miR-342 downregulation in the acquisition of tamoxifen resistance, reintroduction of miR342 using synthetic miRNAs sensitize tamoxifen-resistant cells to tamoxifen-induced cell growth arrest and apoptosis. In order to identify the mediators of mir-342 activity, we used three different algorithms to identify potential targets that are clinically relevant markers of tamoxifen response. Experiments to validate miR-342 targets and their impact on tamoxifen-response will be presented. Our findings suggest that mir-342 may play a significant role in the tamoxifen response and may emerge as a potential therapeutic target to sensitize endocrine refractory breast tumors. Supported by US AMRMC grant W81XWH-08-1-0458 (DC).

# Oncogenic HER2 Evades Tamoxifen Intervention Through Suppression of miR15a and miR16

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**Running Title:** HER2Δ16 and miR15a/16 suppression in tamoxifen resistance

**Key Words:** EGFR-family, endocrine therapy, micro RNAs, BCL-2 family, breast cancer, apoptosis

## ABSTRACT

Tamoxifen is the most commonly prescribed therapy for patients with estrogen receptor (ER $\alpha$ ) positive breast tumors. Tumor resistance to tamoxifen remains a serious clinical problem especially in patients with tumors that also overexpress HER2. Current preclinical models of HER2 overexpression fail to recapitulate the clinical spectrum of endocrine resistance associated with HER2/ER $\alpha$  positive tumors. Here we show that an oncogenic isoform of HER2, HER2 $\Delta$ 16, promotes tamoxifen resistance and estrogen independence of ER $\alpha$  positive MCF-7 xenografts. MCF-7/HER2 $\Delta$ 16 cells evade tamoxifen through a novel mechanism of tamoxifen induced upregulation of anti-apoptotic BCL-2 and suppression of BCL-2 expression restores tamoxifen sensitivity. Equivalent levels of BCL-2 mRNA are observed in tamoxifen sensitive and resistant cells lines; however, tamoxifen resistant MCF-7/HER2 $\Delta$ 16 cells upregulate BCL-2 protein in response to disengaged ER $\alpha$  signaling. MCF-7/HER2 $\Delta$ 16 cells also expressed reduced levels of BCL-2 targeting microRNAs miR15a and miR16. Reintroduction of miR15a/16 reduced tamoxifen induced BCL-2 expression and sensitized MCF-7/HER2 $\Delta$ 16 to tamoxifen. Conversely, suppression of miR15a/16 activated BCL-2 expression and converted cells to a tamoxifen resistant phenotype. Our results suggest that HER2 $\Delta$ 16 expression promotes endocrine resistant HER2/ER $\alpha$  positive breast tumors. We further show that in contrast to wild-type HER2, preclinical models of HER2 $\Delta$ 16 overexpression recapitulate multiple phenotypes of endocrine resistant HER2/ER $\alpha$  positive tumors. The novel mechanism of HER2 $\Delta$ 16 therapy evasion, involving tamoxifen induced upregulation of BCL-2, provides a template for unique therapeutic interventions involving modulation of microRNAs and/or derepressed apoptosis in combination with tamoxifen.

## INTRODUCTION

Nearly, 70% of breast cancer patients develop tumors expressing the estrogen receptor (ER $\alpha$ ) and are candidates for endocrine therapy. The selective ER $\alpha$  modulator, tamoxifen is the most commonly prescribed endocrine therapy and has recently been recommended as a preventative (1). Nevertheless, 30-40% of patients fail adjuvant tamoxifen therapy and nearly all patients with metastatic disease develop tamoxifen resistance (2-4). Unfortunately, *de novo* and acquired tumor resistance to tamoxifen therapy remains a poorly understood and serious clinical problem.

Several clinical studies implicate the HER2 receptor tyrosine kinase as a significant risk for tamoxifen failure. Patients with HER2 expressing tumors account for approximately half of the ER $\alpha$  positive population and over 70% of these patients may exhibit *de novo* tamoxifen resistance (4). Furthermore, a large percentage of HER2/ER $\alpha$  positive tumors are estrogen-independent and therefore continue to grow when patients are estrogen depleted (5). Preclinical models of HER2 overexpression have provided insights into possible mechanisms underlying tamoxifen resistance; however, only the occasional HER2 overexpressing ER $\alpha$ (+) cell line exhibits at best partial tamoxifen resistance and all remain estrogen dependent (6-10).

Recently an oncogenic isoform of HER2 (HER2 $\Delta$ 16) has been identified (11) which is coexpressed with HER2 in primary breast tumors (12). Here we show that expression of HER2 $\Delta$ 16 promotes estrogen independent growth and *de novo* resistance to tamoxifen therapy. We further demonstrate that HER2 $\Delta$ 16 evades tamoxifen therapy through altered regulation of BCL-2 targeting miRNAs. Our results suggest that HER2 $\Delta$ 16 is a clinically important HER2 isoform driving tamoxifen resistant and estrogen independent breast cancer.

## MATERIALS AND METHODS

**Cell lines.** The MCF-7 cell line was purchased from ATCC and cultured according to their instructions and stable MCF-7 cell lines have been described elsewhere (13).

**Tumor formation in nude mice.** Tumor xenografts were generated and analyzed as described elsewhere (13) with the following modification. The minus estradiol groups were implanted with placebo pellets during the entire experiment.

**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.** Cell proliferation was measured as a function of metabolism by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) assay exactly as described elsewhere (14).

**Western blot analysis of cell lysates.** Total cell lysates were prepared and analyzed by western blot exactly as described elsewhere (15). Primary antibodies used for western blot analysis included ER Ab-1 (Neomarkers), HER2 (Neomarkers),  $\alpha$ -tubulin (Upstate), and BCL-2 (Cell Signaling). Secondary antibodies were Alexa-fluor 680 Conjugated Affinity Purified Anti-Rabbit or Anti-Mouse IgG (Invitrogen) detected using an Odyssey Infrared Imaging System (Licor Biosciences, Lincoln, NE).

**Luciferase reporter assay.** Luciferase reporter assay was performed exactly as described elsewhere (14).

**Quantitation of BCL-2 mRNA by quantitative reverse transcription PCR.** Total RNA was extracted using PureLink Micro to Midi Total RNA Purification System (Invitrogen) and cDNA

was synthesized using Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Quantitative RT-PCR (qRT-PCR) was performed in 1X SYBR-Green Master Mix (IQ SYBR, BioRad) with BCL-2 oligonucleotide primers (forward 5'-TGGGATGCCTTTGTGGAAGT and reverse 5'-GAGACAGCCAGGAGAAATCAAAC). The  $\beta$ -actin internal control was analyzed by qRT-PCR as above using  $\beta$ -actin Internal Standards (Ambion, Austin, TX). The qRT-PCR reaction was performed in an iQ5Cycler (Bio-Rad) for 41 cycles. The Ct analysis for each reaction was performed using the iQ5Cycler software and BCL-2 mRNA levels were normalized to  $\beta$ -actin mRNA levels using the  $2^{-\Delta\Delta C_t}$  method.

**Suppression of BCL-2 expression.** Cells were transfected with BCL-2 siRNA SMART-pool or Nonspecific Negative Control Pool (Upstate Biotechnology) exactly as described elsewhere (16).

**Apoptosis assay:** Apoptosis was quantitated by measuring mono- and oligonucleosomes release using the Cell Death Detection ELISA PLUS Kit (Roche).

**Quantification of miR15a and miR16.** Total RNA was isolated using the MirVana PARIS RNA isolation system (Ambion) and cDNA from mature miR-15a and miR-16 was synthesized using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed using TaqMan MicroRNA Assay hsa-miR-15a or hsa-miR-16 probe sets (Applied Biosystems) and the TaqMan Universal PCR Master Mix (Applied Biosystems). For normalization, a  $\beta$ -actin qRT-PCR reaction was performed.



**Suppression of BCL-2 expression with pre-miR15a and pre-miR16.** Cells were transfected with 30 or 50 nM of miRNA Precursor Molecules Pre-miR non-specific control #2, hsa-miR15a, or hsa-miR-16 (Ambion) using Hyperfect Reagent (Qiagen). Cell lysates were analyzed by western blot or if growth assays were performed cells were transfected a second time and the MTT growth assay was performed.

**Inhibition of miR15a and miR16.** Cells were transfected with 50 or 100 nM of miRIDIAN miRNA inhibitor non-specific control #1, hsa-miR-15a, or hsa-mir16 (Dharmacon) using Hyperfect Reagent (Qiagen).

## **RESULTS AND DISCUSSION**

**HER2 $\Delta$ 16 expression promotes estrogen independence and tamoxifen resistance.** HER2 positive breast tumors coexpress the oncogenic HER2 isoform HER2 $\Delta$ 16 and nearly half of HER2 positive tumors are also ER $\alpha$  positive (17). To determine the impact of HER2 $\Delta$ 16 expression on ER $\alpha$  positive breast tumor cells we compared HER2 $\Delta$ 16 and wild-type HER2 in the ER $\alpha$  positive MCF-7 breast tumor cell line. Stable expression of HER2 $\Delta$ 16 resulted in reduced ER $\alpha$  levels; however, equivalent levels of ER $\alpha$  transcriptional activity and regulation was observed in each cell line (Supplementary Fig. S1A,B) suggesting that each cell line retains normal regulation of ER $\alpha$  function.

We next compared the ability of each cell line to form xenograft tumors. MCF-7/Vector xenografts were estrogen dependent and tamoxifen sensitive (Fig. 1A). Consistent with other reports, the MCF-7/HER2 xenografts were estrogen dependent and suppressed by tamoxifen (7,

8) (Fig. 1B). MCF-7/HER2 $\Delta$ 16 xenografts were estrogen responsive but estrogen independent. In addition, MCF-7/HER2 $\Delta$ 16 xenografts were tamoxifen resistant (Fig. 1C).

In an *in vitro* cell proliferation assay estrogen withdrawal or tamoxifen treatment significantly reduced MCF-7/Vector and MCF-7/HER2 cell growth with increased cell apoptosis (Fig. 1D and Supplementary Fig. S1C). In contrast, tamoxifen only marginally inhibited MCF-7/HER2 $\Delta$ 16 cells (Fig. 1D and Supplementary Fig. S1C). Taken together our results demonstrate that HER2 $\Delta$ 16 expression, but not wild-type HER2, renders ER $\alpha$  positive MCF-7 breast tumor cells estrogen independent and tamoxifen resistant. Furthermore, our results suggest that HER2 $\Delta$ 16 expressing MCF-7 xenografts more closely resemble the estrogen independent and tamoxifen resistant phenotype of aggressive HER2/ER $\alpha$  positive tumors observed clinically (4).

**Upregulation of BCL-2 in MCF-7/HER2 $\Delta$ 16 cells promotes tamoxifen resistance.** Tumor cell apoptosis contributes to cellular responses to tamoxifen (13, 18) and overexpression of anti-apoptotic BCL-2 promotes tamoxifen resistance (13). We therefore determined the influence of endogenous BCL-2 expression on tamoxifen activity. Consistent with previous observations, estrogen stimulated BCL-2 mRNA expression whereas tamoxifen suppressed BCL-2 expression to below basal levels (18) (Fig. 2A). Despite tamoxifen induced suppression of BCL-2 mRNA the MCF-7/HER2 $\Delta$ 16 cell line accumulated BCL-2 protein in response to tamoxifen (Fig. 2B and Supplementary Fig. S2A). A similar upregulation of BCL-2 protein was observed in MCF-7/HER2 $\Delta$ 16 cells treated with fulvestrant (ICI 182780) and during estrogen withdrawal (Supplementary Fig. S2B). These results suggest that HER2 $\Delta$ 16 upregulation of BCL-2 may occur in response to disengaged ER $\alpha$  signaling. Importantly, treatment of the MCF-7/HER2 $\Delta$ 16 cell line with BCL-2 targeting siRNA abolished tamoxifen induced BCL-2 expression and

sensitized MCF-7/HER2 $\Delta$ 16 cells to tamoxifen with increased apoptosis (Fig. 2C,D and Supplementary Fig. S2C). Likewise, MCF-7/HER2 $\Delta$ 16 cells were sensitized to tamoxifen when treated with the BCL-2 family inhibitor ABT-737 (19) (Supplementary Fig. S2D). Taken together these results indicate that HER2 $\Delta$ 16 promotes tamoxifen resistance through a unique mechanism involving tamoxifen induced upregulation of BCL-2 protein expression.

**Ectopic miR15a/16 expression sensitizes MCF-7/HER2 $\Delta$ 16 cells to tamoxifen.** We next explored the molecular mechanisms underlying altered BCL-2 expression in tamoxifen resistant cells. One possibility is the involvement of BCL-2 targeting miRNAs miR15a and miR16 (miR15a/16) (20). The levels of miR15a/16 were similar in tamoxifen sensitive cells (Fig. 3A). In contrast, the levels of miR15a/16 in the tamoxifen resistant MCF-7/HER2 $\Delta$ 16 cell line were reduced by 71% and 64%, respectively (Fig. 3A). A reduction in miR15a/16 levels by only 40% is sufficient to relieve repression of BCL-2 protein expression (20). Increasing the expression of miR15a/16 resulted in suppression of BCL-2 expression and sensitized MCF-7/HER2 $\Delta$ 16 cells to tamoxifen with increased apoptosis (Fig. 3B-D). Importantly, miR15a/16 expression was not significantly altered in response to estrogen or tamoxifen (Supplementary Fig. S3A). In addition, HER2 $\Delta$ 16 expression failed to impact miR15a/16 in the ER $\alpha$  negative MDAMB231 cell line which expressed lower levels of miR15a/16 when compared to MCF-7 cells and lacked tamoxifen induced expression of BCL-2 (Supplementary Fig. S3B,C).

**Suppressed miR15a/16 expression promotes tamoxifen resistance.** Pretreatment of MCF-7/Vector and MCF-7/HER2 cells with inhibitors of miR15a/16 resulted in enhanced BCL-2 expression, partial but significant tamoxifen resistance, and reduced apoptosis (Fig. 4A-C).

These results further implicate the BCL-2 targeting miR15a/16 as important regulators of tamoxifen response and suggest that tumor cell suppression of miR15a/16 may represent an important mechanism of tamoxifen resistance.

In summary, our results demonstrate that the HER2 oncogenic isoform, HER2 $\Delta$ 16, promotes estrogen independent growth and cooperates with BCL-2 to evade tamoxifen therapy. We further show that HER2 $\Delta$ 16 expressing cells upregulate BCL-2 expression in response to tamoxifen through a novel mechanism involving suppression of miR15a/16. The covert clinical influence of HER2 $\Delta$ 16 expression in HER2/ER $\alpha$  positive tumors may explain the inability of wild-type HER2 preclinical models to fully recapitulate the aggressive and variable clinical nature of HER2/ER $\alpha$  positive breast tumors (6-10). Breast tumor expression analysis of HER2 $\Delta$ 16 in combination with suppressed miR15a/16 expression may provide improved markers of tamoxifen resistance and novel targets for therapeutic intervention.

## **ACKNOWLEDGEMENTS**

The authors would like to thank June Allison for excellent lab management. This work was supported by US AMRMC grant W81XWH-08-1-0458 (DC). ABT737 was provided by Abbott Laboratories.

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## FIGURE LEGENDS

**Figure 1.** HER2 $\Delta$ 16 expression promotes estrogen independent and tamoxifen resistant growth. (A-C) Graphs representing xenograft tumor kinetics in nude mice injected with (A) MCF-7/Vector, (B) MCF-7/HER2, or (C) MCF-7/HER2 $\Delta$ 16 cells. With the exception of the -E2 treatments all mice were primed with E2 pellets and after 21 days mice with established tumors were left untreated or implanted with TAM pellets. (D) MCF-7/HER2 $\Delta$ 16 cells are E2 independent and TAM resistant *in vitro*. Each cell line was untreated or treated for five days with 100 pM E2 alone or in combination with 1.0  $\mu$ M TAM. MTT assay was used to quantitate cell growth. Results represent mean  $\pm$  SE percent growth inhibition relative to 100 pM E2 alone.

**Figure 2.** BCL-2 mediates tamoxifen resistance of MCF-7/HER2 $\Delta$ 16 cells. (A) Quantitation of estrogen induced BCL-2 mRNA expression. Each cell line treated for the indicated time with 100 pM E2 alone or in combination with 1.0  $\mu$ M TAM. Each sample was normalized to a  $\beta$ -actin internal control and BCL-2 mRNA expression is represented relative to the untreated MCF-7/Vector. (B) Western blot analysis of BCL-2 in estrogen and tamoxifen treated MCF-7 cells. Each cell line was treated as above and cell lysates were analyzed by western blot for BCL-2 expression. (C) Suppression of BCL-2 restores tamoxifen sensitivity. Each cell line was treated with non-specific control or BCL-2 RNAi. Each sample was treated with 100 pM E2 alone or in combination with 1.0  $\mu$ M TAM for 72 hrs. MTT assay was used to quantitate cell growth. Results represent mean  $\pm$  SE percent growth inhibition relative to 100 pM E2 alone. (D) Suppression of BCL-2 sensitizes MCF-7/HER2 $\Delta$ 16 cells to tamoxifen. Each cell line was treated as above and apoptosis was quantitated using a Cell Death Detection ELISA. Results represent

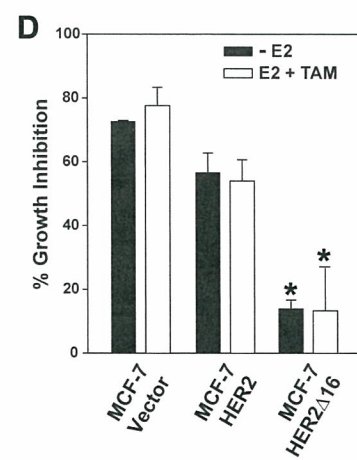
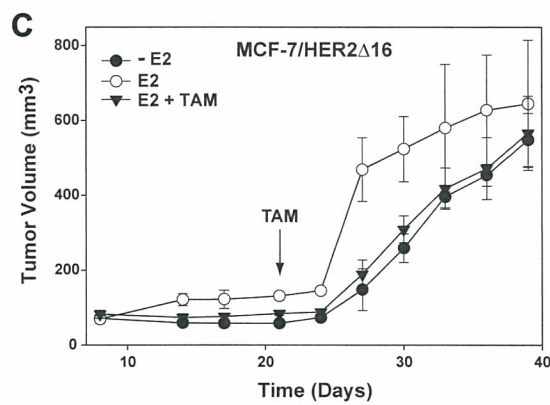
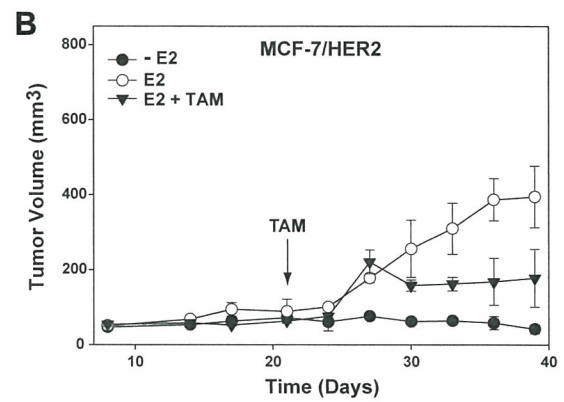
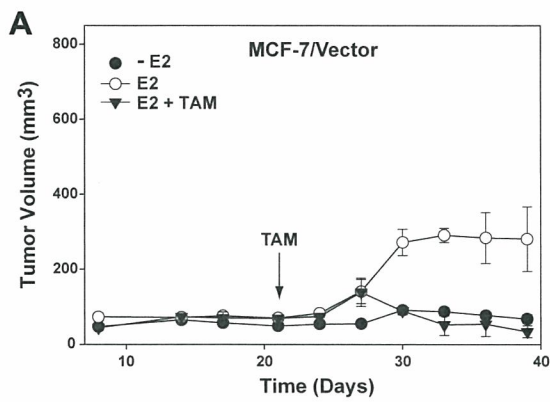


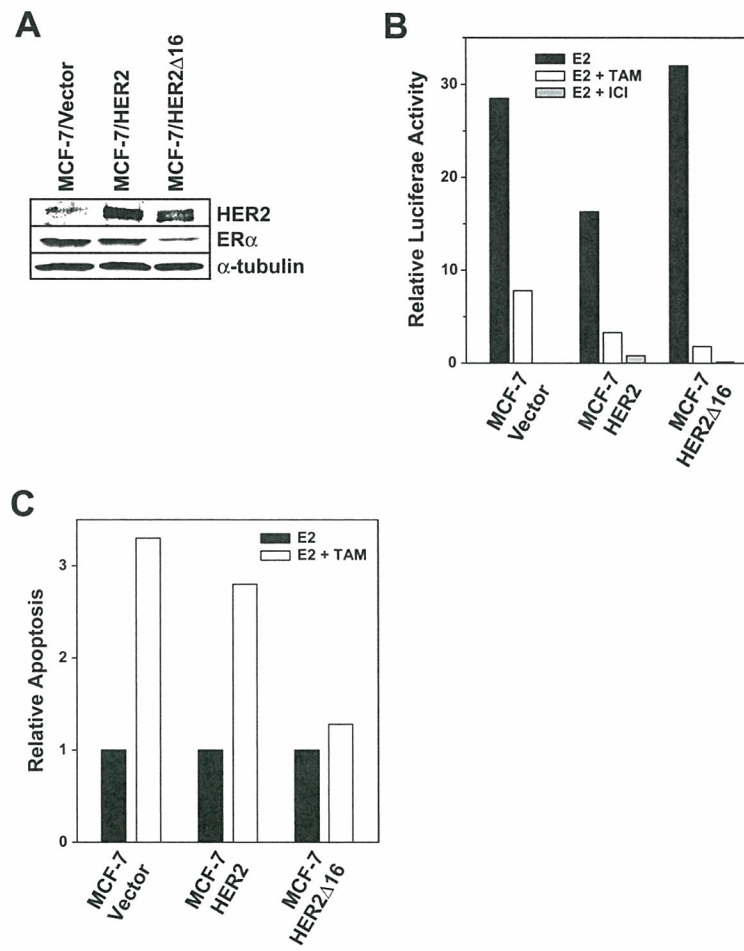
mean  $\pm$  SE apoptosis relative to MCF-7/Vector treated with 100 pM E2 alone. (C,D) Asterisks indicate samples with significant differences.

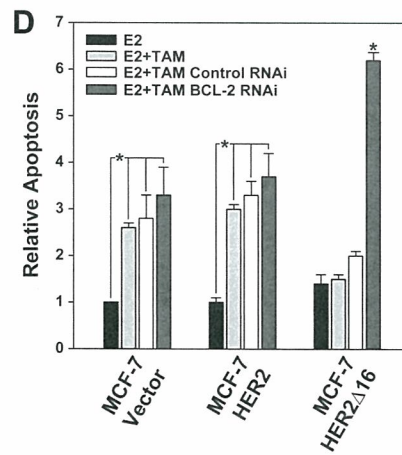
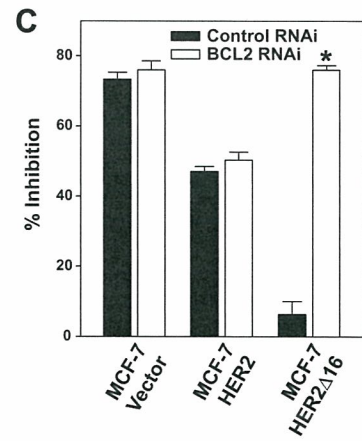
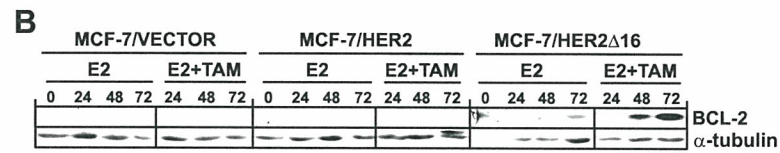
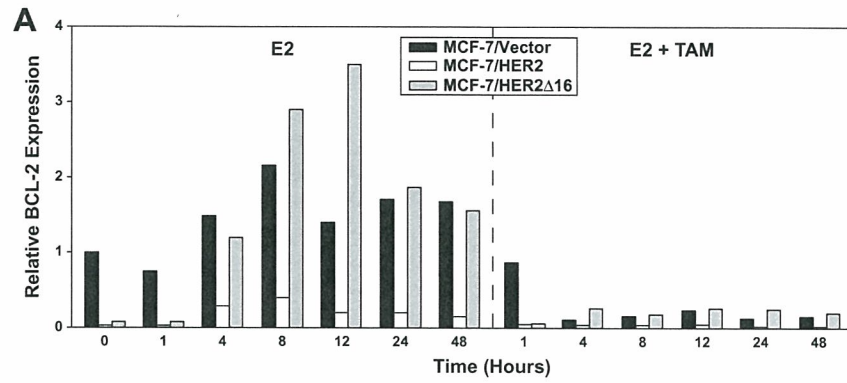
**Figure 3.** Expression of miR15a/16 sensitizes MCF-7/HER2 $\Delta$ 16 cells to tamoxifen. (A) Suppressed expression of miR15a/16 in MCF-7/HER2 $\Delta$ 16 cells. Total RNA was extracted and analyzed for miR15a/16 expression by qRT-PCR. Results are represented as mean  $\pm$  SE expression relative to  $\beta$ -actin. (B) pre-miR15a and/or pre-miR16 suppresses BCL-2 expression. The MCF-7/HER2 $\Delta$ 16 cell line was untreated or treated with 30 nM of the indicated pre-miR and treated with 100 pM E2 and 1.0  $\mu$ M TAM for 48 hrs. Cell lysates were analyzed by western blot and BCL-2 expression relative to the untreated control was quantitated by densitometry. (C,D) pre-miR15a and/or pre-miR16 sensitizes MCF-7/HER2 $\Delta$ 16 cells to tamoxifen. The MCF-7/HER2 $\Delta$ 16 cell line was transfected with pre-miRs as above, treated with 100 pM E2 alone or in combination with 1.0  $\mu$ M TAM for 72 hrs. MTT assay was used to quantitate cell growth or apoptosis was quantitated using a Cell Death Detection ELISA. Data represents mean  $\pm$  SE relative to mock and E2/TAM treated cells. Asterisks indicate samples with significant differences.

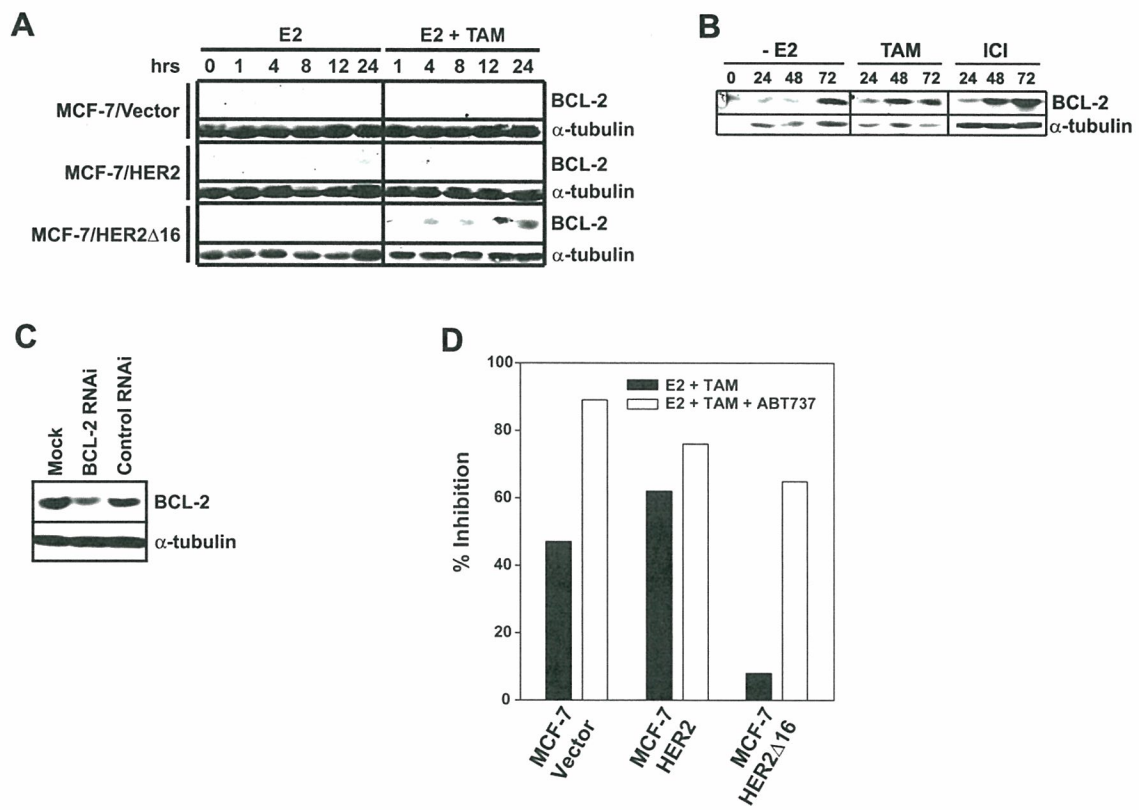
**Figure 4.** Suppressed miR15a/16 expression promotes tamoxifen resistance. (A) Inhibition of miR15a/16 results in enhanced BCL-2 expression. Each cell line was treated with 50 nM of the indicated miR inhibitor for 48 hrs and BCL-2 expression was analyzed by western blot. (B,C) Suppression of miR15a/16 promotes tamoxifen resistance. Each cell line was untreated or treated with the indicated anti-miR and treated with 100 pM E2 alone or in combination with 1.0  $\mu$ M TAM for five days. MTT assay was used to quantitate cell growth and apoptosis was quantitated

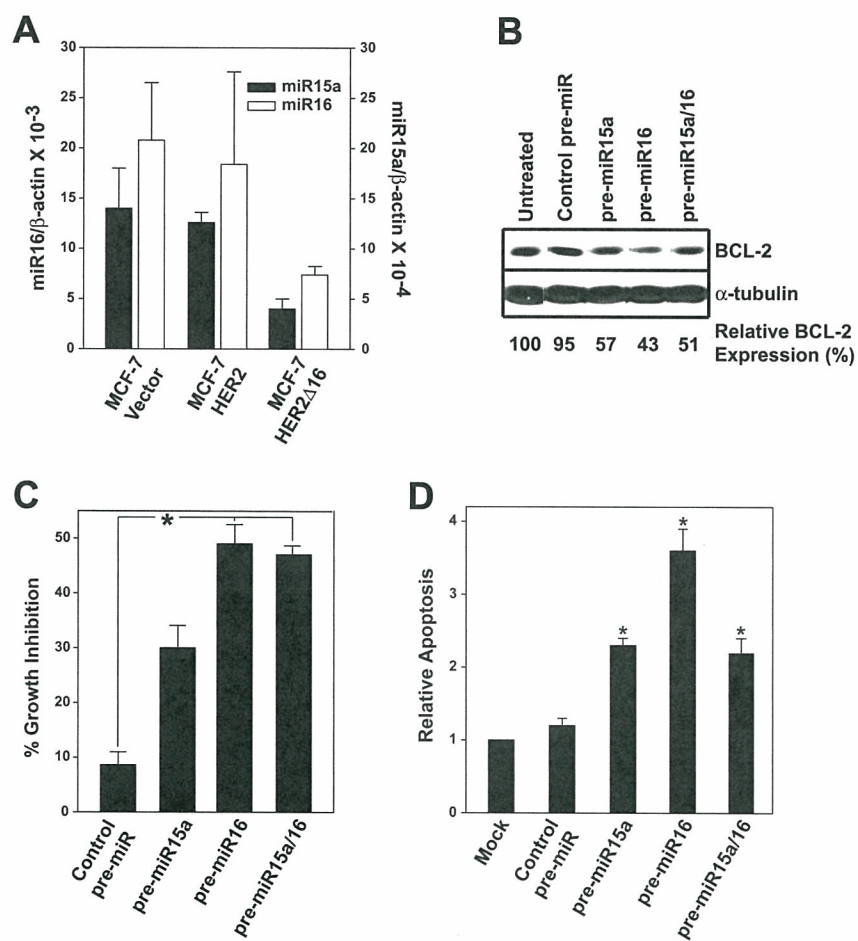
using a Cell Death Detection ELISA. Data is represented as mean  $\pm$  SE relative to mock anti-miR and E2/TAM treated MCF-7/Vector cells. Asterisks indicate samples with significant differences.

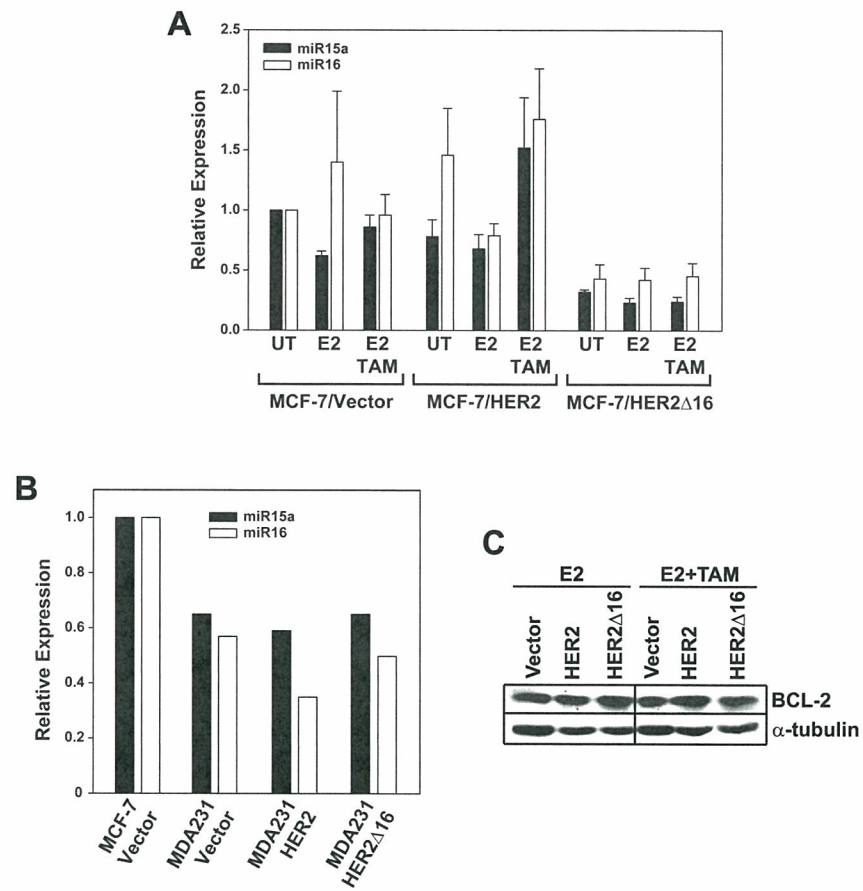




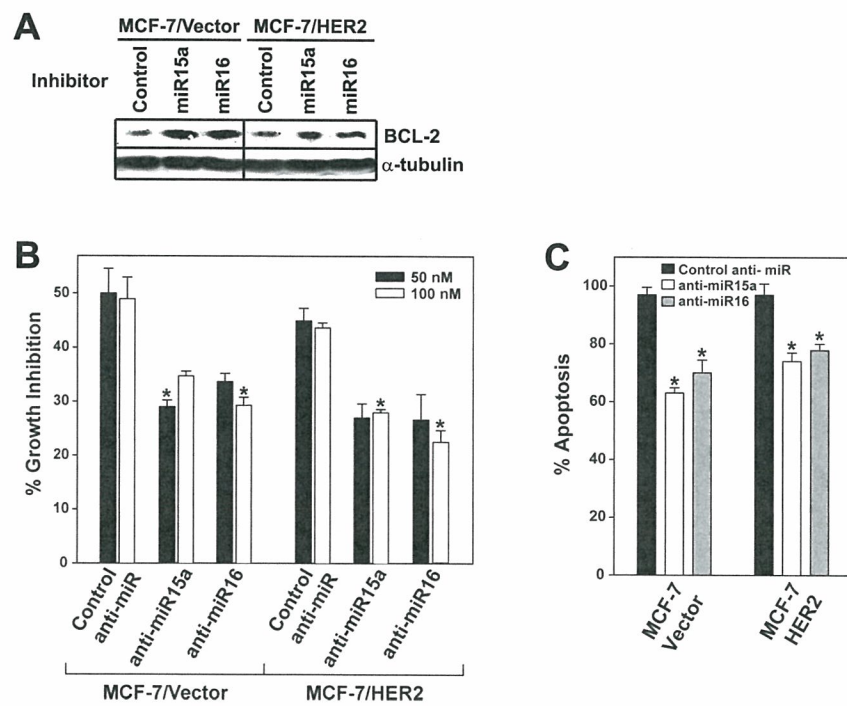












## Appendix 3

List of personnel paid with this research effort:

Diana Cittelly Ph.D 100% effort