ASSESSING THE ROLE OF MICRORNAS IN THE SUPPRESSION OF EPITHELIAL-MESENCHYMAL TRANSITION BY THE FRY TUMOR SUPPRESSOR GENE

by

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ABSTRACT OF THE THESIS

Assessing the Role of MicroRNAs in the Suppression of Epithelial-Mesenchymal Transition by the FRY Tumor Suppressor Gene

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The tumor suppressor gene, *Fry*, induces reversal of epithelial-mesenchymal transition (EMT) phenotype in the breast cancer cell line MDA-MB-231. Based on *in silico* predictions made by Ingenuity Pathway Analysis™ (IPA), we hypothesized that *FRY* and microRNAs interact and these interactions link *FRY* to EMT. In this study we attempted to identify microRNAs that are differentially expressed in breast cancer cells without (MDA-MB-231) and with (231wCFry) ectopic *Fry* expression. Using TaqMan™ MicroRNA Array Cards to screen of over 700 microRNAs, we identified 118 microRNAs that were differentially expressed between cells with and without *Fry* expression. Among the differentially expressed microRNAs, hsa-mir-25, hsa-mir-106b, and hsa-mir-203 were previously associated with EMT. We used IPA to build interaction networks between microRNAs, *FRY,* and two genes *(CACNAID,* and *NDR1),* whose expression levels were tightly correlated with the expression of FRY. This *in silico* approach suggested that hsa-mir-515 may play a role. However, our results indicated that the expression of has-mir-515 was not significantly changed by ectopic expression of *Fry* expression in MDA-MB-231 cells. We next chose to investigate hsa-mir-301b, whose

expression was known to be increased in certain cancers. Our results showed hsa-mir-301b expression is decreased 100-fold in MCF-10A and 231wCFry as compared the to the MDA-MB-231 cell line. This correlation suggested that hsa-mir-301b could play a role in tumorigencity of breast cancer, and ectopic expression of *Fry* may contribute to decreased hsa-mir-301b expression. The microRNA hsa-mir-4728-3p was found to share 83% alignment complementarity to the mRNA nucleotide region 4,561-4,583 of *Fry*. Expression of hsa-mir-4728-3p was decreased 100-fold in 231wCFry and increased in 2.5-fold in MCF-10A compared to MDA-MB-231 cells. These findings were unexpected given previous studies demonstrating a direct relationship between transcription of hsa-mir-4728-3p and *ERRB2*. Given that these cell lines do not express ERRB2 the observed increased expression of mir-4728-3p in MCF-10 cells and decrease in expression in 231wCFry compared to MDA-MB-231 was unexpected. The studies described in this thesis indicated ectopic expression of the *Fry* tumor suppressor gene in the triple negative MDA-MB-231 breast cancer cell line correlates with the expression of numerous microRNAs, including many previously linked to EMT and carcinogenesis.

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Chapter I INTRODUCTION

Breast cancer is the most common malignancy in women, accounting for 31% of all female cancers (Zheng et al., 2009). Several risk factors for breast cancer include: patient age, family history of breast cancer, early menarche, late menopause, prolonged hormone replacement therapy, and genetic mutations (Carlson et al., 2009). One approach to identifying mutated genes underlying differential susceptibility to breast cancer is through genetic linkage analysis. Genetic linkage analysis in high risk breast cancer families identified *BRCA1* and *BRCA2* as tumor suppressor genes (Claus et al., 1996). Germ line mutations in *BRCA1* and *BRCA*2 increase an individual's cancer risk of developing breast cancer usually at an earlier age (Claus et al., 1996).

A second approach in identifying cancer susceptibility genes is to perform linkage analysis in inbred animal models (Graham, 2011). In an example of this approach, our laboratory used F2 genetic backcross progeny of the resistant Copenhagen (Cop) and the susceptible Fischer 344 (F344) rat strains, to identify *Fry*, the mammalian ortholog of the *Drosophila melanogaster furry* gene, as a Cop mammary carcinoma susceptibility (*Mcs)* gene conferring resistance to N'-Methyl-N'-Nitrosourea (NMU)-induced mammary carcinogenesis (Graham, 2011). Our laboratory further showed that FRY protein expression was significantly decreased in breast cancer cell lines and breast cancers, particularly in poorly differentiated breast cancer phenotypes such as high grade and hormone receptor negative breast cancers, further suggesting a role for FRY in the progression of cancer (Graham, 2011).

The hormone receptor negative breast cancers include the highly aggressive triple-negative breast cancer. These cancers the lack the expression of estrogen receptors

(ERs), progesterone receptors (PRs), and hormone epidermal growth factor receptor type 2 (HER2) (Hiller and Quyen 2012). Triple-negative breast cancers are difficult to treat because there are no known endocrine targets nor are there specific receptors to target with existing therapeutic agents (Hiller and Quyen, 2012).

In addition to the observed decrease of FRY expression in aggressive hormone receptor negative breast cancers, the ectopic expression of the wild-type Cop *Fry* in a triple negative breast cancer cell line (MDA-MB-231) enhanced cell polarity, adhesion, and differentiation (Graham, 2011). These phenotypic changes to the opposite of changes observed during EMT, which is characterized by loss of cell adhesion, increased cell mobility and acquisition of invasive and metastatic phenotypes (Graham, 2011). These observations, coupled to the results of gene expression profiling of MDA-MB-231 cells with and without FRY expression, indicated that FRY expression can reverse or suppress EMT.

MicroRNAs are short, non-coding RNA transcripts known to play a role in many biological processes by directly interacting with specific mRNAs through molecular complexes, leading to mRNA degradation or inhibition of their translation into proteins. Binding is dependent upon nucleotide sequence complementarity of the microRNA to its target mRNAs, and the type of Argonaute (Ago) protein deposited on the mRNA target (Liu et al., 2008). In plants, the major mechanism of regulation is through microRNA cleavage of the target mRNA. In animals microRNA silencing is predominantly accomplished by repression of translation and/or destabilization of mRNA, without endonucleolytic cleavage (Liu et al., 2008).

MicroRNAs are shown to act as oncogenes or tumor suppressor genes and can contribute to carcinogenesis (Ilipoulos et al., 2009). MicroRNAs can inhibit the progression of the cell cycle and drive terminal differentiation. The down regulation of important developmental microRNAs may be important in the cascade or progression leading to cancer (O'day and Lai, 2010).

We hypothesized that FRY expression would also mediate its tumor suppressor effects and inhibit EMT, at least in part, by altered expression of microRNAs. *In silico* analysis of FRY signaling pathways using Ingenuity Pathway Analysis (IPA) software further suggested molecular interactions between the *Fry* gene and several microRNAs. We therefore sought to identify microRNAs whose expression is related to ectopic expression of *Fry* and may interact within the EMT pathway.

The first objective of this thesis is to identify differentially expressed microRNAs in MDA-MB-231 breast cancer cells with and without the ectopic expression of FRY. This was accomplished through a high throughput screen for differentially microRNAs TaqMan MicroRNA Array Cards. The second objective is to validate differential expression of candidate microRNAs in MDA-MB-231 breast cancer cells without and with ectopic *Fry* (231wCFry), and in non-tumorigenic MCF-10A cells using quantitative Real-Time PCR (qRT-PCR). For these microRNA candidates we used *in silico* prediction of microRNAs associated with FRY signaling networks using Ingenuity Pathway Analysis Software, and identification of microRNAs with sequence complementarity to the FRY mRNA.

The significance of this thesis work is that it has identified a series of microRNAs that are regulated by FRY expression, including some previously implicated in EMT and

carcinogenesis. The studies lay the foundation for future studies on the role of these microRNAs in FRY mediated suppression of carcinogenesis and provide the molecular basis for the development of novel diagnostic, prognostic, chemo preventive and/or therapeutic strategies for a wide variety of carcinomas. The discovery of specific microRNAs that are increased or decreased as a function of FRY expression could be used be used as a screening method for triple negative breast cancers, and may also suggest novel therapeutic strategies that express or target microRNAs in breast cancer cells.

Chapter II LITERATURE REVIEW

II.A. Current Perspective of Cancer in the United States

As of 2008, in the United States, there were approximately 12 million cases of invasive cancer recorded by the Surveillance Epidemiology and End Results (SEER) (American Cancer Society, 2012). In 2007, the total cost, including the direct medical and indirect mortality costs of cancer, was estimated to be $226.8 billion, with 1 in every 4 deaths in the United States being due to cancer (American Cancer Society, 2012). Cancer is a multistep process which acquires progressively more abnormal growth and differentiation phenotypes. Cancer begins as a result of genetic or epigenetic changes that lead to alterations in normal cell growth control. These initiated cells continue to grow and additional genetic and epigenetic alterations and eventually forms an abnormal mass, known as a tumor (American Cancer Society, 2012). As the tumor progresses it escapes normal growth regulatory mechanisms, eventually becomes malignant. Ten hallmark alterations in cell physiology may together control malignant growth and are common to most human tumors; these are self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, deregulating cellular energetics, avoiding immune destruction, tumor-promoting inflammation, and genome instability and mutation (Hanahan and Weinberg, 2011). Tumor cells are able to generate many of their own growth signals and can therefore reduce dependence on their microenvironments unlike normal cells that require mitogenic growth signals transmitted to them. These signals include diffusible growth factors, extracellular matrix components, and cell-to-cell adhesion/interaction molecules (Hanahan and Weinberg, 2000).

Heterotypic signaling between the diverse cell types within a tumor may be important in explaining tumor cell proliferation (Hanahan and Weinberg, 2000).

II.B. Introduction to Human Breast Cancer

Breast cancer is a collection of malignant diseases that displays distinct histopathological features, genetic and genomic variability, and diverse prognostic outcomes (Creigton et al., 2010). In 2011, it is estimated that 230,480 new cases of invasive breast cancer will be diagnosed in women in the United States (American Cancer Society, 2012). Breast cancer may either start in the duct or lobules of the breast, though a small percentage may begin in other tissues (American Cancer Society, 2012). The two key molecular signatures, progesterone receptor (PR) and HER2 are fundamental in outlining of classification and treatments (Volinia et al., 2012). The frequently described stepwise model of breast tumorigenesis assumes a gradual transition from epithelial hyper proliferation to ductal carcinoma *in situ* (DCIS) and eventually to invasive ductal carcinoma (IDC) (Volinia et al., 2012). This progression model is supported by clinical and epidemiological data and by molecular clonality studies (Volinia et al., 2012). However, molecular genetic analyses have suggested a second model which poses a complex series of events leading to distinct and divergent pathways in the development of invasive carcinoma. This model suggests that progression between low grade ductal and lobular carcinomas is indistinct, and that molecular parameters are better able to predict tumor behavior (Simpson et al., 2005).

II.C. Prevention and Treatment Options for Breast Cancer

Currently, screening for early breast cancer is accomplished through mammography. This diagnostic test has over 95% specificity, but the sensitivity range is

between 67% - 95% and is dependent upon factors such as age, breast density, and the professional experience of the examiner (Britton et al., 2012). Other options include magnetic resonance imaging (MRI) of the breast and breast ultrasound to improve cancer detection, although these techniques are not routinely used for breast cancer screening (Britton et al., 2012). Triple-negative breast cancer, also known as “basal subtype” is an aggressive malignancy that is not responsive to current molecularly targeted therapies because the tumor cells lack ER, PR, and HER2 as therapeutic targets (Volinia et al., 2012). As a result, treatment options are primarily limited to surgery and chemotherapy (Blick et al., 2010). As stated in Blick et al. the recurrence in ER-negative (ER^-) tumors has two hazard peaks, the first in the initial two years after diagnosis and second at approximately five years (Blick et al., 2010). For ER-positive (ER^+) tumors the hazard of recurrences continues at a steady rate for many years (Blick et al., 2010).

Resistance of cancer cells to chemotherapy continues to be a major clinical obstacle to the successful treatment of cancer (Zheng et al., 2009). This resistance is considered to be multifactorial phenomenon involving several major mechanisms, such as decreased uptake of water-soluble drugs, increased repair of DNA damage, reduced apoptosis, altered metabolism of drugs, and increased energy-dependent efflux of chemotherapeutic drugs that diminish the ability of cytotoxic agents to kill cancer cells (Zheng et al., 2009). Failure of treatments could be due to residual tumor cells not eliminated by initial therapy and they may develop acquired resistance (Creigton et al., 2010). The resistance could also be due to a subpopulation of cells, known as tumor initiating cells (TICs), which within tumors show characteristics of stem cells and possess an intrinsic resistance to endocrine and chemotherapy (Creigton et al., 2010).

Clinical evidence shows that TICs become enriched in breast cancer patients after common treatments, indicating there is intrinsic therapeutic resistance of the tumorigenic subpopulation of cells (Creigton et al., 2010). Gene expression profiling studies revealed an association found between TICs and mesenchymal claudin-low subtype, suggesting that epithelial-mesenchymal transition may be involved in regeneration of tumors after initial therapy (Creigton et al., 2010). Thus novel approaches targeting EMT could reduce the inherent resistance of tumors to chemotherapy.

II.D. Epithelial-Mesenchymal Transition

Epithelial-Mesenchymal Transition (EMT) is a process by which cells acquire a stem-like quality, which facilitates dysfunctional cell-cell adhesive interaction, cell-matrix adhesion and junctions (Creigton et al., 2010). EMT is a multi-step process that reorganizes the cytoskeleton, resulting in a loss of apical polarity and the acquisition of spindle-shaped mesenchymal cell morphology (Creigton et al., 2010). In normal epithelial cells junctional complexes function together to prevent cell motility due to the cells being tightly connected to each other by intercellular junctions composed of tight junction, adherens junction, desmosome, and gap junction (Lee and Shen, 2012). In EMT, there is a suppression of epithelial adhesion junctions, gain of mesenchymal markers, cytoskeleton reorganization, increased cellular migration and invasiveness, and resistance to anoikis (Lee and Shen, 2012). Molecularly, EMT is associated with loss of epithelial markers, such as E-cadherin and β-catenin; *de novo* expression of mesenchymal-related proteins: N-cadherin, fibronectin, and the mesenchymal intermediate filament vimentin (Lee and Shen, 2012). EMT is also characterized by

cytoskeleton rearrangement mediated by Rho small GTPases up-regulation and nuclear translocation of transcription factors (Lee and Shen, 2012).

EMT has an important role in morphogenetic events spanning embryo implantation to heart valve formation (Blick et al., 2010). EMT also plays a role in homeostatic mechanisms governing tissue regeneration, inflammation, and wound healing (Blick et al., 2010). Normally, EMT is followed by the reverse process mesenchymal-epithelial transition (MET) to produce structures such as kidney, gastrointestinal tract, lung, and skin (Blick et al., 2010). The three types of EMT that may exist in physiological and pathological conditions are 1) part of the developmental processes, 2) involved in inflammation, tissue remodeling, wound healing, fibrosis, and 3) tumor invasion and metastasis (Lee and Shen, 2012). Additionally, incomplete EMT in an epithelial cell may generate a hybrid metastable cell, which contains both epithelial and mesenchymal traits (Lee and Shen, 2012).

II.E. Implications of EMT in Cancer

EMT may facilitate migration and invasion of cancer cells into the surrounding microenvironment (Creigton et al., 2010). Studies have shown EMT to be rare in primary tumors, but it has been implicated in the invasion-metastases cascade (Creigton et al., 2010). EMT also contributes to disease states, such as fibrosis and cancer, and is thought to contribute to the resistance to treatment via its anti-apoptotic effects (Blick et al., 2010). In epithelial cancers, EMT is characterized by a switch in cell membrane cadherins, change from apical-basal to front-back polarity, and acquiring motility by the restriction of the actin cytoskeleton (Blick et al., 2010). Epithelial carcinoma cells from benign tumors showing altered epithelial polarity and can adopt single cell mesenchymal-

type movement to dissociate from primary tumor sites (Lee and Shen, 2012). This phenotypic conversion may enable tumor cells to dissociate from their original tissue and form metastases in secondary sites, which benefits carcinoma cells for phenotypic plasticity and malignant cancer progression (Lee and Shen, 2012).

II.F. Overview of MicroRNAs

MicroRNAs, synonymous with miRNAs and miRs are small non-coding RNAs that are typically 19-24 nucleotides long. They act as repressive regulatory agents by primarily binding to target mRNA 3' untranslated region (UTR) causing it to be stored or destroyed by DICER mediated mechanisms (Schrauder et al., 2011). MicroRNAs were first described in 1993 by Lee et al. in *C. elegans* (Schrauder et al., 2011). Currently, miRBase release 18 (November 2011) database includes a total of 18,226 microRNAs, with 1,527 of those being from *Homo sapiens*. MicroRNAs are encoded within all parts of the human genome including: exons, intergenic regions, and introns (Schmittgen, 2008).

II.G. Processing of MicroRNAs

The majority of microRNA genes are transcribed by RNA polymerase II, as primary microRNA transcripts (pri-microRNAs), and contain a hairpin structure from which the mature microRNA is processed (Schmittgen, 2008). The pri-microRNA transcripts are similar to mRNA in that they are polyadenylated, contain a 5' cap structure and may undergo splicing (Schmittgen, 2008). In the nucleus pri-microRNA are processed into pre-microRNA by a complex that includes the RNase III enzyme Drosha and double stranded RNA binding domain protein DGCR8, which creates a 3' overhang (Schmittgen, 2008). The pre-microRNAs are transported to the cytoplasm by Exportin 5,

where they are loaded onto a complex that includes RNase II enzyme Dicer and TRBP/Loquacious (Schmittgen, 2008). This ribonucleoprotein complex is responsible for cleaving the hairpin loop from the pre-microRNA to from a double-stranded complex containing the microRNA and microRNA* (star) sequence (Schmittgen, 2008). The star strand is typically degraded. The mature microRNA strand, along with the Argonaute protein 2, is assembled into a ribonucleoprotein complex known as RISC (RNA-induced silencing complex) (Schmittgen, 2008). However, in some cases microRNAs may be derived from both arms of the microRNA precursor such as, mir-30a and mir-30a* (Lee et al., 2010). The RISC complex scans cellular mRNA to locate the microRNA's target sequences to be able to hybridize to the microRNA. Binding can be through either perfect or imperfect complementarity to conserved sequences within the target untranslated region (UTR) (Schmittgen, 2008). Depending on the concordance between microRNA sequence and mRNA the negative regulatory effect can range from weak repression of protein translation to complete cleavage of the mRNA (Schrauder et al., 2011).

II.H. Function of MicroRNAs

Initial studies indicated that the main function of microRNAs was to suppress mRNA translation (O'Day and Lai, 2010). However, recent studies have revealed that changes in protein expression mediated by microRNAs are usually associated with altered mRNA expression as well (O'Day and Lai, 2010). Furthermore, recent findings have shown that microRNAs not only negatively regulate gene expression by 3'UTR base-pairing, but can in some cases up-regulate the translation of their target mRNAs (O'Day and Lai, 2010). The up-regulation of translation is found in quiescent cells,

which exit the cell cycle due to several stimuli such as contact inhibition, loss of adhesion, or serum starvation (Vasudevan et al., 2007). Key components, Ago 2 and fragile-X mental retardation related protein 1 (FXR1), need to be associated with (A+U)-rich elements in the 3' UTR of TNF alpha mRNA for translational up-regulation during serum starvation (Vasudevan et al., 2007). MicroRNA 369-3 is required by direct base pairing to the target mRNA for translation up-regulation of these key components after serum starvation (Vasudevan et al., 2007). One-third of all protein-coding genes are susceptible to microRNA regulation, and a single protein transcript can be targeted by multiple microRNAs. As a result microRNAs may regulate multiple proteins within the same signaling pathway (O'Day and Lai, 2010).

II.I MicroRNAs Role in Cancer

Three seminal observations implicated microRNAs as having a role in cancer. First, the earliest microRNAs discovered, including *let-7*, were shown to regulate cellular proliferation and apoptosis in *C. elegans* and *Drosophila* by binding to complementary target mRNAs (O'Day and Lai, 2010; Brennecke et al., 2003). Second, many microRNAs map to genomic areas that become amplified or deleted in various human cancers (O'Day and Lai, 2010). Third, tumor cell lines and malignant tumors show widespread deregulation of microRNA expression (O'Day and Lai, 2010). The deregulation of microRNAs may be caused by chromosomal deletions, epigenetic silencing through inactivation by hypermethylation or loss of miRNA binding sites in target gene (Sassen et al., 2008). These observations suggested that microRNAs as could be novel targets for cancer therapy (O'Day and Lai, 2010). An example of possible novel targets is the miR-200 family, whose changes in expression level have been associated with enhanced

tumorigenesis (O'Day and Lai, 2010). The region of chromosome 1 that encodes miR-200 cluster was also found to be amplified in epithelial cancers including ovarian, breast, and melanoma cancer (O'Day and Lai, 2010).

Let-7, one of the first microRNA families discovered, is frequently deleted in human cancers and overexpression of this family leads to reduction of tumors in the K-ras murine lung cancer model (Ilipoulos et al., 2009). The growth reduction was observed in tumor xenografts, of both murine and human non-small cell lung tumors when overexpression of let-7g was induced from lentiviral vectors (Kumar et al., 2007). Chromosomal regions showing significant enrichment for microRNAs are commonly amplified or deleted in human cancers, which suggested microRNAs may act as tumor suppressors (suppressor-miRs), promoters of tumorigenesis (onco-miRs) or both (Schrauder et al., 2011).

Since the original identification of microRNAs in serum of cancer patients, numerous studies have confirmed that microRNAs are present in blood, saliva, pleural fluid and urine (Schrauder et al., 2011). Chen et al. detected all microRNAs in the serum of human subjects using the Solexa™ deep sequencing platform (San Diego, CA). They then compared specific expression patterns of serum microRNAs in lung cancer, colorectal cancer, and diabetes patients to provide evidence that serum miRNAs contain fingerprints for various diseases (Chen et al., 2008). These circulating microRNAs can be used as potential non-invasive biomarkers for breast cancer and other diseases. Subsequent studies associated microRNA levels in extracellular fluids with relapse-free survival, overall survival, and response to therapy (Schrauder et al., 2011). The unusual stability of circulating microRNAs in the RNase-rich environment of the bloodstream is

the basis of their values as biomarkers. Initial studies indicated that these microRNAs are protected from degradation by inclusion in lipid or lipoprotein complexes, such as microvesicles, exosomes, or apoptotic bodies (Schrauder et al., 2011).

II.J. MicroRNAs and EMT

MicroRNAs are known to play a role in preventing EMT. The endogenous inhibition of the miR-200 family has been shown to induce EMT by repressing the expression of repression of E-cadherin by ZEB1 and ZEB2 (Korpol and Kang 2008). In mesenchymal-like ovarian cancer cells, the over-expression of miR-429, which belongs to the miR-200 family, will lead to the reversal of mesenchymal phenotype (Zhao et al., 2011). From this observation, it was shown that the epithelial phenotype is under the regulation of miR-200 family, whose expression induces the ‘mesenchymal−epithelial transition phenotype (Zhao et al., 2011).

In human fetal pancreatic epithelial cells, the miR-30 family closely relates to EMT. Depleting the expression of miR-30 family will lead the epithelial phenotype in transition to mesenchymal phenotype, whereas the over-expression of miR-30 family will keep the epithelial phenotype maintenance (Zhao et al., 2011). Further research has shown that the mesenchymal mRNAs, such as vimentin and snail-1, whose translations can be inhibited by miR-30 family, are both targets of the miR-30 family (Zhao et al., 2011).

The microRNA hsa-mir-9 is a MYC/MYCN activated miRNA, whose expression is up-regulated in breast cancer (Ma et al., 2010). Cadherin-1 (CDH1), the target of miR-9, is an E-cadherin-encoded messenger RNA, which could increase cell mobility and invasiveness capabilities (Ma et al., 2010). Hsa-mir-9 mediates down-regulation of the E-

cadherin level, which activates the β-catenin signaling pathway, and further up-regulates the expression level of VEGF in order to induce tumor angiogenesis (Zhao et al., 2011). This indicates that hsa-mir-9 acts as an initial tumor miRNA, involved in the regulation of the signaling transduction pathway and directly controls the expression level of E-cadherin, a key protein in tumor metastasis suppression (Zhao et al., 2011). Hsa-miR-9 could also induce tumor angiogenesis, EMT and the formation of micro-metastasis (Zhao et al., 2011).

II.K. Overview of the *Furry* Gene

In a 2001 paper by Cong et al., *furry* was identified as a new autonomously acting gene, whose mutation resulted in a *tricornered*-like phenotype in the bristles, hairs, and laterals of *Drosophila melanogaster*. The *furry* gene was cloned and characterized using a P insertion allele, RT-PCR, and mutant gene sequencing. The cDNA clone analysis concluded that it was a gene encoding two proteins, including a full length protein of 3479 amino acids and a truncated protein of 1629 amino acids (Cong et al., 2001). Currently, there is no known function of the truncated protein because present studies have only focused on the full length protein.

Furry is a member of a conserved family of proteins found in all eukaryotes including yeast, *C. elegans*, *A. thaliana*, and humans (Cong et al., 2001). The *tricornered* (*trc*) gene encodes the *Drosophila* ortholog of the human NDR kinases and mutations within this gene are recessive lethal in the fly (Fang et al., 2010). *Furry* interacts directly with the C-terminal region of the TRC protein in *Drosophila*, suggesting that NDR kinase family proteins may also interact with the FRY protein *in vivo* (Fang et al., 2010). *Furry* moves distally and proximally in developing bristles and dendrites of neurons, suggesting

involvement in intracellular transport that regulates polarized growth (Fang et al., 2010). One study indicated that in mammalian cells, *FRY* binds to microtubules, further implicating the FRY protein in the process of intracellular transport (Fang et al., 2010). Recently our laboratory demonstrated that ectopic expression of wild type *Fry* leads to the reversal of the EMT phenotype in breast cancer cells lacking *FRY* expression (Graham, 2011).

II.L. Identification of the *Fry* Gene as a Putative *Mcs* Locus

An alternative to genetic linkage analysis in high risk families is to identify cancer susceptibility genes by inbred animal models (Graham, 2011). Genetic linkage studies performed in our laboratory identified several mammary carcinoma susceptibility (*Mcs*) loci in differentially susceptible rat strains (Graham, 2011).

Previous segregation analyses indicated that susceptibility to mammary carcinogenesis is a polygenic trait and it was observed that a cross between the resistant rat strain Copenhagen (Cop) and intermediately-sensitive Fisher 344 (F344) had minimal number of modifiers relative to other crosses in their susceptibility to induced mammary carcinomas (Gould, 1986; Gould and Zhang 1991). Since a low number of minimal number of genetic modifiers can greatly simplify genetic linkage studies of polygenic traits, our laboratory performed genetic linkage studies in ((F344 X Cop) F1 X (F344)) N2 backcross progeny to identify loci that conferred susceptibility to N-Methyl-N-Nitrosourea (NMU)-induced mammary carcinogenesis in the F344 rat (Graham, 2011). The linkage analysis mapped a previously unreported *Mcs* locus to the long arm of rat chromosome 12 (Graham, 2011). Analysis of DNA sequences and expression levels of several known candidate genes within the rat locus ruled out several candidate tumor

suppressor genes within the locus, including several known to be involved in various cancers. Eventually, this candidate gene approach led to the identification of two non-synonymous mutations in a hypothetical F344 rat gene (NCBI Accession XM_221867). This predicted gene encodes the mammalian ortholog of the Drosophila *furry* gene (*Fry*) (Graham, 2011). This gene was also highly similar to the hypothetical gene CG003 in humans (NCBI Accession NM_023037). The mammalian *Fry* is highly conserved among eutherian mammals, with 89% similarity between rats and humans at the amino acid level (Graham, 2011).

Alignment of *Fry* gene orthologs from a variety of species detected two F344-specific SNPs within regions that are highly conserved during evolution (Graham, 2011). Consistent with the possibility that the F344 rat harbors mutant *Fry* alleles, the non-synonymous SNPs affected amino acid residues that were conserved in all species tested except yeast, suggesting that they impact FRY function (Graham, 2011). The first of these changes (codon 661) substitutes an aspartic acid (D) residue in the FRY protein of Cop rat strain and with glutamic acid (E) in the FRY protein of F344 rat strain. The SNP in codon 2170 has a more profound effect, substituting a nonpolar alanine (A) in the FRY protein of Cop rat strain and with a polar serine (S) residue in FRY protein of the F344 rat strain (Graham, 2011). Given FRY's previous role in regulation of serine/threonine protein kinases in *Drosophila* and yeast, our lab sought to determine if the mutation of an alanine to a serine residue at codon 2170 created a *de novo* phosphorylation site in the mutant FRY protein in F344 rats (Graham, 2011). *In silico* analysis using prediction algorithms confirmed that the alanine to serine mutation creates a peptide sequence that has a 98.6% probability of being a substrate for several protein kinases implicated in

carcinogenesis (Graham, 2011). The same *de novo* phosphorylation site would be created by this mutation in the human FRY sequence, although as of yet, the occurrence of this SNP is not specified in the NCBI dbSNP (Graham, 2011). Protein sequence analyses identified a putative ATP-binding domain common to GMP kinases which was highly conserved across FRY proteins, further suggesting that FRY protein may have ATP-binding and kinase activities (Graham, 2011). Together these observations strongly suggested that the SNPs present in the F344 allele will alter the function and/or regulation of the FRY protein in this strain.

II.M. Decrease of FRY Expression in Human Breast Cancer

Analysis of available cancer profiling data for breast cancer in the Oncomine 3.0 database showed that *Fry* gene expression was significantly reduced in human breast cancer as compared to normal mammary tissue cohorts (Graham, 2011). An anti-FRY antibody which recognizes a peptide sequence that is conserved between the human and rat proteins was developed by the Zarbl laboratory (patent pending) (Graham, 2011). This antibody allowed for evaluation of FRY protein expression in cores of tissue microarrays (TMAs) comprising normal breast and tumor tissue tumor by immunohistochemical staining. Quantitative image analysis indicated that FRY protein expression was consistently decreased in poorly differentiated human breast tumors relative to well-differentiated human breast tumor tissue (Graham, 2011). Analysis of *FRY* expression in breast cancer cohorts, confirmed that *FRY* was consistently decreased in the triple negative breast cancer phenotype relative to its expression in cancers with other receptor statuses (Graham, 2011). This observation suggests that decreased *FRY* is associated with the aggressive triple negative mammary tumor phenotype (Graham, 2011).

II.N. Role of FRY in Suppressing Tumorigencity

The decreased expression of FRY in breast cancer cell lines was consistent with the hypothesis that FRY activity also suppresses human mammary carcinogenesis. To test this hypothesis, the wild-type Cop *Fry* allele was ectopically expressed in the triple negative human MDA-MB-231 breast cancer cell line. In contrast to the highly undifferentiated parental cell line, clones expressing the wild-type Cop Fry allele exhibited an organized, differentiated epithelial like, cobblestone growth pattern in monolayer cultures (Graham, 2011). Moreover, unlike parental MDA-MB-231 cells, which showed a disorganized and undifferentiated spindle-like growth pattern in 3D Matrigel™, clones expressing ectopic FRY formed a differentiated, polarized mammospheres in 3D Matrigel™ cultures (Graham, 2011). This finding suggested that the Cop *Fry* allele restored a more normal, differentiated phenotype to the cancer cells *in vitro* (Graham, 2011). Ectopic expression of wild-type Cop *Fry* cDNA also reduced the tumorigencity of MDA-MB-231 breast cancer cells in nude mice, resulting in an 8-fold reduction in tumor volume compared to vector controls 15 days after injection (Graham, 2011). Moreover, in contrast to the MDA-MB-231 cells, the MDA-MB-231 with ectopic FRY cells did not invade into the underlying skeletal muscle and/or fat tissue (Graham, 2011). These findings suggest that expression of the Cop *Fry* gene in human breast cancer cells suppress *in vivo* tumorigencity by inducing a more differentiated epithelial cell phenotype (Graham, 2011).

Chapter III HYPOTHESIS AND OBJECTIVES

III.A. Hypothesis

Reversal of EMT is observed in ectopically expressing *Fry* MDA-MB-231 cells, and *in silico* predictions made by Ingenuity Pathway Analysis™ (IPA) revealed numerous one-step connections between FRY, EMT, and microRNAs. Given the established role of microRNAs in EMT and carcinogenesis, we hypothesized dysregulated expression of microRNAs correlated with the decreased expression of FRY may lead to an EMT phenotype.

III.B. Objectives

1. To identify differentially expressed microRNAs in MDA-MB-231 cells with and without ectopic *Fry* expression. We did this though a comprehensive screen of over 700 microRNAs using TaqMan™ MicroRNA Array cards. A total of 118 microRNAs were found to be differentially expressed compared to the MDA-MB-231 cell line. The three candidates hsa-mir-106b, hsa-mir-25, and hsa-mir-203 have previous links to EMT and thus are the best candidates for elucidating FRY, EMT, and microRNA interactions.

2. To validate differential expression levels of several candidate microRNAs in MDA-MB-231 cells with and without ectopic *Fry* expression. Differential expression of hsa-mir-515-3p, which was predicted to play a role in FRY signaling by IPA, could not be confirmed experimentally. We found that expression of hsa-mir-4728-3p, which was identified by sequence complementarity to the FRY mRNA, was decreased in 231wCFry and increased in MCF-10A compared to MDA-MB-231. Lastly, we found the expression of hsa-mir-301b to be decreased in the non-tumorigenic 231wCFry and MCF-10A cell lines as compared to tumorigenic MDA-MB-231 breast cancer cells.

Chapter IV MATERIALS AND METHODS

IV.A. Reagents and Cell Culture

MDA-MBA-231, 231wCFry, and 231wCVector cells were cultured in Advanced DMEM containing 10% heat inactivated fetal bovine serum (FBS) and 100 ug/ml penicillin-streptomycin solution. Mammary Epithelium Basal Medium (MEBM) was supplemented with MEGM *SingleQuots* (Lonza Basel, Switzerland) for the culture of MCF-10A cells. All cell cultures were maintained at 37ºC in an incubated atmosphere of 5% CO_2 and 95% air. MDA-MBA-231 cells and MCF-10A cells were obtained from American Type Culture Collection (ATCC Manassas, Virginia). 231wCFry and 231wCVector were transfectant cell lines previously created in our lab.

IV.B. Total RNA Extraction

The total RNA was extracted from cell culture samples using mirVana microRNA Isolation Kit and protocol (Ambion Austin, TX). To begin, the culture medium was aspirated and the cells were rinsed with 1X phosphate buffered saline (PBS). The cells were trypsinized, pelted, supernatant removed, and then re-suspended in 1 mL of 1X PBS at a density of 10^6-10^7 per ml. The cells were disrupted in 400 µl lysis/binding buffer. A 40µl aliquot of the microRNA homogenate additive was added and the cells were left on ice for 10 minutes. Next 400 µl Acid-Phenol: Chloroform was added and the mixture was vortexed until a milky colored solution formed. The emulsion was then centrifuged at 10,000 rpm for five minutes at room temperature. The aqueous phase was transferred to fresh tube and 475 µl 100% EtOH was added. This mixture was pipetted onto a filter cartridge and centrifuged for 15 seconds at 10,000 rpm. The flow through was discarded and 700 µl of wash solution 1 was added and the filter cartridge was again centrifuged.

Next 500 µl of wash solution 2/3 was added and centrifuged. The filter cartridge was transferred to a new collection tube and 100 µl pre-heated ($95^{O}C$) elution solution was used to elute the RNA from the cartridge.

IV.C. RNA Integrity

The integrity of RNA samples was determined using the Agilent RNA 6000 Nano Assay (Agilent Waldbroon, Germany). The concentration of RNA in each sample was between 100ng/ul-200ng/µl, measured using NanoDrop spectrophotometer obtained from Thermo Scientific (Wilmington, DE). Briefly, samples were inserted into the thermocycler and incubated at $70^{O}C$ for two minutes and then put on ice for two minutes. The samples were added to the RNA chip containing gel/dye mix and the RNA 6000 Nano Marker. The chip is then placed into the Bioanalyzer (Agilent Waldbroon, Germany).

IV.D. TaqMan MicroRNA Array Cards

Two sets of arrays were purchased from ABI; the first was TaqMan Human MicroRNA Array Card A, which contained well studied microRNAs and the second was TaqMan Human MicroRNA Array Card B, which contained newly identified microRNAs (Life Technologies Carlsbad, CA). The purified RNA was converted into cDNA using Megaplex pools provided by ABI (Life Technologies Carlsbad, CA). For the reverse transcriptase (RT) reaction, the following reagents were combined in a microcentrifuge tube: Megaplex RT primers (10X), dNTPs with dTTP (100mM), MultiScribe Reverse Transcriptase (50 U/µl), 10X RT Buffer, $MgCl_2$ (25mM), RNase Inhibitor (20 U/µl), and Nuclease-free water. The tube was inverted to mix the contents and then centrifuged briefly. A 4.5µl aliquot of the RT reaction was combined with 3µl

of total RNA (MDA-MB-231, 231wCFry, 231wCVector, and MCF-10A) in MicroAMP tubes. The tubes were inverted to mix, spun briefly and then incubated on ice for five minutes. The Megaplex reverse transcription was performed at maximum ramp speed using GeneAmp 9700 PCR System (Life Technologies Carlsbad, CA) using the following thermal-cycling conditions: 40 cycles (16°C 2min, 42°C 1 min, 50°C 1 sec), Hold (85°C 5 min), and Hold (4°C, Infinity).

The cDNA targets were then pre-amplified to increase the quantity of the desired cDNA for gene expression analysis using the TaqMan MicroRNA Arrays. For pre-amplification the following were combined in a microcentrifuge tube: TaqMan PreAmp Master Mix (2X), Megaplex PreAmp Primers (10X), Nuclease-free water, and RT product. The tube was inverted and spun before incubation for five minutes on ice. The pre-amplification was performed using GeneAmp PCR System 9700 (Life Technologies Carlsbad, CA) using the following thermal-cycling parameters: Hold (95°C 10 min), Hold (55°C 2 min), Hold (72°C 2 min), 12 Cycles (95°C 15 sec, 60°C 4 min) Hold (99.9^{o}C 10 min), and Hold (4°C infinity).

The PCR reaction mix contained: TaqMan Universal PCR Master Mix (2X), Nuclease-Free water, and the pre-amplifed product diluted with 75 µl ddH_2O. This PCR reaction mix was then added into each port of the TaqMan MicroRNA Array Card. The array was centrifuged and sealed. The array was run on the 7900T ABI system using the default 384-well TaqMan Low Density Array thermal-cycling conditions: Hold (50°C 2 min), Hold (94.5^{o}C 10 min), and 40 Cycles (97°C 30 sec, 59.7^{o}C 1 min). The expression level of the microRNAs was calculated using the delta-delta CT method.

IV.E. miRCURY LNA Universal microRNA PCR

A two part kit and primers for determining expression level of single microRNAs was purchased from Exiqon (Woburn, MA). The template RNA samples were first diluted to a concentration of 5ng/µl using nuclease-free water. The sample was then combined with the following reagents for the first-strand cDNA synthesis: 5X Reaction buffer, Nuclease-free water, Enzyme mix, and synthetic RNA spike-in. The contents were vortexed and spun before being placed in GeneAMP PCR System 9700 (Life Technologies Carlsbad, CA). The thermal cycle was incubation for 60 minutes at 42°C, heat-inactivation of the reverse transcriptase for 5 minutes at 95°C, and then immediately cooled to 4°C.

Before using the cDNA for qRT-PCR the samples were diluted. We found that a dilution of 40X gave results within 45 amplification cycles. The diluted cDNA was combined with SYBR Green master mix and PCR primer mix (Exiqon Woburn, MA). The real-time PCR reaction was in a 394-well plate. The cycling temperatures were modified on 7900T ABI (Life Technologies Carlsbad, CA): Hold (95°C 10min) and 45 cycles (95°C 10 sec, 60°C 1 min). The cycles were increased to 45 because the samples were still amplifying at cycle 40 and the increase allowed for calculation of an improved threshold level.

Chapter V RESULTS

V.A. RNA integrity

The integrity and purity of total RNA extracted from MDA-MB-231, 231wCFry, 231wCVec, and MCF-10A was checked using the Agilent 2100 program (Waldbroon, Germany). The presence of intact total RNA in the samples was indicated by the presence of sharp, 28S and 18S rRNA bands (Figure 1). Analysis indicated the 28S band were twice as dark as the 18S bands, which was the expected ratio for intact RNA samples (Figure 1). Secondly, Agilent software was used to calculate an RNA integrity number, which ranges from 1 (degraded) to 10 (intact). The RNA integrity numbers for our samples was above nine signifying that our RNA was of high quality.

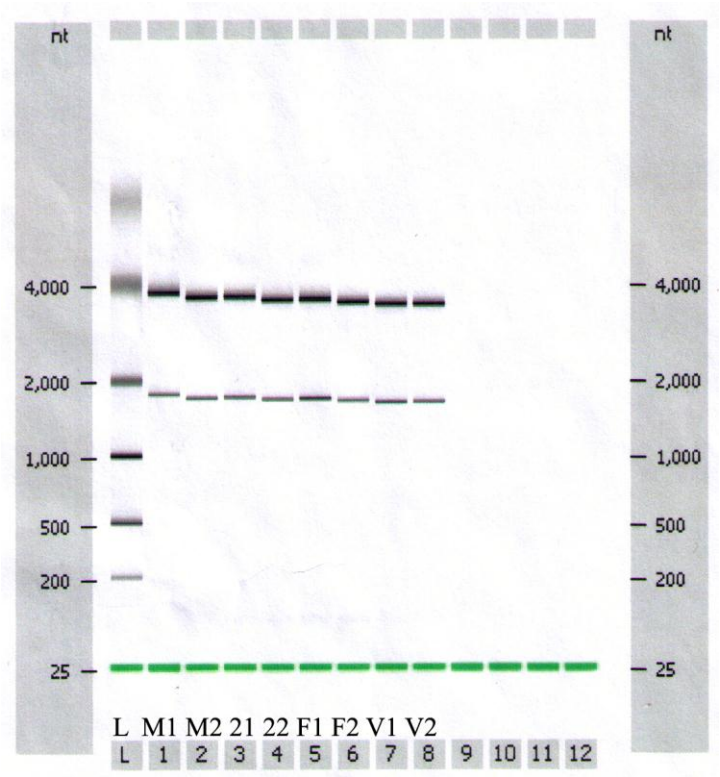

Figure 1: Examination of RNA integrity. Agilent Bioanalyzer was used to visualize the 28S rRNA and 18S rRNA bands. L is the RNA ladder, M1 & M2 are MCF-10A, 21 & 22 are MDA-MB-231, F1 & F2 are 231wCFry, and V1 & V2 are 231wCVector.

V.B. TaqMan Quantitative Real Time-PCR

To determine the expression levels of microRNAs in the following cell lines: MCF-10A, MDA-MB-231, 231wCFry, and 231wCVector, we used quantitative Real-Time PCR, also known as qRT-PCR. This process amplifies and quantifies the cDNA simultaneously (Applied Biosystems, 2012). By using this method one can quantify the number of target microRNAs in each sample by computing values against endogenous microRNA controls. Measurement of microRNA expression levels using TaqMan™ MicroRNA Array Cards, occurs in three steps, the first of which entails reverse transcription (RT) using Megaplex pools of primers, in which the cDNA is reverse transcribed from total RNA samples using specific miRNA stem-loop RT primers (Applied Biosystems, 2012). The second step is pre-amplification, in which PCR products are uniformly amplified from cDNA templates using Megaplex PreAmp Primers (Applied Biosystems, 2012). The third step is Real-Time PCR, in which each TaqMan MGB probe anneals specifically to its complementary sequence between the forward and reverse primer sites (Applied Biosystems, 2012). This probe has the reporter dye (FAM™ dye) linked to the 5' end of the probe. At the 3' end is both a minor groove binder (MGB) to allow the design of shorter probes with greater specificity and a non-fluorescent quencher (NFQ) (Applied Biosystems, 2012). When the probe is intact, the proximity of the quencher dye to the reporter dye causes the reporter dye signal to be quenched by resonance energy transfer. The AmpliTaq Gold® DNA polymerase extends the primers bound to the cDNA template, resulting in nick translation by the inherent 5' nuclease activity of the polymerase. Once the hybridized probes are cleaved by AmpliTaq Gold® enzyme, the quencher is separated from the reporter dye, increasing the

fluorescence of the reporter dye (Applied Biosystems, 2012). The fluorescence signal generated by PCR amplification indicates the gene expression level in the sample.

V.B.1. TaqMan MicroRNA Array Cards

The TaqMan MicroRNA Array Card A and Card B are each comprised of each had 377 microRNAs for expression profiling. RNA extracted from cells according to the manufacturer's protocol and transferred to the Bionomics Research Technology Core (BRTC) at the Environmental and Occupational Health Sciences Institute, a joint institute of UMDNJ and Rutgers University. MicroRNA profiling analyses were performed in duplicate using the same RNA isolates (technical replicates). The data was analyzed by delta delta Ct method.

The microRNA expression data between the original and repeat array showed poor reproducibility for each cell line with respect to the expression levels of individual microRNAs. Only 45 microRNAs on Card A and 73 microRNAs on Card B met the criteria for differential expression relative to the levels in MDA-MB-231 cell line (Appendix 1 and Appendix 2). The following is the criteria we used to filter the microRNA data: 1) microRNAs which had a Ct value greater than 35 in MDA-MB-231 cell line were removed to limit background noise. 2) ΔCt values of MDA-MB-231 duplicates that were not within one ΔCt value of each other were removed to exclude erroneous technical replicates that may be caused by inefficiency of cDNA measurement. We used the ΔCt value to allow for quantitative differences to be corrected by the endogenous control measurement. 3) MicroRNAs whose fold-change for the control 231wCVector cell line that was not 1.0 (+/- 0.25) were removed. Without the third criterion, we would not be certain if the fold-change was due to the ectopic expression of

FRY, or if the fold-changes arose because of the vector insertion. The top four microRNAs candidates that may be linked to ectopic FRY expression from Card A have all been previously implicated in cancer (Table 1). In particular hsa-mir-106b, hsa-mir-25, and hsa-mir-203 may have a direct interaction between FRY and EMT due to expression changes in 231wCFry (Table 1).

Our results indicated that one of the Card B replicates yielded Ct values above 35 for all but 10 of the 377 microRNAs represented on the card. These findings suggested that either the card was faulty, or that the cDNA was not properly added to the card, creating high noise levels. Therefore, only data from the second Card B was considered for evaluation. The top 12 candidates from Card B were up-regulated at least 3 fold in 231wCFry relative to the tumorigenic MDA-MB-231 cells (Table 2).

Table 1: Top microRNA candidates showing differential expression between tumorigenic and non-tumorigenic mammary tumor cell lines

Card A: miRNA	231wCVector	231wCFry	MCF-10A
U6	1.000	1.000	1.000
hsa-miR-106b	1.035	0.513	0.517
hsa-miR-25	1.033	0.510	0.768
hsa-miR-203	0.762	0.255	33.266
hsa-miR-142-3p	1.029	0.391	0.064

The fold changes in TaqMan MicroRNA Array Card A were calculated using the delta delta Ct method. There was only one replicate on Card A per microRNA. A second Card A was run for a total of two technical replicates. U6 was the endogenous control from which the data was normalized. The microRNAs marked in red were previously implicated to play a role in EMT.

Table 2: Top microRNA candidates showing differential expression between tumorigenic and non-tumorigenic mammary tumor cell lines.

Card B: miRNA	231wCVector	231wCFry	MCF-10A
U6	1.000	1.000	1.000
hsa-miR-1253	1.042	4.036	4.335
hsa-miR-1285	0.917	4.050	4.070
hsa-miR-1825	0.918	3.437	7.611
hsa-miR-432#	1.022	4.181	5.359
hsa-miR-454#	0.941	3.904	1.064
hsa-miR-520c-3p	0.968	4.222	1.048
hsa-miR-572	0.929	3.800	2.001
hsa-miR-604	0.977	7.765	4.323
hsa-miR-635	1.022	3.635	1.571
hsa-miR-638	0.918	3.910	8.592

The fold changes in TaqMan MicroRNA Array Card B were calculated using the delta delta Ct method. There was only one replicate on Card B per microRNA. U6 was the endogenous control from which the data was normalized. Because these are uncommonly studied we chose microRNAs that showed the highest expression changes in 231wCFry compared to MDA-MB-231 cell line.

V.C. Exiqon Quantitative Real-Time PCR

We next evaluated a second method for measuring microRNA expression in the cell lines with and without ectopic FRY. The second method is from Exiqon (Woburn, MA) which uses Locked Nucleic Acid Technology™ (LNA). The LNA nucleosides are a class of nucleic acid analogues in which the ribose ring is "locked" into a N-type conformation, by a methylene bridge connecting the 2'-O atom and the 4'-C atom (Exiqon, 2012). The main feature of LNA is the ability to use short unique sequences to recognize complementary nucleic acids with high affinity (Exiqon, 2012). Other advantages of LNA include: high thermostability, enhanced selectivity, and nuclease resistance (Exiqon, 2012). Unlike the ABI primers, the Exiqon protocol first adds a poly A tail to the mature microRNA template. The cDNA is synthesized using a poly T primer with a 3' degenerate anchor and a 5' universal tag. The cDNA template is then amplified using microRNA-specific and LNA™ enhanced forward and reverse primers (Exiqon, 2012). SYBR Green dye is used during RT-PCR to detect all amplified double-stranded DNA. This alternative approach was used to quantify expression the expression of candidate microRNA in the following cell lines: MCF-10A, MDA-MB-231, 231wCFry, and 231wCVector.

V.C.1. Expression level of hsa-mir-515 in 231wCFry and MCF-10A cells using Exiqon microRNA LNA™ PCR primer sets

Ingenuity Pathway Analysis™ (IPA) predicts molecular interactions between microRNAs and target molecules by searching microRNA literature and databases such as TargetScan, whose predictions are based on the targets conserved sites that match the seed region of the microRNA (Ingenuity, 2012; TargetScan, 2012). IPA then further

filters the search by searching canonical pathway literature, which is predicted to interact with the target molecule (Ingenuity, 2012). Using the Ingenuity Pathway Analysis™ software, our lab designed an interacting network to include *FRY*, *CACNA1D* calcium channel and the *NDR1* kinase gene (Graham, 2011). *NDR1* and *CACNA1D*, we both found to be highly correlated with *FRY* expression in the gene expression profiling (Oncomine 3.0) studies (Graham, 2011). Using Ingenuity Pathway Analysis, new networks were designed that linked microRNAs to these genes (Graham, 2011). The human microRNA, hsa-mir-515 was predicted to interact with all three of these genes; however the mechanism behind the interaction is undetermined (Figure 2).

We therefore used Exiqon LNA™ primers designed for hsa-mir-515-3p and hsa-mir-515-5p to determine if the expression level of hsa-mir-515 is correlated with FRY expression in the four cell lines**.** The results indicated that between the two mature forms, hsa-mir-515-3p yielded more amplification in the four cell lines: MCF-10A, MDA-MB-231, 231wCFry, and 231wCVector (Figure 3). The first experiment showed that hsa-mir-515-3p amplification to also be better defined than hsa-mir-515-5p in the aforementioned cell lines (Figure 3). The results showed an increase in expression of hsa-mir-515-3p in 231wCFry. A second set of cells were extracted for total RNA and the experiment was repeated for N=6. However, these finding could not be verified using RNA extracted from a second set of cells, even though the experiment was repeated (Figure 4 and Figure 5). In contrast to the first experiment which showed increased expression of hsa-mir-515-3p, the second experiment showed a decrease of hsa-mir-515-3p in 231wCFry cells as compared to MDA-MB-231 (Figure 6). From these results we conclude that additional factors, such as cell passage number may have contributed these differential findings.

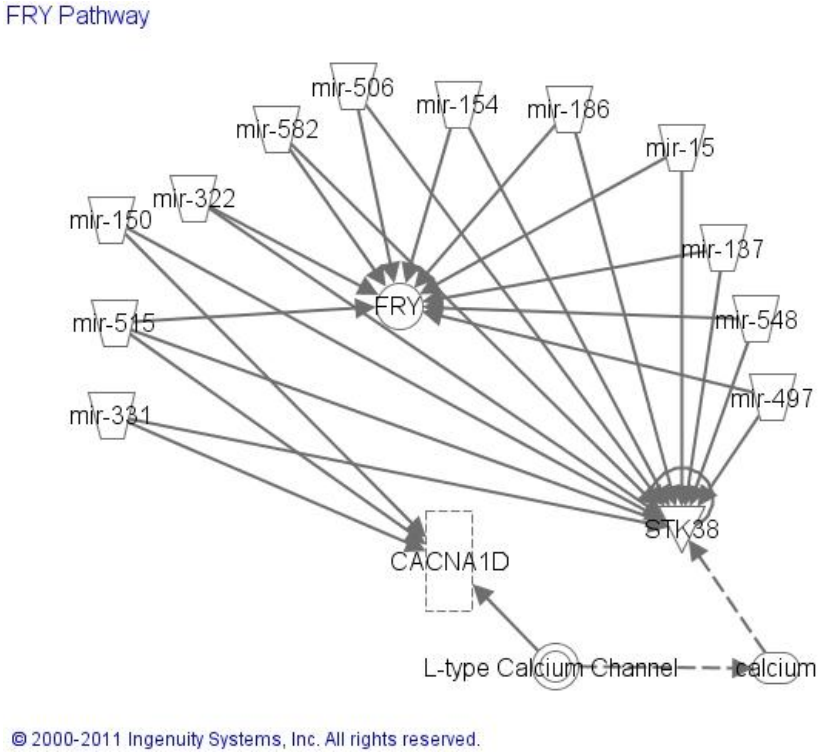

Figure 2: Pathway linking hsa-mir-515 to *FRY, CACNA1D,* and *NDR1* (Graham, 2011). The diagram shows that the three genes, *FRY*, *CACNAID*, and *NDR1 (STK38)* are predicted to interact together through the microRNA hsa-mir-515.

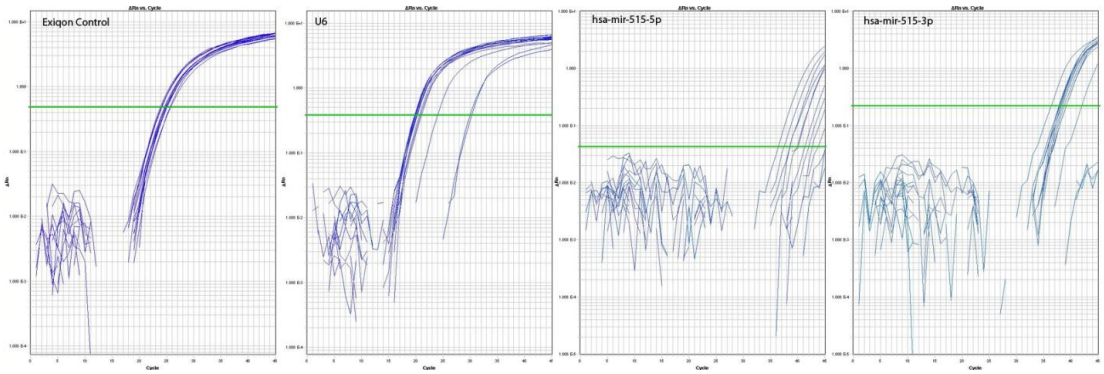

Figure 3: Amplification of hsa-mir-515-3p and hsa-mir-515-5p. **Δ** Rn versus Cycle graphs. The RNA Spike-in control read during the amplification and labeled as Exiqon Control. U6 is used as the endogenous control to determine amplification levels of hsa-mir-515-3p and hsa-mir-515-5p. The cDNA was diluted 40X and cycles extended to 45 for a better threshold level.

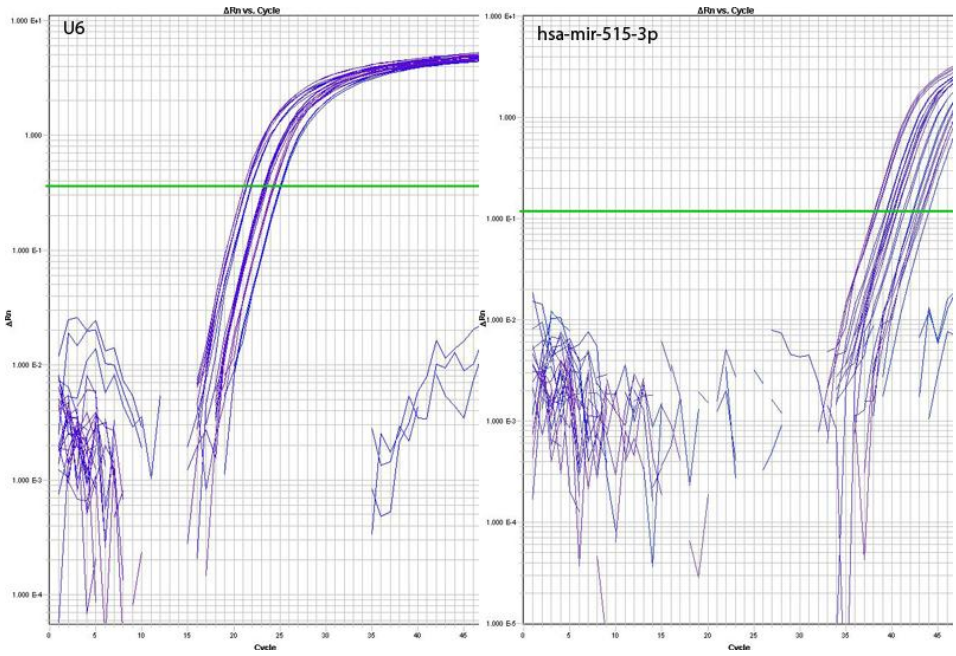

Figure 4: First Cell Set Amplification of hsa-mir-515-3p. Δ Rn versus Cycle graphs. U6 is used as the house keeping endogenous control to determine amplification levels of hsa-mir-515-3p. The cDNA was diluted 40X and cycles extended to 45 for a better threshold.

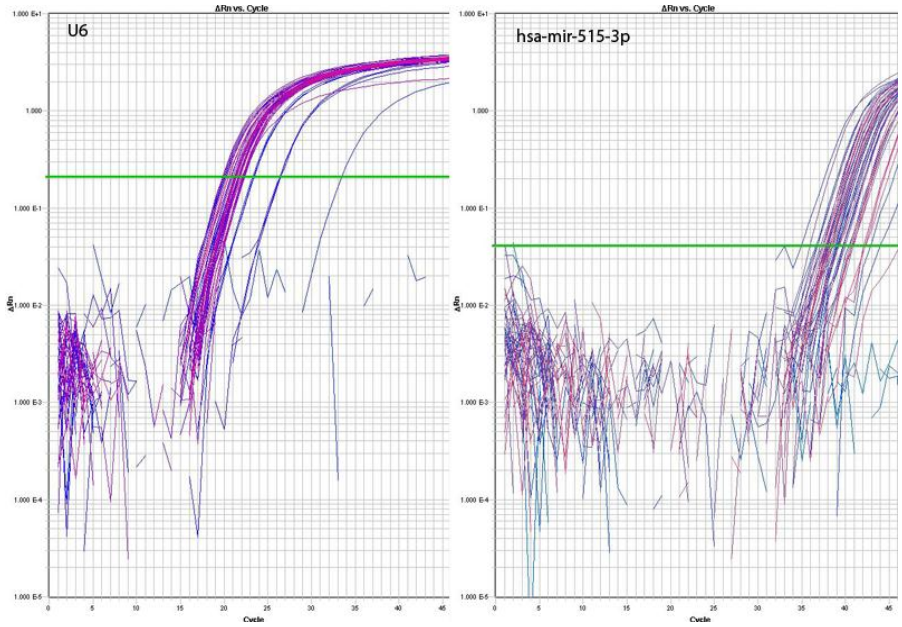

Figure 5: Second Cell Set Amplification of hsa-mir-515-3p Δ Rn versus Cycle graphs. U6 is used as the house keeping endogenous control to determine amplification levels of hsa-mir-515-3p. The cDNA was diluted 40X and cycles extended to 45 for a better threshold level.

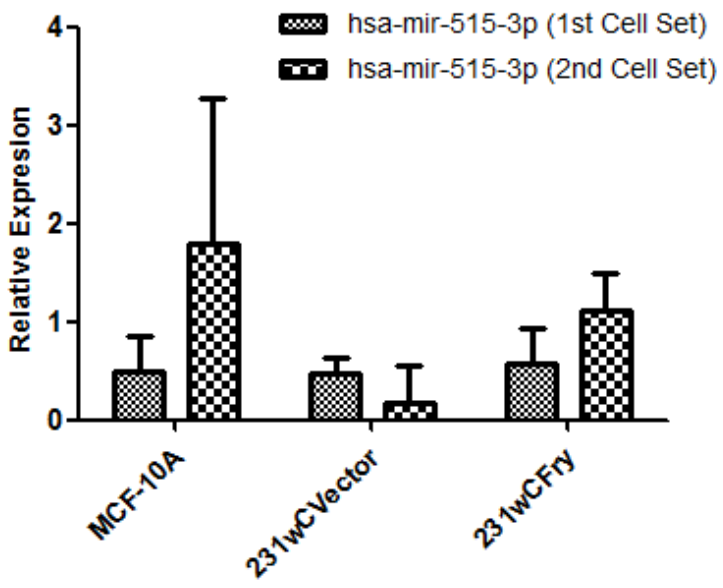

Figure 6: **Relative Expression of hsa-mir-515-3p in 231wCFry and MCF-10A cells**. The first set of cells extracted for RNA showed a decrease in microRNA level in ectopically expressing FRY cells; however the second set of cells extracted for RNA showed an increase in fold change for the microRNA level in ectopically expressing FRY cells. A t-test did not show any statistical significance.

V.C.2. Expression Level of hsa-mir-4728-3p and hsa-mir-301b in 231wCFry and MCF-10A Cells Using Exiqon microRNA LNA™ PCR primer sets

Another approach to identifying microRNAs that regulated FRY signaling is to identify those that directly target the FRY mRNA molecule. Increased expression of such microRNAs would reduce FRY protein expression in cells and thus contribute to EMT and carcinogenesis. We therefore sought microRNAs that showed nucleotide sequence complementarity with the human *Fry* mRNA. We began the search for microRNAs that target FRY mRNA using the Basic Local Alignment Search Tool (BLAST) and the miRBase microRNA sequence database; we identified hsa-mir-4728-3p as sharing 83% sequence complementarity to the *Fry* mRNA nucleotide sequence at 4,561 to 4,583. Not all nucleotides of a mature microRNA are available for base pairing with their target

mRNA. Regulatory effect has been observed for microRNA-mRNA pairing with as little as 8 base-pairs between miRNA and its target mRNA (Dong et al., 2009). The lack of specificity in perfect base pairing creates enormous difficulty to understand the mechanism in target recognition. Higher frequency of pairing is in the “seed” region, often defined as the 2nd-8th nucleotide from the 5' end of the miRNA (Dong et al., 2009). As a result, a BLAST analysis may overestimate the strength of the potential binding between a microRNA and a target mRNA. However, given the high degree of complementarity, the uniform distributions of mismatches across the microRNA sequence, and the fact that a previous studies linked hsa-mir-4728-3p to *ERRB2* expressing breast cancers, we considered hsa-mir-4728-3p as a reasonable candidate targeting the FRY mRNA (Persson et al., 2011).

The results of the experiment showed the expression in 231wCFry is decreased, while the expression in MCF-10A is increased compared to MDA-MB-231 cells (Figure 8). We can predict from these results that the ectopic expression of FRY is interacting with a pathway that may decrease the expression of hsa-4728-3p, while the increase of expression in MCF-10A cell may be explained by its non-tumorigenic properties to which the MCF-10A expression levels were compared. However, both these results are still unexpected due to the fact that none of the cell lines express ERRB2 (Neve et al., 2006).

The expression of hsa-mir-301b in both 231wCFry and MCF-10A was increased in comparison to the MDA-MB-231 cells on TaqMan™ MicroRNA Array Card A. To confirm these results we used individual primers from Exiqon. However, the expression of hsa-mir-301b was decreased in both MCF-10A and 231wCFry cells (Figure 7 and

Figure 9). Previous studies indicated that hsa-mir-301b is overexpressed hsa-mir-301b overexpressed in colorectal cancer (Wang et al., 2010). It is plausible that FRY mediates its tumor suppression function, at least in part, by inhibiting the expression of hsa-mir-301b. The regulation of this microRNA by FRY thus merits further study.

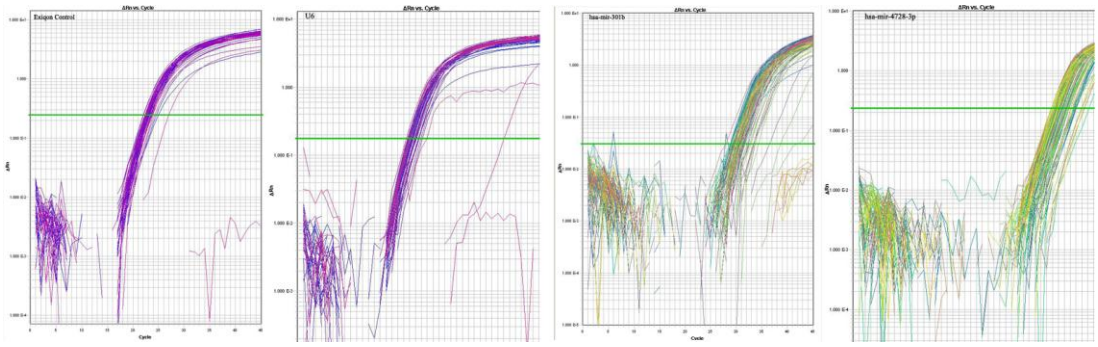

Figure 7: Amplification of hsa-mir-301b and hsa-mir-4728-3p. **Δ** Rn versus Cycle graphs. The RNA-Spike in control read during the amplification and labeled as Exiqon Control. U6 is used as the endogenous control to determine amplification levels of hsa-mir-301b and hsa-mir-4728-3p by the delta-delta CT method. The cDNA was diluted 40X and cycles extended to 45 for a better threshold calculation.

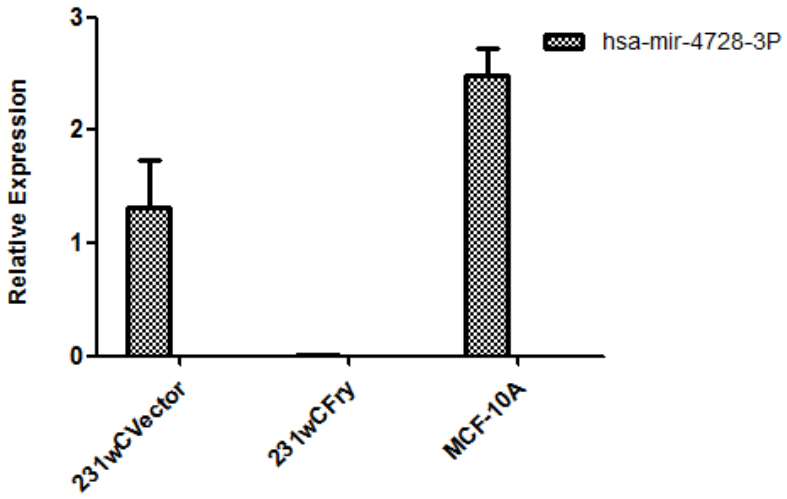

Figure 8: Expression levels of hsa-mir-4728-3p in 231wCFry and MCF-10A cells. In hsa-mir-4728-3p the fold expression in 231wCVector is 1.3, 231wCFry 100-fold decrease, and MCF-10A up-regulated by 2.48 fold in comparison to MDA-MB-231. Using t-test this result was found to be significant $p<0.01$

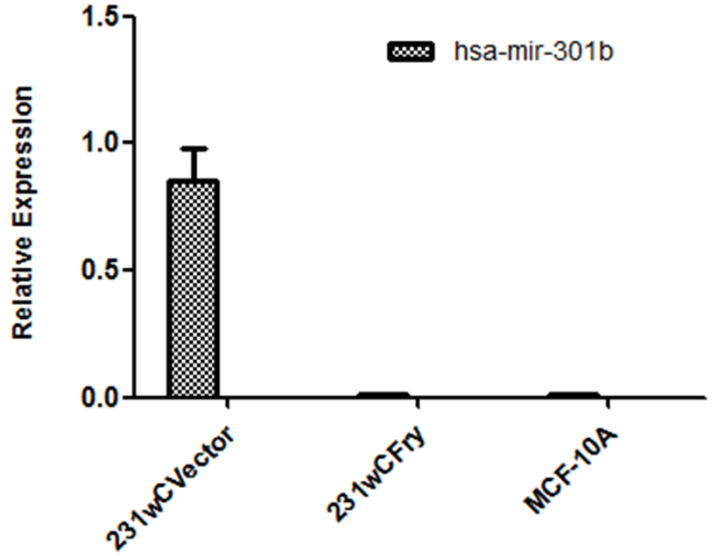

Figure 9: Expression levels of hsa-mir-301b in 231wCFry and MCF-10A cells. Fold levels calculated by delta-delta CT of hsa-mir-301b in 231wCVector are 0.85, in 231wCFry 100-fold decrease, and MCF-10A 100-fold decrease compared to MDA-MB-231. Using t-test this result was found to be significant $p<0.01$

Chapter VI DISCUSSION

VI.A. Determination and Accuracy of MicroRNA Expression Levels

In research, genome-wide analysis by microarray technology has provided deeper biological insights for a decade, while in the clinical field, the US Food and Drug Administration (FDA) approved MammaPrint as the first *in vitro* diagnostic multivariate index assay (IVDMIA) in 2007 (Sato et al., 2009). Recently, microarray technology has been utilized to analyze a comprehensive microRNA expression profile. Sato et al. compared repeatability and comparability of microRNA microarrays using various platforms (Agilent, Ambion, Exiqon, Invitrogen and Toray) (Sato et al., 2009). They also compared the quantitativity of microarray data generated for microRNA expression from the various platforms with that of quantitative RT-PCR (TaqMan) method, which remains the standard method of microRNA measurement (Sato et al., 2009).

Gene expression analysis from microarray data is generally normalized under an assumption that the total amount of mRNA is constant between different samples (Sato et al., 2009). However, microRNA microarray data generated from the same amount of total RNA can not necessarily be normalized because the amount of microRNA varies depending upon cell types, such as normal vs. cancer (Sato et al., 2009). Because reliable housekeeping microRNAs have not been discovered, the TaqMan assay produces Ct values without normalization. Data is obtained using the same amount of total RNA leading to higher variability between the values (Sato et al., 2009). However, the Exiqon system uses endogenous, small non-coding RNAs (snRNAs) that are constitutively expressed in a wide variety of tissues to normalize microRNA expression data, allowing for a more accurate comparison among cell lines. The primer sets for specific snRNAs

have been validated as reference genes for use with the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon, 2012). The major challenge of microRNA research is accuracy of measuring specific levels of microRNA, true for essentially all microRNA measurement platforms based on qRT-PCR or microarray technologies (Lee et al., 2010). There is a lack of not only inter-platform consistency, but also intra-platform reproducibility on microRNA measurement (Lee et al., 2010). This may be due to inadequate probe or primer specificity due to the short length and high sequence similarity amount between microRNA species (Lee et al., 2010).

The TaqMan qRT-PCR method uses a stem-loop primer that anneals to the 3' end of the microRNA to generate cDNA for qPCR, while the Exiqon platform uses polyadenylase to generate poly A tails and then uses anchored poly T primer to generate cDNA templates for microRNA profiling (Lee et al., 2010). The Exiqon platform also uses nucleotide analogs to enhance the specificity and selectivity of their microRNA qRT-PCR primers (Lee et al., 2010). Changes at the 3' or 5' end of mature microRNA by one or two nucleotides is shown to be enough to drastically affect the measurement results (Lee et al., 2010). The preferred method between the two platforms is Exiqon due to its reliance on the sequence integrity of the 3' end. Additionally, LNA™ modified nucleotides can be used at a lower melting temperatures (Tm) to enhance the primer specificity that perform better to detect the sequences of the microRNAs (Lee et al., 2010). To overcome the disadvantages of microRNA arrays, one could use deep sequencing, which is less subject to background and cross-hybridization problems. Deep sequencing measures the copy number rather than relative abundances of previously discovered microRNAs (Wu et al., 2011). Deep sequencing measures absolute abundance

of a microRNA and is not limited by array contents, allowing for discovery of novel microRNAs or other small RNA species (Wu et al., 2011).

VI.B. Expression Level of MicroRNAs in Presence of Ectopically Expressed *FRY*

In slico analysis of gene networks revealed no direct connections between EMT-associated molecules and *FRY*. The analysis did find 224 one-step connections between *FRY, CACNA1D, NDR1*, and molecules involved in EMT, many of which are also linked to microRNAs (Graham, 2011). These observations indicated that *FRY* may be affecting EMT at the level of signaling, and suggested several novel biomarkers of *FRY* expression and function (Graham, 2011). We therefore assessed the expression levels of individual microRNAs that were differentially expressed between MCF-10A mammary epithelial cells, MDA-MB-231 breast cancer cells and clones of the latter with ectopic expression of FRY. MicroRNAs were selected for further study on the basis of expression analysis from the TaqMan Assay Cards, validation of Ingenuity Pathway Analysis using Exiqon, and a homology search by BLAST. MicroRNAs showing differential expression were selected for validation using individual qRT-PCR amplifications.

VI.B.1. Link Between hsa-mir-25 and hsa-mir-106b to TGFβ Signaling and EMT

The first two candidates hsa-mir-25 and hsa-mir-106b showed differential expression in 231wCFry compared to MDA-MB-231 on TaqMan MicroRNA Array Cards, these candidates are part of the mir-106b-25 cluster (Petrocca et al., 2008). This cluster accumulates in different types of cancer, including gastric, prostate, and pancreatic neuroendocrine tumors, neuroblastoma, and multiple myeloma (Petrocca et al., 2008).These microRNAs are located in the intron of the host gene *MCM7*, where they are co transcribed in the context of MCM7 primary transcript (Petrocca et al., 2008).

Although amplification of this region in gastric cancer has been reported in several studies, MCM7 overexpression is a bad prognostic indicator in prostate and endometrial cancer (Petrocca et al., 2008).

The functional implications of miR-106b-25 overexpression in gastric cancer are tightly linked with the TGFβ tumor suppressor pathway (Petrocca et al., 2008). Overexpression of hsa-mir-106b down-regulates the cell cycle inhibitor p21, impairing TGFβ-dependent cell cycle arrest, whereas hsa-mir-25 silences BIM expression, which is essential for TGFβ-dependent apoptosis (Petrocca et al., 2008). Furthermore, down-regulation of hsa-mir-25 in ovarian cancer cells induced apoptosis, whereas overexpression of hsa-mir-25 enhanced cell proliferation indicating it directly regulates apoptosis by targeting BIM (Zhang et al., 2011). Our TaqMan microRNA Array data shows down-regulation in 231wCFry cells in comparison to tumorigenic MDA-MB-231 cells, which supports hsa-mir-25 anti-proliferation role. Recently, it has been found that Six1 activates the tumor promotional arm of TGF-β signaling by up-regulating the miR-106b-25 microRNA cluster (Smith et al., 2012). A significant correlation between hsa-mir-106b, Six1, and activated TGF-β signaling was found in human breast cancers, and high levels of hsa-mir-106b in breast tumors significantly predicts shortened time to relapse (Smith et al., 2012). The miR-106b-25 cluster is sufficient to induce an EMT and a tumor initiating cell phenotype, and that it is required downstream of Six1 to induce these phenotypes (Smith et al., 2012). Previously our labs Ingenuity Pathways Analysis demonstrated that a top canonical pathways significantly affected by ectopic FRY expression was TGF-β signaling (Graham, 2011). Additionally, TaqMan microRNA Array data demonstrates that hsa-mir-106b is down-regulated in 231wCFRY cells, which

may support the hypothesis, that ectopic expression of FRY reverses EMT though microRNA interactions.

VI.B.2. Overexpression of hsa-mir-203 leads to MET

Our third candidate hsa-mir-203, was differentially expressed in 231wCFry in comparison to MDA-MB-231 on TaqMan MicroRNA Card. Hsa-mir-203, a tumor suppressor is often silenced in different malignancies (Viticchiè et al. 2011). It was shown hsa-mir-203 is down regulated in clinical primary prostatic tumors compared to normal prostate tissue, and in metastatic prostate cancer cell lines compared to normal epithelial prostatic cells (Viticchiè et al. 2011). Hsa-mir-203 overexpression is sufficient to revert epithelial to mesenchymal transition (EMT) in metastatic prostate cancer cell lines (Viticchiè et al. 2011). Hsa-mir-203 is a transcriptional repression target of the EMT activator ZEB1, and its down regulation was observed during metastatic invasion in pancreatic and colorectal cancers (Viticchiè et al. 2011). Additionally, it was shown that reintroduction of hsa-mir-203 in cell culture is sufficient to block proliferation and to revert the mesenchymal to an epithelial-like phenotype (Viticchiè et al. 2011). This particular study is important in understanding our TaqMan microRNA Array results. The decrease in expression in 231wCFry cells compared to MDA-MB-231 may be due to the reversal of EMT phenotype, but the increase in expression in MCF-10A may be due the fact that it is normal mammary epithelial cell line and therefore the level of tumor suppressor hsa-mir-203 would naturally be higher than in the tumorigenic MDA-MB-231 cell line.

VI.B.3. Role of hsa-mir-142-3p in RAC1 Regulation

RAC1 is a GTPase that regulates a diverse array of cellular events, including control of cell growth, cytoskeletal reorganization, migration and invasion in addition to the activation of protein kinases (Wu L et al., 2011). The expression of hsa-mir-142-3p is down regulated in hepatocellular carcinoma tissues. Normally, hsa-mir-142-3p inhibits colony formation, migration and invasion by targeting RAC1; suggesting hsa-mir-142-3p is a tumor suppressor (Wu L et al., 2011). However, high hsa-mir142-3p expression is associated with a poor prognosis in esophageal squamous cell carcinoma (ESCC), suggesting that it is involved in the progression of ESCC (Lin et al., 2012). Our TaqMan MicroRNA Array data demonstrates down regulation of this microRNA in 231wCFry and MCF-10A in comparison to the tumorigenic MDA-MB-231 cell line. Since the above literature suggests the role of hsa-mir-142-3p as either an oncogene or tumor suppressor in carcinomas it should be determined if the levels of this microRNA are directly influenced by FRY in breast cancer and if so, does the down regulation of this microRNA support an oncogenic role of hsa-mir-142-3p.

VI.B.4. Inconclusive Role of hsa-mir-515-3p in 231wCFry and MCF-10A

A recent paper on germ cell tumor cell lines described the potential involvement of hsa-mir-515 in resistance to cisplatin, a chemotherapeutic agent used in treatment of solid tumors, including breast cancer (Port et al., 2011). The study alluded to the importance of understanding the gene networks through which a 2-3 fold up-regulation of this microRNA induces the resistance of cell lines to cisplatin. Elevated levels of this microRNA have also been found to be overexpressed in oral squamous cell carcinoma (Scapoli et al., 2010).

As stated previously, using Ingenuity Pathway Analysis software, our lab designed a pathway to include *FRY, CACNA1D,* and *NDR1* genes. Using pathway analysis we discovered that hsa-mir-515 interacts with all the three molecules (*FRY, CACNA1D, and NDR1*). We were however unable able to confirm conclusively whether the expression of this microRNA changed among cell lines. Instead, we found that there was no significant fold change. However, future experiments may show a different mechanism of interaction between the three molecules and hsa-mir-515.

VI.B.5. Expression of hsa-miR-4728-3p is decreased in 231wCFry and increased in MCF-10A

Since microRNAs bind to target mRNAs, we decided to see if there were any microRNAs sharing complementary sequence homology with the human *Fry* gene. We did a BLAST search using miRBase and found hundreds of microRNAs that shared some homology, but the one that had the greatest score was hsa-mir-4728-3p, with an alignment score of 83 and Expect Value (E) of 0.13. The alignment score is the sum of the score for each aligned pair, the greater the score the better the alignment (BLAST, 2012). E-Value is representative of the number of times this match or a better match would be expected in a search of the BLAST database, the lower the E-value the greater the similarity is between the microRNA and *Fry* gene (BLAST, 2012). Additionally, as the E-Value approaches zero, the match increases in significance (BLAST, 2012).

A recent publication linked hsa-mir-4728 to breast cancer. Persson et al. identified this new microRNA using extensive next-generation sequencing of small RNAs from tumor and normal breast tissue (Persson et al., 2011). What is particularly interesting is that they found a potential dual function for the *ERBB2/HER2* gene

involving hsa-mir-4728-3p. Overexpression of *ERRB2* oncogene is found in about 30% of breast cancer patients and correlates with poor prognosis because it enhances the metastatic potential of cancer cells (Kim et al., 2009). The group identified 39 new microRNAs in breast cancer subtypes showing high-level genomic amplifications (Persson et al., 2011). Hsa-mir-4728 is located in the intron of the *ERRB2* gene, and both mature hsa-mir-4728-3p and hsa-mir-4728-5p were found among Ago2-associated RNAs in MCF7 cells (Persson et al., 2011). The hsa-mir-4728-3p sequence ends just before the 5' splice site of exon 24, which suggests that this microRNA is a half-mirtron, and that processing may depend on splicing of the *ERBB2* RNA (Persson et al., 2011).

Persson et al. further analyzed expression of this microRNA in ERBB2 amplification cell lines (BT-474, JIMT-1, and SK-BR-3) versus the control cell lines (MCF7 and MCF10A) (Persson et al., 2011). They confirmed the results by looking at breast tumors that were $ERBB2^{+}$ and $ERBB2^{-}$; those overexpressing ERBB2 also overexpressed hsa-mir-4728 and *ERBB2* exon 24 (Persson et al., 2011). Since hsa-mir-4728 is encoded within an intron of *ERBB2*, the authors speculated that expression of hsa-mir-4728 may explain overexpression of ERBB2 (Persson et al., 2011). Using TargetScan, the authors predicted that hsa-mir-4728 may target cellular processes such as GTPase mediated signal transduction, MAPK1, SOS1, and central components of transduction signals that control cell growth and differentiation (Persson et al., 2011). These findings may suggest that hsa-mir-4728 is part of a negative feedback loop that controls or fine tunes the function of its host gene.

Kim et al. overexpressed ERRB2 in MCF-10A cells, this induces up-regulation of matrix metalloproteinase 9 (MMP-9) leading to invasion and migration of MCF-10A

cells. Strong ERRB2 expression in MCF-10A has shown to cause a marked reduction of E-cadherin and induction of vimentin, suggesting EMT may play a role in invasion and migration induced by ERRB2 in MCF-10A cells (Kim et al., 2009). The data demonstrated that ERBB2 induced MMP up-regulation. Additionally, invasive and migratory abilities of MCF10A cells require activation of p38 MAPK and PI3K/Akt signaling pathways (Kim et al., 2009). Interestingly, top canonical pathways that are significantly changed by FRY, based on genes that were commonly changed in the *Fry* knockdown MCF-10A (10A-shFRY) isogenic set of cell lines, included PI3K/Akt signaling as well (Graham, 2011).

Previous literature has suggested a direct relationship between transcription of hsa-mir-4728-3p and *ERRB2*. Compared to MDA-MB-231 the increased expression in MCF-10A cells and further decrease of hsa-4728-3p expression in 231wCFry was unexpected due to both cell lines being negative for ERBB2 expression. This may suggest that in MDA-MB-231 cells, hsa-mir-4738-3p can be expressed separate from *ERRB2*. Additionally, the expression of *Fry* may be correlated to the decreased expression of hsa-mir-4728-3p leading to reversal of EMT. Future studies must be done to determine if there is a link between hsa-mir-4728-3p, EMT, and *FRY*.

VI.B.6. Supporting Oncogenic Role of hsa-miR-301b in 231wCFry and MCF-10A

Overexpression of hsa-mir-301b has been found in colorectal cancer and localized in pancreatic tumor cells (Wang et al., 2010 and Lee et al., 2009). Hsa-mir-301 overexpression has been implicated as a negative prognostic indicator in lymph node negative (LNN) invasive ductal breast cancer (Wei et al., 2011). Wei et al. established hsa-mir-301 as an important oncogenic gene by demonstrating attenuation decreased cell

proliferation, clonogenicity, migration, invasion, tamoxifen resistance, tumor growth, and microvessel density in breast cancer cells. Hsa-mir-301 is located in an intron of the SKA2 gene, which is responsible for kinetochore assembly, and both genes were found to be co-expressed in primary breast cancer samples (Wei et al., 2011).

In our studies expression of hsa-mir-301b was decreased in both MCF-10A and 231wCFry cells. This supports hsa-mir-301b as oncogenic due to the decrease in MCF-10A, a non-tumorigenic cell line and 231wCFry, which shows a suppression of the EMT phenotype. The decrease in expression of hsa-mir-301b is important to investigate further because the expression of FRY, a tumor suppressor, may be partly causing down regulation of this microRNA. If this result is seen with other oncogenic microRNAs within 231wCFry cells, it will continue to support data demonstrating that *Fry* is a tumor suppressor.

VI.C. Summary and Conclusions

In summary, our current findings identified microRNAs that are differentially expressed in MDA-MB-231 cells with and without ectopic *Fry* expression. In a screen of over 700 microRNA's using TaqMan Array MicroRNA cards, the three microRNAs most likely to play a role between FRY and EMT are hsa-mir-106b, hsa-mir-25, hsa-mir-203. From the list of uncommonly studied microRNAs on Card B the top 10 highly expressed microRNAs in 231wCFry cell compared to MDA-MB-231 may be found to play a role with *Fry* expression in future studies.

We found that although hsa-mir-515 was predicted to be part of the *FRY*, *CACNAID*, and *NDR1* pathway that the expression level does not change in 231wCFry nor MCF-10A compared to MDA-MB-231 cells. We report that levels of hsa-mir-301b

are decreased 100-fold in expression in both MCF-10A and 231wCFry in comparison to the tumorigenic MDA-MB-231 cells. This result suggests that hsa-mir-301b plays a role in tumorigencity of breast cancer and that expression of ectopic FRY may play a role in decreased expression of hsa-mir-301b. For hsa-4728-3p we observed the expression to be decreased 100-fold in 231wCFry and increased 2.50-fold in MCF-10A compared to tumorigenic MDA-MB-231 cells. Previous literature had suggested a direct relationship between transcription of hsa-mir-4728-3p and *ERRB2*. However, the decrease of hsa-mir-4728-3p expression in 231wCFry may suggest that in MDA-MB-231 cells, hsa-mir-4738-3p is expressed separately from *ERRB2*. Additionally, we pose that the increased expression of *FRY* may be correlated to the decreased expression of hsa-mir-4728-3p leading to suppression of EMT. The research in this thesis confirmed that ectopic expression wild type *Fry* in a triple negative breast cancer cell line modulates the expression of multiple microRNAs, including several previously implicated in carcinogenesis, tumor suppression and EMT. Further investigation of these differentially expressed microRNAs in triple negative breast cancers will be useful as screening methods and may also aid in the discovery of novel therapeutic strategies that express or target microRNAs in breast cancer cells.

VI.D. Future Experimental Directions

Although, we were unable to observe a change of expression in endogenous hsa-mir-515-3p, the connection found through Ingenuity Pathway Analysis could be further explored through knockdown of hsa-mir-515-3p, using small interfering RNA (siRNA) in MDA-MB-231 cells with and without FRY expression. Transfection by siRNA leads to the degradation of the targeted microRNA inhibiting its translation. This will help to

elucidate if there is an *in vitro* association between FRY and hsa-mir-515-3p expression in MDA-MB-231 cells, originally predicted by the IPA Pathway. The siRNA transfection could also elucidate an *in vitro* connection between hsa-mir-4728-3p and hsa-mir-301b with ectopic FRY.

The future studies could use Exiqon LNA Cancer Focus microRNA PCR Panels, which would be used to compare microRNA expression profiles in MDA-MB-231 breast cancer cells with and without ectopic *Fry*, and MCF-10A normal breast epithelial cells with and without FRY knockdown. The comparison of the microRNA expression profiles in tumor cells with and without ectopic FRY will identify candidate microRNAs that might contribute to the suppression of EMT.

Additional experiments may use miRCURY LNA microRNA Family inhibitors, to determine if knockdown of candidate microRNAs expressed in normal mammary epithelial cell expressing FRY induce tumorigenic phenotype by reversal of EMT. We may use miRCURY LNA Target Site Blockers to inhibit any microRNA binding to the *Fry* gene to determine if FRY expression is activated and driven by binding of microRNAs. We can then use gene expression profiling to compare patterns of gene expression induced by ectopic expression of candidate microRNAs in tumor cells and knockdown of microRNAs in normal cells. These studies will confirm the effect of microRNAs on EMT and identify gene networks regulated by their expression.

Together these studies could determine if *Fry* expression leads to altered expression of microRNAs or is mediated by microRNAs and the role of the affected microRNAs on epithelial cell differentiation, regulation of EMT, and carcinogenesis.

Furthermore, these studies will identify microRNAs and/or microRNA regulated genes that can be targeted by, or used as novel mechanistically-based therapeutic agents.

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CHAPTER VII APPENDICES

APPENDIX 1: TaqMan MicroRNA Array Card A Fold Change Data

Card A: miRNA	231wCVector	231wCFry	MCF-10A
U6	1.000	1.000	1.000
hsa-let-7b	0.758	0.526	4.106
hsa-let-7e	0.763	0.508	2.054
hsa-miR-100	0.763	0.517	0.743
hsa-miR-106b	1.035	0.513	0.517
hsa-miR-10a	0.762	0.511	0.320
hsa-miR-125a-5p	0.754	1.026	2.081
hsa-miR-130a	0.754	0.509	0.750
hsa-miR-130b	1.025	0.774	1.023
hsa-miR-137	0.760	0.515	6.116
hsa-miR-142-3p	1.029	0.391	0.064
hsa-miR-149	0.771	0.786	1.497
hsa-miR-150	0.780	0.519	0.759
hsa-miR-15b	0.757	0.510	1.230
hsa-miR-181a	1.023	0.781	2.036
hsa-miR-182	0.768	0.509	1.538
hsa-miR-184	1.056	1.283	1.968
hsa-miR-193a-5p	0.767	1.019	4.090
hsa-miR-197	0.766	0.507	0.763
hsa-miR-203	0.762	0.255	33.266
hsa-miR-21	0.759	0.507	0.741
hsa-miR-210	0.770	0.772	1.486
hsa-miR-218	1.035	0.784	1.037
hsa-miR-22	1.237	1.017	1.526
hsa-miR-25	1.033	0.510	0.768
hsa-miR-27a	1.035	0.507	0.750
hsa-miR-27b	1.017	0.787	0.758
hsa-miR-29c	0.760	0.517	1.519
hsa-miR-301a	1.030	0.776	0.753
hsa-miR-335	0.752	0.514	0.373
hsa-miR-342-3p	0.773	0.519	6.111
hsa-miR-362-3p	0.770	0.780	1.030
hsa-miR-374a	1.031	0.778	1.507
hsa-miR-374b	0.756	0.511	1.512
hsa-miR-422a	1.246	1.035	2.030
hsa-miR-452	0.763	0.765	2.072
hsa-miR-494	0.755	0.388	1.025
hsa-miR-502-3p	0.764	1.024	1.519
hsa-miR-503	0.771	0.788	1.530
hsa-miR-518f	1.025	0.372	0.764
hsa-miR-532-5p	0.769	1.032	1.513
hsa-miR-545	1.256	1.037	1.012
hsa-miR-642	0.780	0.525	0.385
hsa-miR-652	0.753	0.506	0.507
hsa-miR-98	0.727	0.499	2.488
hsa-miR-99a	0.762	0.510	0.758
hsa-miR-99b	0.793	0.509	0.775

APPENDIX 2: TaqMan MicroRNA Card B Fold Change Data

miRNA:CardB	231wCVector	231wCFry	MCF-10A
U6	1.000	1.000	1.000
hsa-let-7f-1#	1.058	1.983	8.556
hsa-let-7f-2#	0.950	2.008	8.497
hsa-let-7g#	1.000	4.028	9.350
hsa-miR-100#	0.953	0.998	2.195
hsa-miR-106b#	0.944	1.985	1.080
hsa-miR-1180	0.957	1.993	2.147
hsa-miR-1208	0.959	2.020	4.135
hsa-miR-1233	0.967	2.032	4.228
hsa-miR-1238	0.846	1.963	1.222
hsa-miR-124#	1.201	2.255	2.155
hsa-miR-1247	0.859	3.816	4.344
hsa-miR-1253	1.042	4.036	4.335
hsa-miR-126#	0.940	0.976	0.531
hsa-miR-1260	0.919	1.990	2.086
hsa-miR-1270	0.938	1.982	28.661
hsa-miR-1271	0.940	2.032	2.225
hsa-miR-1274A	0.929	0.960	0.531
hsa-miR-1275	0.921	1.882	4.260
hsa-miR-1276	0.902	1.964	4.031
hsa-miR-1285	0.917	4.050	4.070
hsa-miR-1290	0.970	1.966	1.077
hsa-miR-1296	0.916	1.935	4.228
hsa-miR-1303	0.859	1.788	3.994
hsa-miR-130b#	0.918	2.004	4.187
hsa-miR-132#	1.157	2.296	0.020
hsa-miR-135b#	0.959	2.008	2.170
hsa-miR-148b#	0.932	0.973	1.064
hsa-miR-151-3p	0.943	0.968	2.133
hsa-miR-15a#	0.946	2.024	4.365
hsa-miR-16-1#	0.922	0.960	2.117
hsa-miR-181a-2#	0.948	1.994	2.161
hsa-miR-1825	0.918	3.437	7.611
hsa-miR-191#	0.927	1.996	2.123
hsa-miR-19b-1#	0.936	1.972	2.157
hsa-miR-206	0.961	0.247	2.180
hsa-miR-22#	0.930	1.947	2.161
hsa-miR-221#	0.930	1.971	2.132
hsa-miR-222#	0.940	0.973	1.070
hsa-miR-26b#	0.947	1.982	8.676
hsa-miR-27b#	0.941	1.915	1.059
hsa-miR-29b-1#	0.897	0.965	2.099
hsa-miR-30a-3p	0.947	1.956	0.275
hsa-miR-30a-5p	0.934	0.975	2.152
hsa-miR-30d	0.934	1.957	4.344
hsa-miR-30e-3p	0.928	1.960	0.538
hsa-miR-34b	0.930	1.957	0.526
hsa-miR-378	0.938	0.972	1.058
hsa-miR-425#	0.951	0.987	2.149
hsa-miR-432#	1.022	4.181	5.359

hsa-miR-454#	0.941	3.904	1.064
hsa-miR-516-3p	0.894	1.912	2.008
hsa-miR-520c-3p	0.968	4.222	1.048
hsa-miR-524	1.141	0.419	0.197
hsa-miR-545#	0.951	0.990	2.149
hsa-miR-550	0.905	0.963	2.126
hsa-miR-559	0.765	0.002	1.223
hsa-miR-564	0.975	1.833	4.284
hsa-miR-572	0.929	3.800	2.001
hsa-miR-589	0.936	1.931	2.091
hsa-miR-590-3P	0.918	1.971	4.225
hsa-miR-604	0.977	7.765	4.323
hsa-miR-624	0.887	0.904	4.118
hsa-miR-625#	0.933	1.932	0.518
hsa-miR-628-3p	0.941	2.018	2.176
hsa-miR-629	0.946	2.024	1.084
hsa-miR-635	1.022	3.635	1.571
hsa-miR-638	0.918	3.910	8.592
hsa-miR-639	0.862	1.828	1.851
hsa-miR-744#	0.954	1.968	8.821
hsa-miR-9#	0.943	0.985	1.073
hsa-miR-92a-1#	0.927	1.947	2.135
hsa-miR-93#	0.943	0.986	1.066
hsa-miR-941	0.938	2.021	2.133
hsa-miR-99b#	0.942	1.001	2.152