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Role of Micro RNA 148/152 Family in Cancer Progression

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ROLE OF MICRO RNA 148/152 FAMILY IN CANCER PROGRESSION

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DEDICATION

I would like to dedicate this thesis to my family, especially my husband Kishore, for their unending and unwavering love and support.

ACKNOWLEDGEMENTS

I would like to start by expressing my gratitude towards my mentors, Dr. Michael Shtutman and Dr. Mythreye Karthikeyan for their guidance, encouragement, and assistance throughout the entirety of this research project. I will always be thankful to Dr. Hosfeth, the program director and Dr. Pittman, my committee chairman for their excellent and candid advice.

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ABSTRACT

Micro RNA are small single stranded RNA that regulate the expression of various genes. MiRNA guide the mRNA disintegrating RISC complex to the complimentary sequence in the target mRNA. Each micro RNA has multiple targets and can play a different biological role depending on the population of targets at the particular stage of the cell or a physiological state. Several miRNA show elevated or decreased levels of expression in various cancers because of their role in tumor initiation and progression. MiRNA belonging to mir148/152 family are examples of such MicroRNA. This family includes miR148a, miR148b and miR152. MiR148a and miR152 are down-regulated in various cancers while there is no significant change in the expression of miR148b. Prior studies indicate a role for miR152 and mir148a in suppressing tumor growth in various cancers. However, the mechanism of action and targets remain to be identified.

Interestingly, miR148b and miR152 are encoded within the first intron of COPZ1 and COPZ2 genes respectively. This study started with the understanding of the role of miR152 in cancer progression. COPZ2 gene expression is lower in malignant tissues of different tumor types when compared to benign tumors suggesting a similar expression pattern for miR152. We have identified several candidate targets for miR152 in the TGF- β pathway including DNMT1, LTBP1, SERPINE1 and the Rho GEF -LARG. This data led to the hypothesis that miR152 expression correlates with tumor progression and

that miR152 suppresses epithelial to mesenchymal transition (EMT) and EMT related events through regulation of specific EMT mediators and effectors. However, all the members belonging to this family have the same seed sequence that binds to the target genes, suggesting that they play similar roles and have common targets.

In this thesis, I have verified LTBP1 and PAI1 as novel, common targets for the miR148/152 family. Additionally, reintroduction of these microRNA into prostate cancer cell lines can decrease the migratory capabilities and increase the adhesive characteristics. This biological role of these miRNA can be exploited in the form of replacement miRNA in cancer therapy.

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LIST OF ABBREVIATIONS

EMT	Epithelial to Mesenchymal Transition
mRNA	Messenger RNA
miR.....	Micro RNA
miRNA	Micro RNA
qPCR.....	Quantitative Polymerase Chain Reaction
PCR.....	Polymerase Chain Reaction
RT-qPCR.....	Reverse Transcriptase – Quantitative Polymerase Chain Reaction
RT-PCR.....	Reverse Transcriptase Polymerase Chain Reaction

CHAPTER 1

INTRODUCTION

Cancer is a complex disease that is characterized by uncontrolled cellular proliferation, unlimited replicative potential, resistance to apoptosis and invasive capabilities.[1, 2] It is the leading cause of death in the United States of America, next only to heart disease, claiming more than half a million lives each year [3]. Cancer is a multistep process in which cells acquire a series of mutations which cause uncontrolled cell growth resulting in the formation of a tumor[2]. Eventually, these cancerous tumors undergo metastasis, a process during which some cells break away from the tumor and invade other parts of the body[4]. The initiation of cancer is associated with many genetic and epigenetic alterations which accumulate over time and drive the advancement from normal cell to a malignant phenotype.[2, 5] This facilitates changes in the expression levels of the genes that are part of important regulatory pathways in place to prevent the transformation of a normal cell into a malignant phenotype. Gene expression can be altered by many mechanisms that cause transcriptional or translational regulation. Post transcriptional or translational repression by micro RNA is one such mechanism, which is being extensively studied due to its emerging role in cancer maintenance and progression.

1.1 Epithelial to Mesenchymal Transition

Cancers of the epithelial tissue like breast and prostate are the most common forms of cancer observed in the clinic. In majority of the cases, cancers of epithelial tissue are not

life threatening and surgical removal of the tissue in an early stage can often cure the cancer. When the cancer metastasizes, it leads to poor prognosis, often resulting in death, as it disrupts vital organs and tissues like liver, lungs, brain and bone marrow. Therefore, it is important to understand the progression of metastasis to prevent and treat invasive malignancies.

Epithelial to Mesenchymal Transition (EMT) is the process by which epithelial cells morph into mesenchymal cells due to loss of polarity and cell-cell adhesion, and gain migratory and invasive properties [6]. While EMT is an important program that is required for embryonic development, wound healing and tissue regeneration, it plays a more ominous role in cancer progression, where EMT is associated with the conversion of localized, early tumors to invasive malignancies [7, 8]. EMT is initiated by a number of biochemical changes in the polarized epithelial cells that are attached to the basement membrane via its basal surface[9]. This results in the alteration to mesenchymal phenotype with enhanced migratory capabilities, invasiveness, increased resistance to apoptosis, and elevated levels of ECM components like fibronectin [10]. Degradation of underlying basement membrane and the migration of a mesenchymal cell from the epithelial layer in which it originated marks the completion of EMT [10].

Many molecular processes are involved in this process; activation of transcription factors, expression of specific cell-surface proteins, production of ECM-degrading enzymes, reorganization of cytoskeletal proteins, and changes in the expression of specific microRNAs [11]. While some transcription factors, cell surface proteins and enzymes have been extensively studied and used as biomarkers to assess the stage of EMT, new

components, like certain micro RNA, that can either hinder or facilitate the process of EMT are being discovered and studied every day.

1.2 Micro RNA

Micro RNA are short, single-stranded non-coding RNA, which are about 19-24 nucleotides in length. The main known function of micro RNAs is the down-regulation of target gene expression by translational repression or degradation of mRNAs upon binding to the consensus sequence on the target mRNA [12, 13]. Every miRNA can potentially regulate the expression of numerous protein-coding genes and ~30% genes are regulated by microRNAs [14]. Even though more than a 1000 microRNAs are expressed in human cells, not all of them are equally important. High throughput screenings of diverse systems have identified certain miRNAs as principal regulators of major cell functions in various physiological (cell cycle, differentiation, development, and metabolism) [15-17] and pathophysiological (immune and neurodegenerative disorders, diabetes and cancer) [18, 19] settings.

1.2.1 Biogenesis

Genes coding for the miRNA are evolutionarily conserved and can be located in the introns or exons of protein coding genes (70%) or the intergenic regions (30%)[20, 21]. MiRNA biogenesis and maturation is compartmentalized between the nucleus and cytoplasm[22]. MiRNA genes are transcribed by RNA polymerase II to generate long primary transcripts called pri-miRNAs [23]. Intronic miRNAs or Mirtrons are derived from splicing of host gene's mRNA or by cropping during transcription [24, 25]. Drosha, an RNase III-type endonuclease, processes the pri-miRNAs to hair-pin pre-miRNA, approximately 70 nucleotides in length [26]. The pre-miRNA hairpin is then exported from

the nucleus into the cytoplasm, where it is processed by another endoribonuclease, Dicer, into the mature double stranded, but unstable 19-25 nucleotide (miRNA) [27, 28]. Depending on the stability, one of the two strands in the duplex is incorporated into the RNA-induced silencing complex (RISC), a multiple-protein nuclease complex [29]. The incorporated strand is called the guide strand and the other strand is called the passenger strand [28].

1.2.2 Mechanism of Gene Regulation by miRNA

The approximately 6 nucleotide long sequence 5' end of the miRNA, called the seed sequence, has a complementary sequence on the mRNA[30]. Once incorporated into a RISC, the miRNA guides the entire RISC complex to the target mRNA by imperfect pairing, i.e., they are exactly not complementary[29]. It can then regulate the target genes by degradation of the mRNA through direct cleavage or by inhibiting protein synthesis by inhibition of translation initiation or elongation[31]. Putative targets of a miRNA can be computationally predicted by matching seed sequences of miRNAs to target mRNAs.

1.2.3 MiRNA and cancer

A number of miRNAs have been shown to be deregulated in cancer [15, 18, 32-35]. The deregulation of miRNAs can be a result of deregulation of upstream regulators of the miRNAs that has a direct result of changing the expression of their target proteins. As a result, important pathways that are in place to prevent cancer progression can be deregulated. An miRNA expression profile or signature can be attributed to certain malignancies and can also be used for diagnosis and to predict the outcome of the disease [36-38]. Two broad classes of genes, oncogenes and tumor-suppressor genes play an

important role in the induction and progression of cancer. MiRNA can also be grouped in these two categories, based on their targets and their expression profiles.

1.3 MiR148/152 family

MiR148/152 family comprises of three micro RNAs; miR148a, miR148b and miR152. All three micro RNA have the same seed sequence and have different bases in three positions; 9, 10 and 20. MiR148/152 family members have been studied individually, and in some cases miR148a and miR152 have been studied together and it has been shown that MiR148a and miR152 are down regulated in a number of cancers [39-45] and have also been known to play a role in immune response[46]. The most important targets that have been validated for miR148a and miR152 are DNA methylases, DNMT1 and DNMT3b. This has an important effect on global methylation that can silence non-target genes like E-cadherin (CDH1) and GSTP1 [40, 41, 44, 47, 48].

1.3.1 MiR148a

MiR-148a is the most comprehensively studied of the three miRNA belonging to this family. The gene coding for miR148a is intergenic and is present on chromosome 7[49]. It has been identified as a tumor suppressor miRNA due to its targets and the observation that it is downregulated in multiple cancers like prostate, breast colorectal and gastric cancers[42-45, 50, 51]. DNMT1, DNMT 3b, CDC25B, MSK1, Bcl-2, IGF-IR, IRS1, ERBB3, TGIF2, PXR, CAND1, p27, HLA-C, ACVR1, HLA-G, WNT10B, PTEN, and ROCK1 are some of the validated targets of miR148a, illustrating its role in various molecular mechanisms like cell growth and proliferation, migration, invasion, apoptosis, cell cycle progression, etc. [39, 42, 48-60]. Its function in normal tissue has been examined but not extensively. miR148a expression is lower in mesenchymal stem cells when

compared to the embryonic stem cells[54]. It is most abundant in the liver tissue[61] and it has been shown that miR-148a abrogates Met/Snail signaling in metastatic hepatocellular carcinoma by downregulating Met, thereby having a weakening effect on the EMT program[62].

1.3.2 MiR148b

MiR148 is an intronic miRNA with the gene being encoded within the first intron of COPZ1 gene on chromosome 12[49]. It has been shown to be downregulated in some liver cancer cell lines, oral, pancreatic, colon and gastrointestinal cancers [63-67], while it has been shown to be unchanged or upregulated in breast and prostate cancers [68, 69]. It has been speculated that the presence of the miRNA within the intron of COPZ1 is responsible for its positive expression in some cancers despite having the same seed sequence as tumor suppressor miRNA (miR148 and miR152). CCKBR and CCK2R, which are involved in cell proliferation have been validated as targets in gastric and colorectal cancers respectively, and MCL1 is another probable target [49, 67, 70].

1.3.3 MiR152

MiR152 is also an intronic miRNA and the gene is encoded within the first intron on COPZ2 gene on chromosome 17. COPZ2 gene encodes for a coatamer protein complex subunit $\zeta 2$ and is not a tumor suppressor, but MiR152 has been shown to be downregulated along with its host gene, COPZ2, in breast and prostate cancers[69]. It has been perceived that COPZ2 is downregulated due to the presence of gene for miR152 in its intron. MiR152 is also downregulated in ovarian, gastric, colorectal and endometrial cancers [40, 47, 71]. CSF1, E2F3, MET, Rictor and MLH1 have been validated as targets in at least one cancer

type. By downregulating these targets, miR152 plays an important role in cell growth, motility and adhesion [47].

1.4 Previous Studies and Objective of the Research

The study of this family of miRNA did not start with micro RNA screen but with a genome wide genetic suppressor element (GSE) screen performed by Shtutman et.al. , to identify the genes on which cancer cells are dependent on for their survival. This screen identified that many cancer cell lines were dependent on COPZ1 for their survival [69]. COPZ1 gene encodes for the ζ subunit of the COP1 coatomer complex [72]. An isoform of this subunit is encoded by COPZ2 gene. Further analysis determined that this dependence on COPZ1 by cancer cells is due the downregulation of COPZ2 gene in various cancer cell lines. In normal cells, the loss of COPZ1 is not detrimental as COPZ2 gene compensates for the loss and ensures continued COP1 function. However, in various cancer cell lines, COPZ2 is downregulated and ζ 2 isoform is not produced to compensate for the loss, causing cancer cells to be dependent on ζ 1 for continued COPI function. This determined that this dependence on COPZ1 is not a classic ‘oncogene addiction’ phenomenon. Instead, the authors called it ‘isoform addiction’. A high level of COPZ2 expression is not detrimental to tumor growth indicating that COPZ2 is not a tumor suppressor[69]. Both COPZ1 and COPZ2 genes encode precursors for miR148b and miR152 within their introns. As discussed earlier, miR152 has been shown to have tumor suppressor activity, indicating the most plausible reason for COPZ2 downregulation.

As shown in Figure 1.1, COPZ2 expression is downregulated in breast (MDAMB-231), fibrosarcoma (HT1080) and prostate (PC3) cancer cell lines when compared to a

normal fibroblast cell line (BJ-HTERT). Biogenesis of intronic miRNA indicate that miRNA transcript is derived from the host transcript [24, 25]. MiRNA micro array expression profiling has revealed that the expression of an miRNA correlates with the expression of its host gene[21]. In concert with that, Shtutman et.al. have shown that miR152 expression correlates with the expression of COPZ2 (Figure 1.2). Analysis of microarray data from GEO database revealed that COPZ2 was strongly downregulated in metastatic prostate cancer and melanoma when compared to their respective benign tumors [69]. This indicates a similar expression pattern for miR152.

Members of the miR148/152 family have the same seed sequence, suggesting that they play a similar biological role in cancer progression. Decreased expression on miR152 in advanced stage tumors and with a number of predicted targets being involved in EMT indicates a possible role for this miRNA family in EMT. We have used prostate cancer model for the experiments here with a hope of extending these results to other carcinoma types in the future.

1.4.1 Hypothesis and Specific Aims

Hypothesis: Members of miR148/152 family inhibit cancer progression and are hence downregulated in cancer.

Specific Aim 1: To discover novel targets of miR148/152 family with a possible role in cancer progression. (Chapter 2)

Specific Aim 2: To analyze the possible biological role of Mir148/152 family in cancer progression. (Chapter 3)

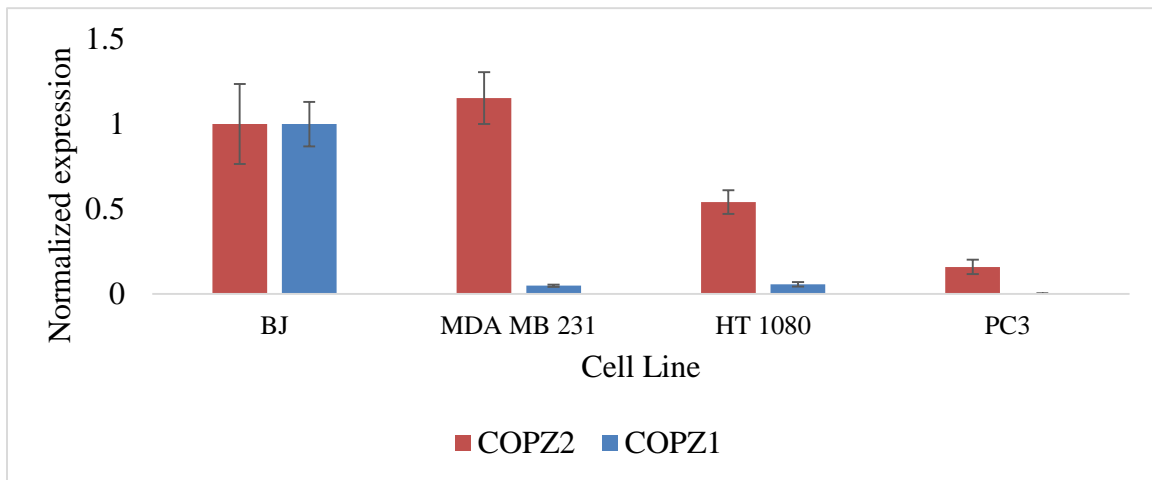


Figure 1.1: Downregulation of expression of COPZ2 in various cancer cell lines compared to the expression of COPZ1. qRT-PCR analysis of expression of COPZ1 and COPZ2 genes in various tumor cell lines and BJ-hTERT fibroblasts. Expression is normalized using GAPDH and is presented relative to expression in BJ-hTERT cell line.

Tumor Cell line	Tumor Type	COPZ2		miR152	
		Mean	SD	Mean	SD
BJ-hTERT	Immortalized fibroblasts	1.000	1.000	6.89E-02	1.00
A549	Lung	0.084	1.28E-02	0.30	1.05E-02
HT1080	Fibrosarcoma	0.088	2.86E-03	0.34	5.80E-02
WM793	Melanoma	0.310	7.76E-03	1.78	1.12E-01
MCF7	Breast Cancer	0.010	4.28E-04	0.14	2.27E-03
MDA MB 157	Breast Cancer	0.004	1.24E-03	0.05	3.30E-03
MDA MB 231	Breast Cancer	0.019	5.98E-04	0.05	1.10E-02
PC3	Prostate Cancer	0.001	2.13E-05	0.01	7.91E-04
Raji	Burkitt Lymphoma	0.004	5.79E-04	0.11	5.47E-03
Ramos	Burkitt Lymphoma	0.010	6.06E-05	0.10	1.10E-03
Jurkat	T-Cell Leukemia	0.008	7.09E-03	0.10	2.86E-04

Figure 1.2: Expression of COPZ2, and miR-152 in human tumor cell lines. Expression is presented relative to BJhTERT. (Adapted from Shtutman et.al., 2010 [69])

CHAPTER 2

NOVEL TARGETS OF miRNA 148/152 FAMILY

2.1 INTRODUCTION

A single miRNA has multiple targets which regulate different regulatory pathways depending on the mRNA population of the cell at a given time[15]. A number of targets have been investigated and validated for miR148/152 family in different cancers [12-22]. Due to the predicted involvement of this family in the regulation of EMT, we have decided to look for novel targets that are involved in EMT. This led to a list of genes that are not only important for EMT but also for other cellular functions; ARHGEF12 or LARG, LTBP1 and PAI1 (SERPINE1) [73-94].

LTBP1 gene codes for latent transforming growth factor - beta binding protein 1. It is required for $\alpha v\beta 6$ -mediated TGF- β activation, one of the three activation mechanisms of TGFb [73-76]. LTBP1 has been shown to play a role in cancer progression mostly via the stromal cells or extracellular matrix [77, 78]. LTBP1, being a modulator of TGFb levels and activity, plays a role in TGFb mediated EMT [79, 80].

SERPINE1 gene encodes for Plasminogen activator inhibitor-1 also known as endothelial plasminogen activator inhibitor or serpin E1 protein. PAI-1 is a serine protease inhibitor which principally inhibits tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), which are key enzymes for the initiation of

fibrinolysis [81]. PAI-1 not only regulates the lysis of blood clots, but also a wide variety of biological reactions occurring in extracellular matrices such as tumor metastasis, neovascularization, inflammation, and cell migration[82]. TGFb induced EMT was inhibited upon the introduction of PAI1-siRNA indicating a role for PAI1 in the progression of EMT[83]. Decrease in PAI1 levels correlates with reduced epithelial cell migration and tumor progression [84-86].

LARG gene codes for Rho guanine nucleotide exchange factor 12 protein. Like other Rho GTPase proteins, it has functions in a number of cellular processes. In contrary to other targets of the miR148a/152 family, LARG has been shown to be a tumor suppressor gene in breast and colorectal cancers[87].

Rictor codes for Rapamycin-insensitive companion of mTOR protein. RICTOR is a component of the mTOR-containing complex, mTORC2, which directly regulates the phosphorylation of Akt, which mediates a variety of biological responses including cell growth, proliferation and survival [88, 89]. RICTOR has been shown to be upregulated in various cancers [90-92]. Increased RICTOR expression has been shown to be associated with EMT [93, 94].

2.2. MATERIALS AND METHODS

2.2.1 Cell lines

Two different prostate cancer cell lines were used in this study, DU145 and PC3. Four stable cell lines each of PC3 and DU145, overexpressing the pre- MiRNAs 148a, 148b, 152 and a cell line with empty vector control were established using lentiviral infection in Dr. Shtutman's lab. The plasmids carrying the respective microRNA also have an insert

for Green Fluorescent Protein and the cells are sorted using Fluorescence-activated Cell Sorting to obtain a pure population of cells overexpressing the MicroRNA. The expression of microRNA in the cell lines is then validated using qPCR. These stable cell lines will be used for all subsequent experiments to study the role of the MicroRNA in cancer. All the cells were grown in DMEM/High glucose medium (HyClone™) with 10% FBS serum (HyClone™), 1% penicillin (100 units/ml), 1% streptomycin (292 µg/ml) and glutamine (2 mM). A standard, sterile cell culture technique was used to culture the cells. Typically, cultures grown to 70-80% confluency were used for the experiments. Cell lines were maintained in culture for about two months before starting a fresh culture.

The normal foreskin fibroblast line, BJ-hTERT immortalized by re-expression of the hTERT protein, along with transformed lines, BJ-ELB and BJ-ELR, which overexpress RAS and myc, are also used in some studies. BJ fibroblasts were cultures in DMEM/High glucose medium with 10% FBS, 2mM L-Glutamine, 10mM sodium pyruvate and 20% medium 199.

2.2.2. Identification of targets

An initial list of genes involved in EMT was obtained from PCR microarray by Sabiosciences™. The genes were then individually checked for miR148a, miR148b and miR152 binding sites using TargetScanHuman™.

2.2.3. Cell lysis and RNA preparation

Total RNA was extracted using Trizol purification method. For a 100 mm dish, media was aspirated and 1 ml of Trizol reagent was used scrape the cells and placed in an Eppendorf tube. 200 µl of chloroform was added mixed and incubated for 2–3 minutes and

centrifuged at 12000 g for 15 min at 4°C. The aqueous layer was transferred to a fresh tube, 500 µl of isopropanol was added and incubated for 10 minutes at room temperature. The samples were centrifuged at 12000 g for 10min at 4°C. Supernatant was discarded and the pellet was then washed with 1 ml 75% EtOH, spun at 7500 g for 5 min. The supernatant is removed and the pellet is dried at room temperature for 10-15 minutes. The pellet is then resuspended in 20 – 50 µl nuclease free water and the RNA quantity and quality was determined at 260/280 nm using a spectrophotometer (Nanodrop™ Technologies).

2.2.4. Modification and amplification of mature miRNA's

The RNA obtained from Trizol purification was used. This method was only used for evaluating the expression of miRNA even though the nascent mRNA is also modified during this procedure. Mature miRNAs were first modified by the addition of a polyA using E-PAP enzyme from NEB Inc. 1 µg of total RNA is mixed with 2 µl E-PAP buffer, 2 µl ATP and 3.5 µl E-PAP enzyme. The final volume is made up to 20 µl using nuclease free water and incubated at 37°C for one hour.

2.2.5. Reverse transcription

Two different reverse transcription methods were used, one for miRNA and the other for mRNA. For miRNA, reverse transcription was performed using Bioline Tetro cDNA synthesis kit and 20 µl of E-PAP treated RNA. For ac sample, 1 µl universal RT primer and 1 µl dNTP mix was added and incubated at 65°C for 10 minutes and on ice for 2 minutes. Then, 4 µl 5X RT buffer, 1 µl RNase inhibitor and 1 µl reverse transcriptase were added and incubated at 45°C for 60 minutes and at 70°C for 15 minutes. The final product is the diluted 1:10 and used for qPCR.

Maxima First Strand cDNA Synthesis Kits for RT-qPCR was used for reverse transcription of mRNA. For each sample, 2 µl of the 5X reaction mix and 1 µl Maxima™ enzyme mix is added and made up to 20 µl using nuclease free water. The samples are then placed in a thermal cycler to be incubated at 25°C for 10 minutes, 50°C for 30 minutes and 85°C for 5 minutes. The reaction is then diluted 1:10 and used for RT-PCR or qRT-PCR or stored at -20°C for short term storage.

2.2.6. RT-PCR:

Primers are designed using NCBI primer blast and ordered from IDT. They are diluted to 100 µM and stored at -20°C. Primer mixes containing both forward and reverse primers are made with the concentration of each primer being 10 µM. The cDNA generated from reverse transcription is used for PCR. The experiment is performed in duplicates using Promega™ PCR master mix. For each sample, 12.5 µl master mix, 5 µl cDNA, 2.5 µl nuclease free water and 5 µl primer mix is added. The samples are then subjected to initial denaturation (95°C for 2 minutes) and 25 cycles of denaturation (95°C for 45 seconds), annealing (55°C for 45 seconds) and extension (75°C for 20 seconds) followed by final extension (75°C for 5 minutes). The samples are then run on a 2% agarose gel and bands are visualized using Enduro™ Gel Documentation System. ImageJ™ is used to quantify the bands. Expression levels are normalized to GAPDH expression. Primers and their sequences are listed in Table 2.1.

2.2.7 qRT-PCR:

The reactions are performed in quadruplicates in a 384 well plate using SYBR Green PCR master mix. For each reaction, 0.5 µl of primer mix, 5 µl SYBR Green and 4.5

µl cDNA was added. Applied Biosystems 7900 HT Fast Real Time PCR system was used for quantification and amplification. Expression levels are normalized to house-keeping genes, GAPDH or RPL13A.

2.2.8 Statistical Analysis:

Differences between the groups was compared using two-tailed paired student's t test. Family-wise error rate was corrected using Bonferroni correction. P-value less than 0.05 was considered statistically significant.

2.3. RESULTS

2.3.1 Putative targets of miRNA148/152 family

A list of putative targets was obtained with the help of SabioscienceTM's database and TargetscanTM. Concurrent with the hypothesis that miR148/152 family plays a role in EMT, various genes involved in EMT were blasted for probable binding sites of miR148a, miR148b and miR152. Targetscan's ability for target prediction was evaluated by first looking for the binding sites of the validated target of this family, DNMT1 and RICTOR. Owing to the similarity in their sequences, it was expected that possible targets will show binding sequences to more than one miRNA belonging to this family. Consistently, it has been observed that the targets had binding sequences for all three miRNA belonging to miR148/152 family. Three novel targets have been identified using this protocol; LARG, LTBP1 and PAI-1 (SERPINE1) (Figure 2.1). Though RICTOR was only validated as a target of miR152, TargetScanTM predicted it as a putative target for the other two miRNA in this family.

2.3.2 Validation of putative targets

Potential targets were validated using RT-PCR and qRT-PCR in the eight stable cell lines (Figures 2.2); DU145 and PC3 cell lines each overexpressing one of the miRNAs of miR148/152 family or the control miRNA. DNMT1, being a validated target, was used as a positive control (Figure 2.3-2.4). Accordingly, DNMT1 was downregulated in all the cell lines, albeit to a different level. Expression of DNMT1 was also evaluated in a series of BJ cell lines; BJhTERT, a normal immortalized cell line, BJ-ELB, partially transformed cell line and BJ-ELR, a fully transformed cell line. These cell lines have decreasing COPZ2 levels in the order of increasing tumorigenicity, suggesting decreasing levels of miR152. Figure 2.4 shows the increase in the expression of DNMT1 in the same order. RICTOR, which is a validated target of miR152, was downregulated in both PC3 and DU145 cell lines overexpressing miR152. Interestingly, RICTOR was also downregulated with the overexpression of the other two miRNAs (Figure 2.5). Two of the predicted targets, LTBP1 and LARG, are also downregulated in all the cell lines (Figures 2.6, 2.7). PAI-1, another potential target was downregulated in all the cell lines, except the PC3 cell line overexpressing miR148b (Figure 2.8).

2.4 Discussion

DNMT1, a DNA methylase, is an important validated target of miR148a and miR152. It has also been shown that there is inverse correlation between the expression of DNMT1 and miR148a/miR152 (Figure 2.4) and that DNMT1 hypermethylates and thereby silences miR148a and miR152 [95]. miR148b has been shown to be silenced as a result of

hypermethylation of the promoter region in pancreatic cancer [45] and a number of metastatic cancers. The reintroduction of miR148a into the cancer cells with epigenetic inactivation inhibited their motility, reduced tumor growth, and inhibited metastasis formation in xenograft models [96]. Hypermethylation of the CpG islands in the promoter region of miR152 by DNMT1 has been shown to be responsible for the silencing of miR152 in endometrial cancer [47] and it has been associated with poor clinical outcome in acute lymphoblastic leukemia (AML) [97]. Increased DNA methylation of miR148a and miR152 promoter regions has been found in colorectal, gastric and endometrial carcinomas when compared to the normal tissues [98].

Any alteration in the methylation pattern has been shown to accompany the process of tumorigenesis be it hypomethylation or hypermethylation [96, 99]. Gene expression profiling of breast cancer model system suggested that there was DNA hypermethylation silenced a number of genes involved in cell adhesion and tight junction formation; CDH1 (E-cadherin), CGN, and CLDN4, in basal breast cancer cells that have undergone EMT. Furthermore, it has been suggested that TGF β -Smad2 signaling and DNMT1 form a positive regulatory loop to maintain epigenetic silencing during epithelial to mesenchymal transition [100]. Comparison of the gene expression profiles between invasive mesenchymal-like MIII cells and the noninvasive epithelial MII cells revealed relatively high expression levels of the downstream targets of TGF β signaling, such as MMP2, SERPINE1, and TGF β I, in MIII cells. Furthermore, expression of TGF β 1 and the TGF β -activating proteins LTBP1, LTBP2, LTBP3, LTBP4, and THBS1 was also dramatically increased in MIII compared with MII cells [100].

Transforming growth factor-beta (TGF-beta) is a multifunctional regulatory cytokine that controls many aspects of cellular function. One of the biological effects of TGF-beta is the inhibition of proliferation of most normal epithelial cells and this suggests a tumor suppressor role for TGF-beta. During the early phase of epithelial tumorigenesis, TGF-beta inhibits primary tumor development and growth by inducing cell cycle arrest and apoptosis via SMAD signaling. In later stages of tumor progression when tumor cells become resistant to growth inhibition by TGF-beta, due to inactivation of the TGF-beta signaling pathway or aberrant regulation of the cell cycle, the role of TGF-beta becomes the one of tumor promotion. TGF β overexpression along with increased nuclear phospho-Smad2 has been observed in advanced tumors [101], and Smad2 has been shown to be involved in the EMT process [102]. To understand if miR152 has any influence on TGF β signaling, we performed a preliminary experiment where we tested the effect of miR152 overexpression on p-smad2/3 levels upon TGF β treatment in DU145 cells. DU145 cells overexpressing miR152 showed decreased induction of p-smad2/3 signaling (Figure A.1 in appendix A). Further experiments with the miR148b and miR148a need to be done to assess the effect of miR148/152 family on TGF β signaling.

LTBP1 expression negatively correlates with the expression of miR148/152 family (Figure 2.5). PAI-1 expression is significantly decreased in all tested cell lines except miR148b overexpressing PC3 cell lines (Figure 2.6), where surprisingly, it is elevated. Also, PC3 cell lines have endogenous miR148b and overexpression might not have changed the expression pattern. LTBP1 regulates the bioavailability of TGF β and downregulation could result in decreased TGF β activity. PAI-I and RICTOR, downstream targets of TGF β signaling, could have decreased levels of expression as a result. RICTOR

has been shown to be induced by TGF β during TGF β mediated EMT, upon which it pairs with ILK, a key intracellular mediator of EMT [93].

Western blots for potential targets can indicate the effect of the miRNA148a/152 family on the protein levels of the potential targets. No conclusion can be made about the targets until a luciferase assay is performed where the potential miRNA binding region is deleted from the promoter and effect on expression is assayed. DNMT1, LTBP1, PAI-1 and RICTOR play a role in epithelial to mesenchymal transition. At least three of these targets are associated with TGF β , which is an important driver of EMT. This suggests a role for miR148/152 family in EMT suppression.

Table 2.1: Primer sequences for RT-PCR and qRT-PCR of the indicated genes

Gene	Sense	Antisense
GAPDH	CCCCGGTTTCTATAAATTGAGCC	ACCAGAGTTAAAAGCAGCCC
RPL13a	AGATGGCGGAGGTGCAG	GGCCCAGCAGTACCTGTTTA
Universal RT	AACGAGACGACGACAGACTTT	
GAPDH		CCATGTAGACCCCTTGAAGA
miR148a		GTGCACTACAGAACTTT
miR148b		TCAGTGCATCACAGAACTTT
miR152		TCAGTGCATGACAGAACTTT
DNMT1	TGTCCAAGAAGAGCAACGG	AGAGCAGGTTGATGTCTGCG
RICTOR	CGCTCTCTGAAGAACCTCCG	TTGATACTCCCTGCAATCTGGC
LTBP1	AACAAGGCAGGCAAGTTGTG	TCTGACATGGCGGAACACAG
LARG	ACACAGTCTACTATCACCGACA	TGCAATGCGCTCAACTTTCTG
PAI-1	AGAGCGCTGTCAAGAAGACC	ACGTAGGATGGGGGATGGTG

Table 2.2 Summary of targets

Target	MiRNA 148a		MiRNA 148b		MiRNA 152	
	PC3	DU145	PC3	DU145	PC3	DU145
DNMT 1	↓	↓	—	↓	↓	↓
RICTOR	↓	↓	—	↓	↓	↓
LTBP1	↓	↓	↓	↓	↓	↓
LARG	↓	↓	↓	↓	↓	↓
PAI1	↓	↓	↑	↓	↓	↓

Figures

a)

Arhgef12(LARG): Binding site – 1153-1160

```
5' ...UUUGACUCUAAUUUUUGCACUGA...
      |||                |||
3'  GGUUCAAGACAGU-----ACGUGACU
```

b)

LTBP1: Binding site: 34-40

```
5' ... UAAGCCCAUAUACUCUGCACUGU...
      |||
3'   GGUUCAAGACAGUACGUGACU
```

c)

SERPINE1: Binding site – 1657-1663

```
5' ...UUUCUUUUUUGAUUUUGCACUGG...
      |||
3'   GGUUCAAGACAGUACGUGACU
```

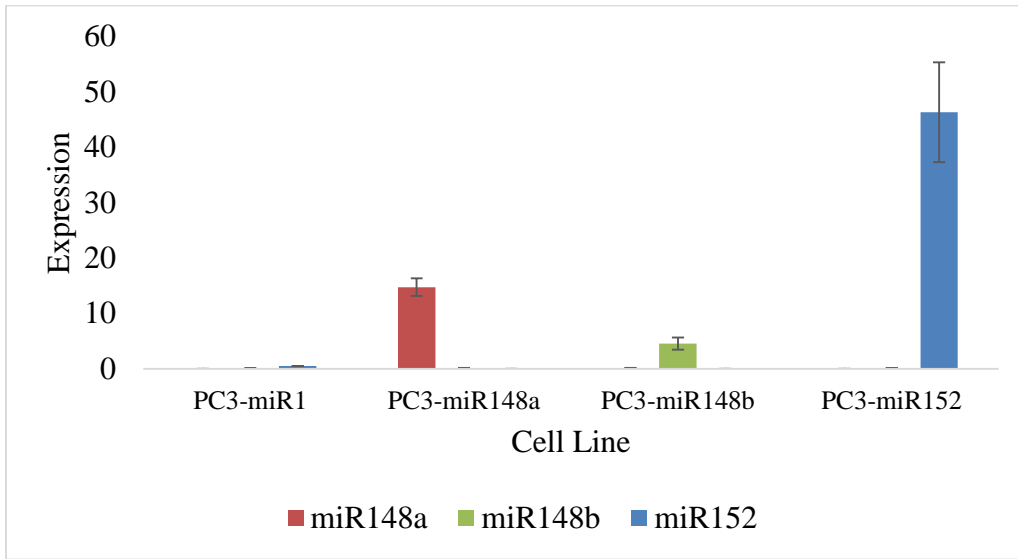
d)

RICTOR: Binding sites – 269-275, 1631-1637

```
5' ...GUAUGCAAGAGCCAAGCACUGAA...
      |||
3'   GGUUCAAGACAGUACGUGACU
5' ...UGAAUUGUUCUUAUGUGCACUGU...
      |||
3'   GGUUCAAGACAGUACGUGACU
```

Figure 2.1 miR148/152 binding sites of putative targets. An initial list of genes involved in EMT was obtained from PCR microarray by Sabiosciences™. The genes were then individually checked for miRNA 152 binding sites using TargetScanHuman™.

a)



b)

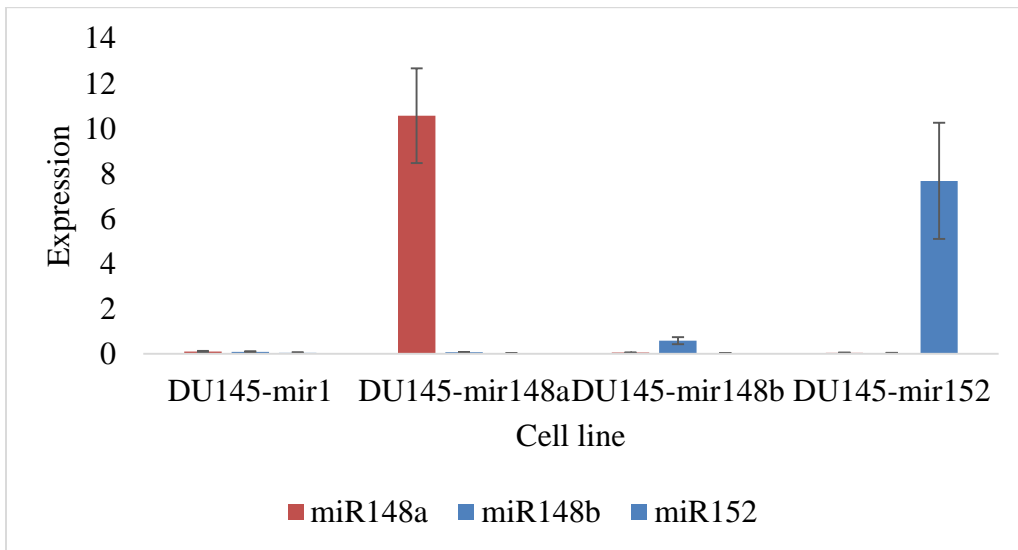
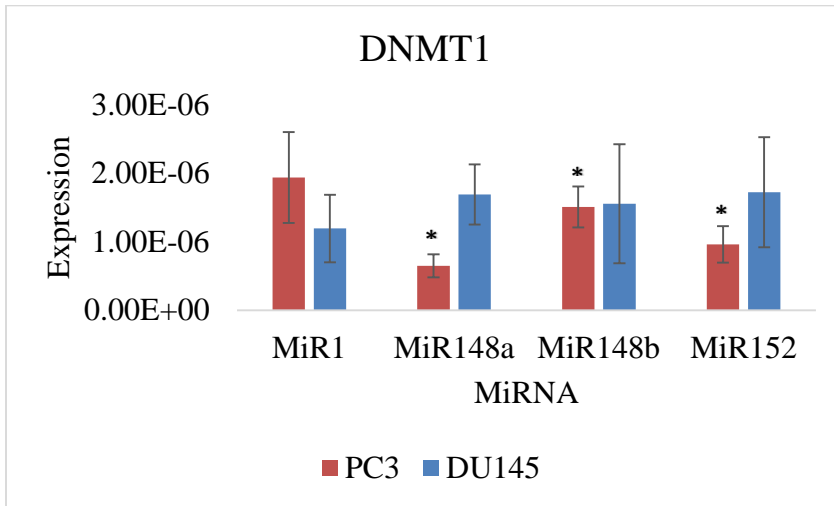


Figure2.2 Expression of miR148a, miR148b and miR152 in a) PC3 and b) DU145 stable cell lines overexpressing individual members of miR148/152 family. qPCR data is normalized to RNU6 and represented as mean of expression and \pm SD.

a)



b)

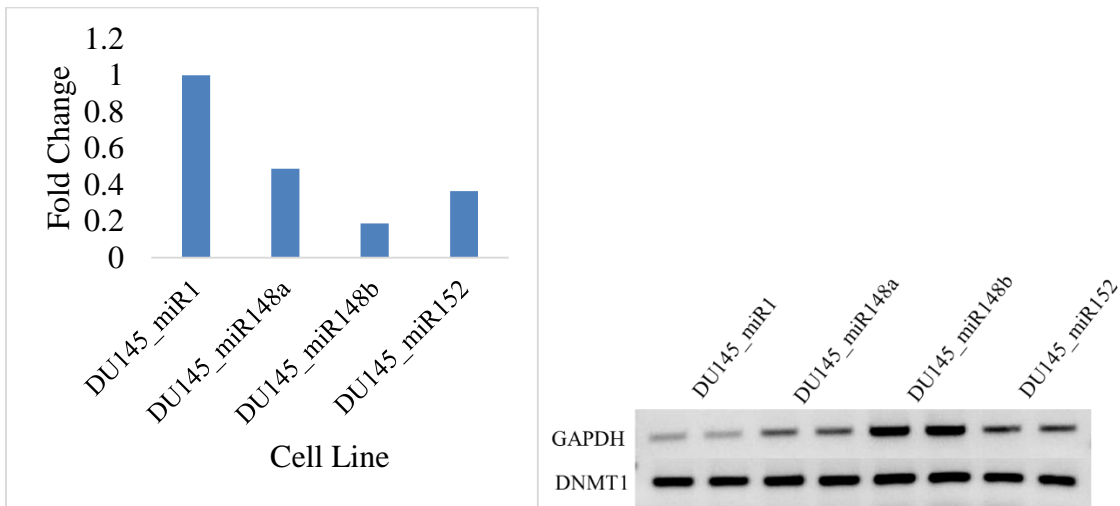


Figure 2.3. Mir148/152 family downregulates the expression of DNMT1 in PC3 cell lines. Expression of DNMT1 in stable cell lines is verified by a) qRT-PCR and b) RT-PCR. RPL13A and GAPDH were used for normalization of qPCR and PCR data respectively. * indicates significant downregulation ($P < 0.05$) of DNMT1 in miRNA overexpressing cell lines when compared to the control cell line.

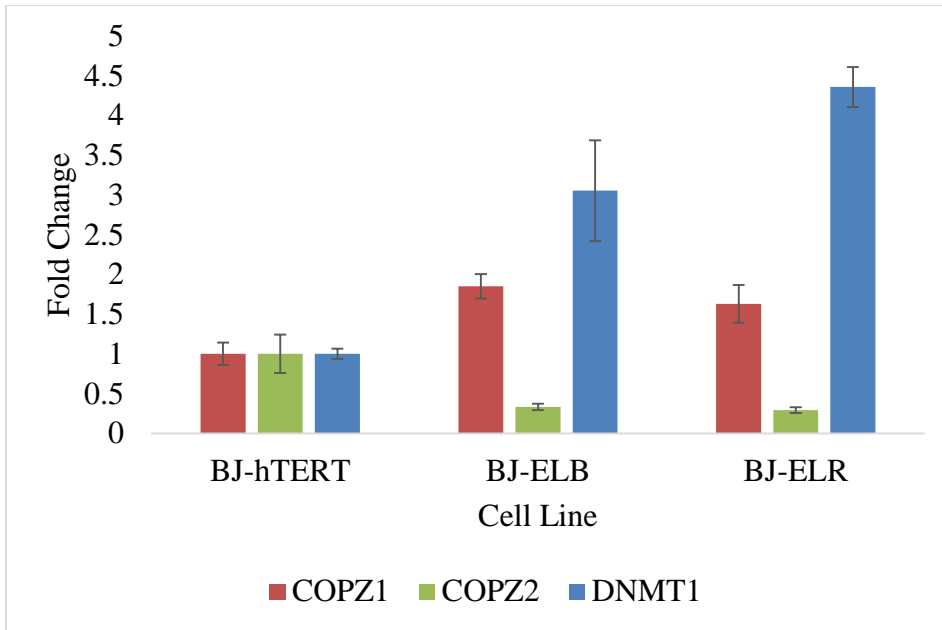
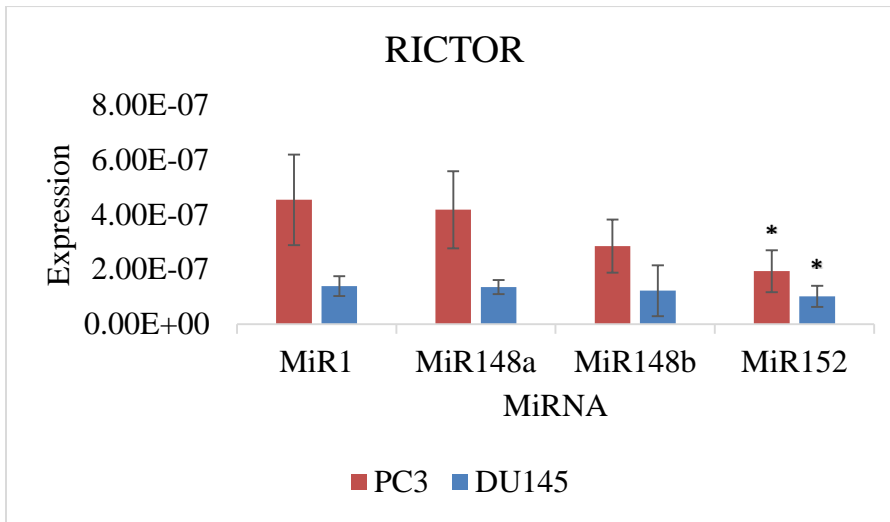


Figure 2.4 DNMT1 expression inversely correlates with COPZ2 expression. The qPCR expression data from transformed cell lines, BJ-ELB and BJ-ELR is shown relative to normal fibroblast cell line, BJhTERT; mean and \pm SD is represented here.

a)



b)

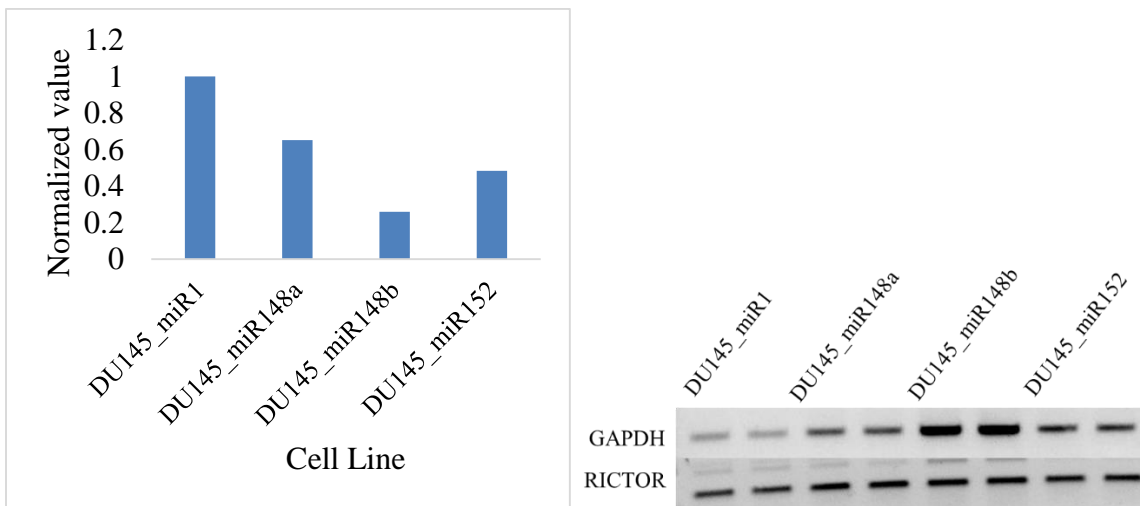
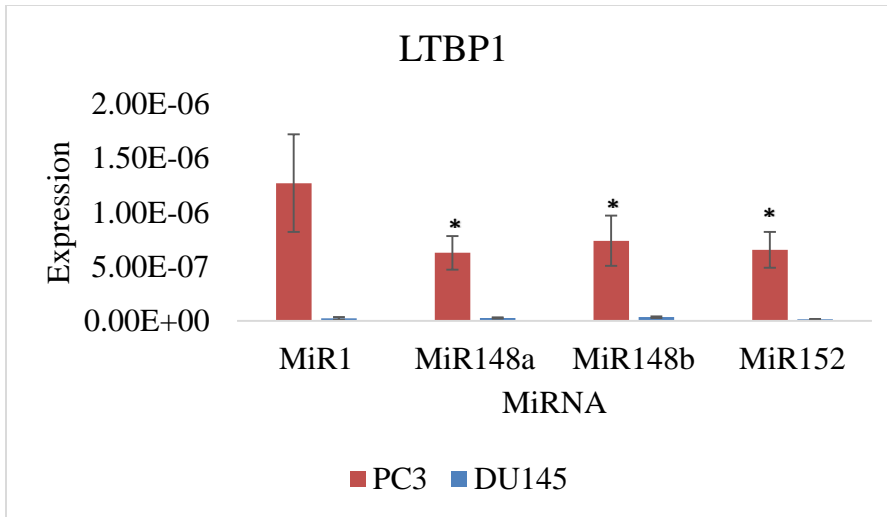


Figure 2.5 MiR152 downregulated the expression of RICTOR. Expression of RICTOR in stable cell lines is verified by a) qRT-PCR and b) RT-PCR. RPL13A and GAPDH were used for normalization of qPCR and PCR data respectively. * indicates significant downregulation ($P < 0.05$) of RICTOR in miRNA overexpressing cell lines when compared to the control cell line.

a)



b)

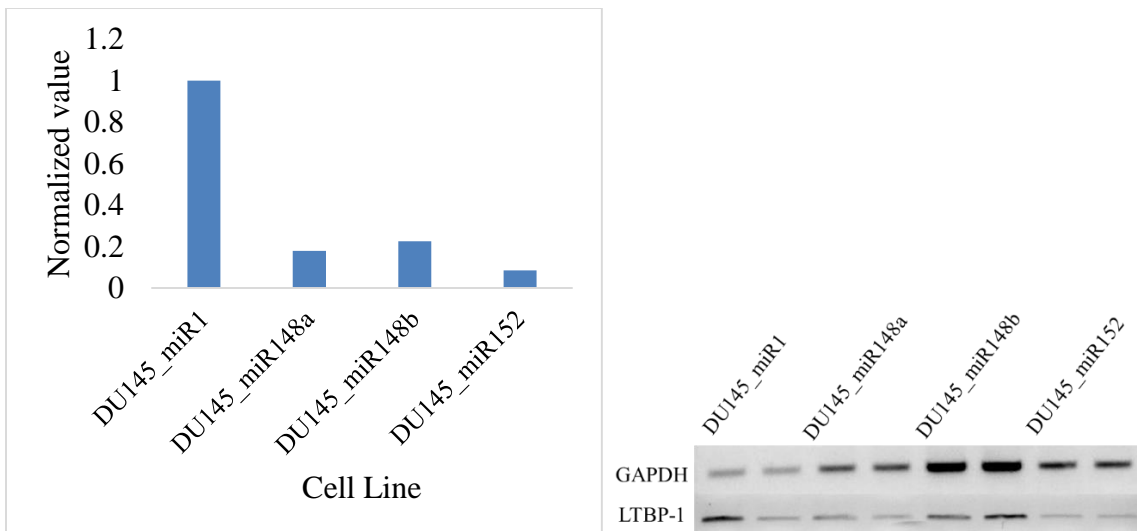


Figure 2.6 Mir148/152 family downregulated the expression of LTBP1 in PC3 cell lines. Expression of LTBP1 in stable cell lines is verified by a) qRT-PCR and b) RT-PCR. RPL13A and GAPDH were used for normalization of qPCR and PCR data respectively. * indicates significant downregulation ($P < 0.05$) of LTBP1 in miRNA overexpressing cell lines when compared to the control cell line.

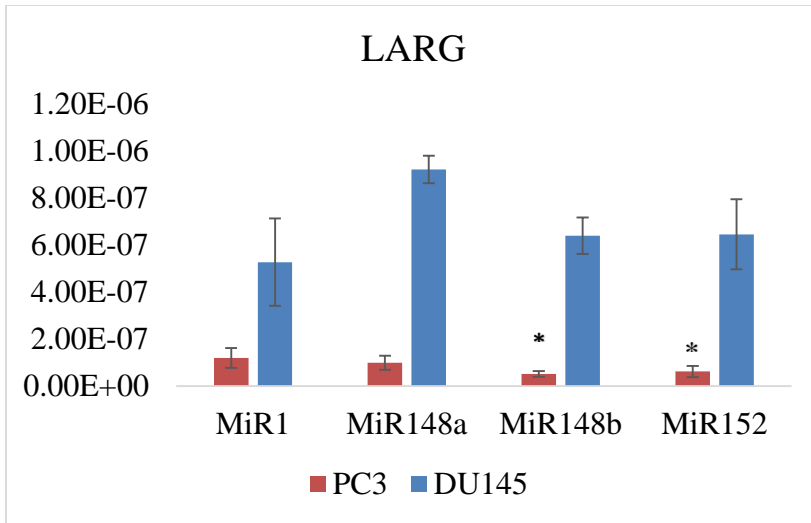


Figure 2.7 Mir148/52 family downregulated the expression of LARG in PC3 cell lines. Expression of LARG in stable cell lines is verified by qRT-PCR. RPL13A was used for normalization of qPCR data. * indicates significant downregulation ($P < 0.05$) of LARG in miRNA overexpressing cell lines when compared to the control cell line.

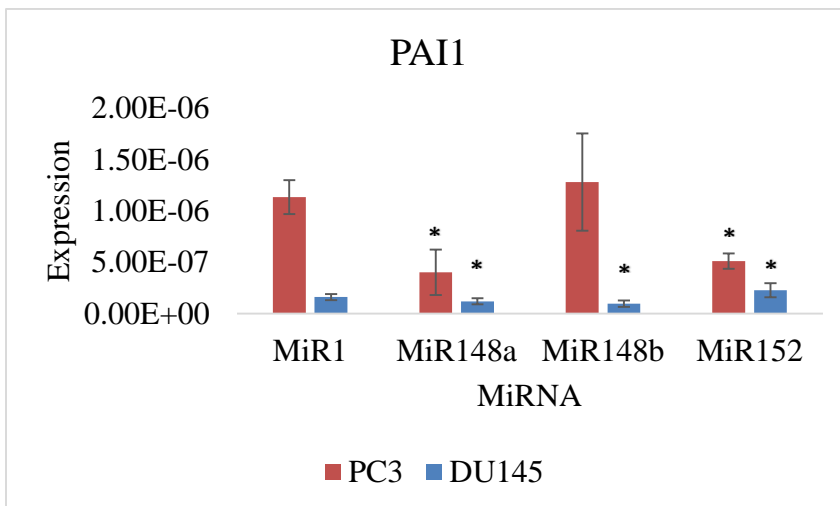


Figure 2.8 Mir148a and miR152 downregulate the expression of PAI1. There is increased expression in PC3_miR148b cell lines. Expression of PAI1 in stable cell lines is verified by qRT-PCR. RPL13A was used for normalization of qPCR data. * indicates significant downregulation ($P < 0.05$) of PAI1 in miRNA overexpressing cell lines when compared to the control cell line.

CHAPTER 3

ROLE OF MIRNA 148/152 FAMILY ON EMT

2.1 INTRODUCTION

Metastasis is associated with poor prognosis and recurrence of malignancy. Epithelial to mesenchymal transition has been shown to play an important role in metastasis[103]. When undergoing the process of EMT, the epithelial cells lose their characteristic differentiated properties like cell–cell adhesion, planar and apical–basal polarity, and lack of motility, and acquire mesenchymal features, including motility, invasiveness and an increased resistance to apoptosis[11, 104]. The process of EMT is associated with changes in the mRNA and protein levels of many genes that are associated with morphological changes. For example, there is decreased expression of E - cadherin, an epithelial cell-cell adhesion molecule and increased expression on N - cadherin, which plays a role in adhesion to endothelial cells[11, 105]. A number of transcription factors regulate the expression of such genes.

To evaluate the role of miRNA 148/152 family in EMT, we studied the migratory and adhesive capabilities of the cell and also the expression levels of important transcription factors involved in EMT – SNAI1, SNAI2, TWIST1 and ZEB. We have also looked at E-cadherin expression levels to investigate one of the morphological changes accompanying EMT. E-Cadherin is downregulated by all the transcriptional factors mentioned above

SNAI1 (SNAIL) and SNAI2 (SLUG) belong to the Snail family of zinc-finger transcription factors[106]. The amino terminus contains SNAG (Snail/Gfi) domain which causes transcriptional repression. The zinc fingers mediate binding to the DNA promoters containing the E-box sequence (CAGGTG) and facilitating transcriptional repression or activation[107]. Apart from E-cadherin, SNAI1 also down-regulates the expression of other epithelial molecules, like Claudins, Occludins and Muc1 and induces the expression of genes associated with a mesenchymal and invasive phenotype, such as fibronectin and MMP9[107].

TWIST-1 is a highly conserved transcription factor that belongs to the family of basic helix–loop–helix (bHLH) proteins[108]. Twist-1 also binds to the DNA promoters containing E-box domain and is involved in the regulation of genes involved in cell proliferation and migration, like, Tbx20, Sema3C, Gadd45a, and Rab39b[109]. ZEB1 is a member of the zinc-finger E-box-binding homeobox factor (ZEB) family and binds to the same consensus sequence as the other three transcription factors. Apart from E-cadherin, ZEB1 is also involved in the repression of other epithelial components like Crumbs 3 and PATJ (Pals-1 associated tight junction protein)[95].

2.2. MATERIALS AND METHODS

2.2.1 Cell Lines

Cell lines described in Chapter 2 were used.

2.2.2 PCR/qPCR

Methods described in the materials and methods section of Chapter 2 were used for RNA purification, reverse transcription, PCR and qPCR.

2.2.3 Wound Healing Assay

Control and test cell lines were grown to >90% confluency in duplicates in a 12 well dish. A scratch was made using a 20 μ l pipette tip. A line was drawn to mark the area for photographs. Olympus IX81 microscope was used to take phase contrast photographs at 10x at regular intervals.

2.2.4 Trans-well migration Assay

Corning 24 well plates with trans-wells are used for this experiment. The trans-wells are coated with one of the three ECM components; collagen, fibronectin and laminin diluted in serum-free media. This is done by incubating the plates with 10 μ g/ml ECM component on the either side of the well for 2 hours at 37°C. The wells are then washed with 1X PBS and stored at 4°C or used for experiment immediately. Upon trypsinization, cells are washed with serum-free media and counted. Depending on the experiment, 20,000 or 40,000 cells are plated onto the upper chamber in 100 μ l of serum free media. Media with 10% FBS is placed in the lower chamber to act as a chemo-attractant. 100pm TGF β was added to the wells when required to assess its influence on the migration. The plates were incubated at 37°C overnight. One of the two following methods was used to quantify the number of migrated cells; 1. The migrated cells are fixed in methanol and stained with eosin Y and methylene blue. The filters are mounted onto a slide, photographed and cells are counted using Cell counter plugin in Image J software. 2.

Migrated cells are fixed onto the filter using 4% paraformaldehyde and stained with 2mg/ml crystal violet. Crystal violet was solubilized in 2% SDS and the intensity was read at 595nm using Synergy™ microplate reader. A filter which was subjected to a similar treatment but with no cells were added was used as a blank.

2.2.5 Adhesion Assay

Adhesion assay is performed in 96 well plates in triplicates. Wells are coated with 10 µg/ml collagen diluted in serum-free media for one hour and blocked with 5% Bovine Serum Albumin) BSA in PBS for 45 minutes. 5% BSA in PBS was used as control to account for the background adhesion. Cells are detached from the plate using 0.5M EDTA and washed in serum free media and 40,000 cells in 50 µl serum free media are plated onto the collagen coated and BSA blocked wells. This assay was performed for three time points; 15 minute, 30 minute and 60 minute. After the required amount of time, media is removed and cells are fixed with 4% paraformaldehyde, stained with crystal violet and washed. Crystal violet was solubilized in 2% SDS and the intensity was read at 595nm using Synergy microplate reader. Two blanks, one each for coated and uncoated wells were used.

2.3 RESULTS

2.3.1 Effect of miR148/152 family on migration

EMT is characterized by increased migratory capabilities of the transformed cells. *In vitro* migration assays were performed on DU145 and PC3 cells overexpressing individual members of miR148/152 family. Since this study was first started to understand the role of miR152 in tumor progression, initial assays were performed only using DU145

cells overexpressing miR152. Scratch assay performed using DU145_miR152 cells did not show any significant difference in the rate of wound closure (Figure 3.1). Single cell migration through ECM component, collagen showed significant decrease in migration, while migration in the absence of an ECM component or through fibronectin and laminin did not show any remarkable difference (Figure 3.2). All three miRNA caused decreased migration in DU145 cell lines while in PC3 cell lines, miR148a showed little change in the migration rate (Figure 3.3).

Results from chapter 1 led to a theory that miR148/152 family plays a role in the suppression of TGF β induced EMT. To this end, we have performed preliminary studies where trans-well migration was accompanied by TGF β treatment of the cells. External compensation of TGF β caused increase in the migration rate of all the cell lines and the percentage of increase was in lieu with the increase in the control cell lines (Figure 3.3).

2.3.2 Effect of miR148/152 family on adhesion

During EMT, cells lose their adhesive properties to detach from the basement membrane and surrounding cells. To verify the effect of miR148/152 family on adhesive properties of the cells, adhesion assay using collagen coated wells was performed in the DU145 stable cell lines. All three miRNA resulted in increased adhesion of the DU145 cells (Figure 3.4). Further studies need to be done with varying concentrations of collagen, and also in the presence of other ECM components. All the experiments need to be repeated using PC3 cell lines before arriving at a conclusion.

2.3.3 Effect of miR148/152 family on the expression of EMT related genes

The expression of various transcription factors that play an important role in induction and progression of EMT and E-Cadherin, cell-cell adhesion molecule, was studied using qRT-PCR. None of these genes is consistently up or downregulated in both PC3 and DU145 cell lines. ZEB1 was downregulated in PC3 cell lines (Figure 3.6) while SLUG was downregulated in DU145 cell lines (Figure 3.7). E-cadherin being the target of all these transcription factors, is expected to be downregulated in these cell lines. Surprisingly, E-cadherin is downregulated in DU145 cell lines and upregulated in PC3 cell lines. However, western blot for the protein levels of E-cadherin revealed no significant difference in the protein levels. Since, mRNA expression levels of these genes do not give a clear indication of their expression levels, further analysis of their protein levels needs to be performed to make any tangible conclusions.

2.4 DISCUSSION

Cell proliferation, migration, invasion and decreased adhesion are the common characteristics of metastatic cells. Two types of migration assays are performed here. Scratch assay represents collective cell migration that typically occurs in the tissues while, trans-well migration assay characterizes single cell migration, and to an extent, invasion. However, the results of both these assays can be influenced by cell proliferation in addition to migration. Previously, MiR152 and miR148a has been shown to have an impact on cell proliferation [43, 69]. Migration through collagen coated filter for a shorter time point of 6 hours did not show a significant difference in the migration rate of DU145 cells overexpressing miR152. The migration assays, when performed under the influence of an

anti-proliferative agent like Mitomycin-C can determine if the migratory effect seen here is due to migration. Dose dependent adhesion assays using different concentrations of collagen can strengthen the conclusion that miR148/152 family increases the adhesion of the cells to collagen. There was decreased in the expression of SLUG and ZEB1 as shown by the qPCR data in all the cell lines. However, analysis of protein levels of all transcriptions factors, SNAIL, SLUG, TWIST1 and ZEB1 along with the study of nuclear localization of these factors would provide a better understanding of the effect of miR148/152 family on the regulation of EMT.

PC3 is an E-cadherin null cell line [110, 111] and overexpression of miR148a and miR152 seems to increase E-cadherin levels while miR148b expression results in decreased E-cadherin levels. E-cadherin expression in DU145 cell lines is an anomaly in this study. While qPCR shows decreased mRNA levels, there is no significant change in the protein levels. Due to the lack of correlation between mRNA and protein levels, it is difficult to come to a conclusion. However, it has been shown that in DU145 cells, P-cadherin, and not E-cadherin is important for maintaining adherens junctions. Also, the depletion of any of the cadherin-associated proteins like p120ctn, β -catenin or α -catenin, is sufficient to disrupt adherens junctions in DU145 cells and increase migration and cancer cell invasion [112]. Analysis of the protein levels of the aforementioned proteins can give a better understanding of the change in migration rate caused by miR148/152 family.

2.5 CONCLUSION AND FUTURE STUDIES

LTBP1 and LARG are potential new and common targets of all the members of the miR148/152 family. RICTOR has been validated as a target of miR152 but it is a possible

new target of miR148a and miR148b. PAI1 has not shown consistent downregulation with both DU145 and PC3 cell lines overexpressing miR148b. Therefore, PAI1 can be a potential new target of miR148a and miR152, if not miR148b. PAI1 plays an important role in the regulation of MMP10 mediated collagen remodeling to facilitate migration and invasion. Absence or downregulation of PAI1 expression results in decreased migration. Further studies to investigate the role of miR148/152 family on invasion through collagen mediated by MMP10 would provide conclusive results. RICTOR is a validated target of miR152 and can be a potential target of miR148a.

The expression pattern and validated targets of miR148a and miR152 are very similar. MiR148b and miR152 are both located in the genes (COPZ1 and COPZ2) coding for the isoforms, of same protein (ζ subunit) and are probably regulated along with the protein coding gene, it is necessary to express one of the two miRNA for the survival. It can be speculated that COPZ1 gene evolved to compensate for the down regulation of COPZ2 gene. It is possible that the expression level of the members of miR148/152 family as a whole has an impact on the target regulation and biological role and could be functioning as a rheostat.

There is decreased single cell migration with the overexpression of miRNAs, while there was no effect on collective cell migration as seen by the scratch assay results. Also, there is increased adhesion to collagen matrix. Cell lines that express two or more of these miRNA can be used to perform knockdown studies which would provide better understanding of the role of endogenous miRNA. In-vivo metastasis studies can be performed to further characterize the anti-metastatic effects of miR148/152 family and also to explore their potential as replacement miRNA for cancer therapy.

Table 3.1 Primer sequences for qRT-PCR of the indicated genes

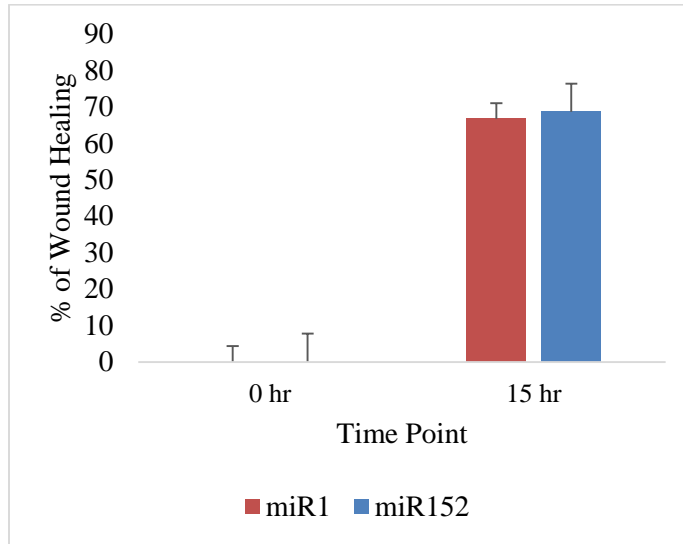
Gene	Sense	Antisense
GAPDH	CCCCGGTTTCTATAAATTGAGCC	ACCAGAGTTAAAAGCAGCCC
RPL13a	AGATGGCGGAGGTGCAG	GGCCCAGCAGTACCTGTTTA
E-Cadherin	GATAATCCTCCGATCTTCAATCCC	CAATATGGTGTATACAGCCTCCC
SNAI1	TCGGAAGCCTAACTACAGCGA	AGATGAGCATTGGCAGCGAG
SNAI2	TGTGACAAGGAATATGTGAGCC	TGAGCCCTCAGATTTGACCTG
TWIST1	GCGTCCCCTAGCAGGC	AAGTGCGCCCCACGC
ZEB1	AAGGGCAAGAAATCCTGGGG	TCTGGTCCTCTTCAGGTGCC

Table 3.2 Summary of EMT genes

Target	MiRNA 148a		MiRNA 148b		MiRNA 152	
	PC3	DU145	PC3	DU145	PC3	DU145
E-cadherin	↑	↓	↓	↓	↑	↓
SNAIL	↑	—	↓	↓	↓	↓
SLUG	↓	↓	↑	↓	↓	↓
TWIST1	↓	↓	↑	↓	↓	↓
ZEB1	↓	—	—	↓	↓	↓

Figures

a)



b)

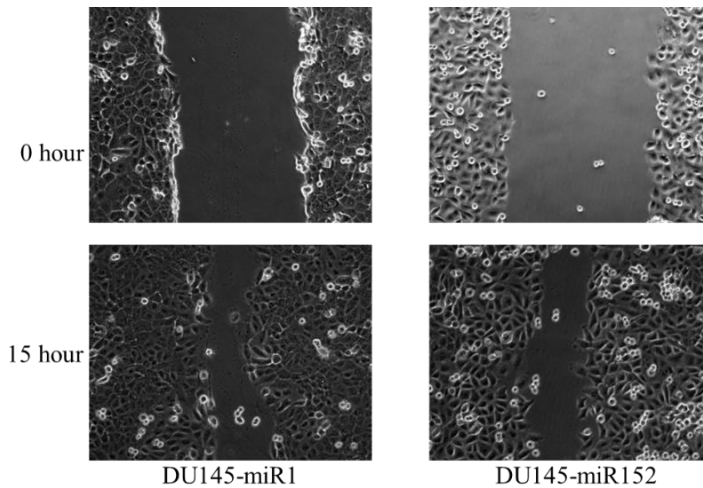


Figure 3.1 miR152 does not effect on the collective migration of DU145 cells. Cells were plated in duplicates and allowed to reach ~90 – 100% confluence. After the scratch, the plates were incubated at 37°C and pictures were taken using 10 X objective on an IX81 Olympus microscope at 0 hr and 15 hrs. a) Percentage of wound healing was then assessed and data shown with SEM (N=2), b) representative pictures of the wound closing taken at two different time points.

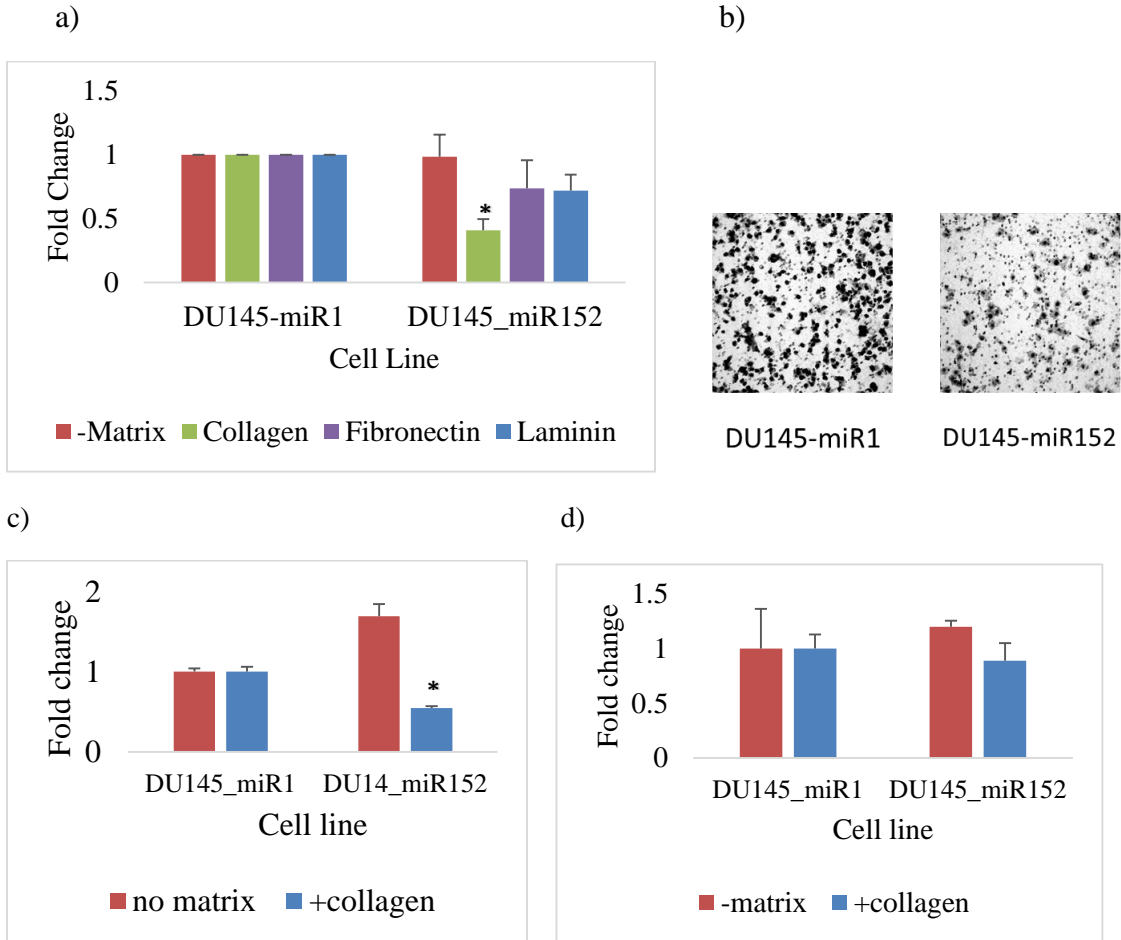
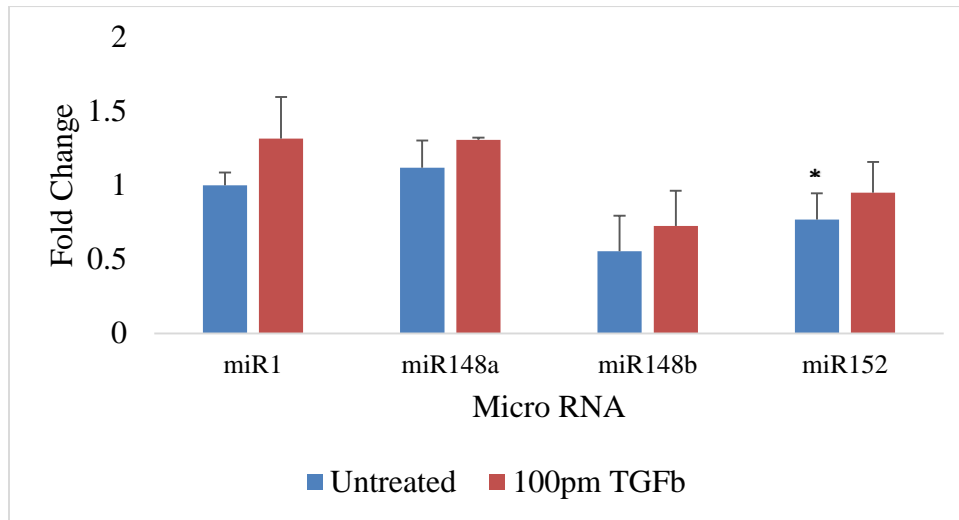


Figure 3.2 mi152 impedes migration through collagen. a) Overnight migration through uncoated trans-wells and trans-wells coated with individual ECM components; collagen, fibronectin and laminin. b) Representative pictures of collagen coated trans-wells. c) Overnight migration through collagen coated wells (N=2) d) 6 hour migration through collagen coated wells. DU145-miR152 and DU145-miR1 cells in 100 μ l serum-free media were loaded in the upper chamber of the trans-well and 400 μ l serum containing media serving as a chemoattractant was placed in the bottom chamber. After incubation at 37°C and the migrated cells which are attached to the filter facing the lower chamber were stained with eosin Y and methylene blue, photographed with 10X objective and scored using ImageJ™. * indicates significant decrease (P<0.05) of migration in miRNA overexpressing cell lines when compared to the control cell line.

a)



b)

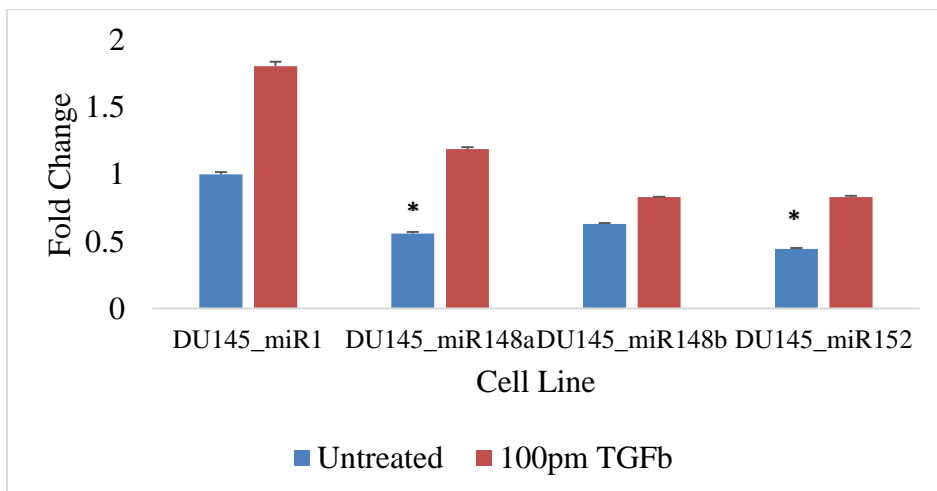


Figure 3.3 mi148/152 family impedes migration through collagen in DU145 stable cell lines and exogenous TGF β treatment restores migration. Overnight migration through trans-wells coated with collagen in a) PC3 cells b) DU145. After incubation at 37°C and the migrated cells which are attached to the filter were stained crystal violet, and the intensity of the dye dissolved in SDS was read using plate reader. Migration experiment in a similar manner was also carried out in the presence of 100 pm TGF β . * indicates significant decrease ($P < 0.05$) of migration in miRNA overexpressing cell lines when compared to the control cell line. (N=3)

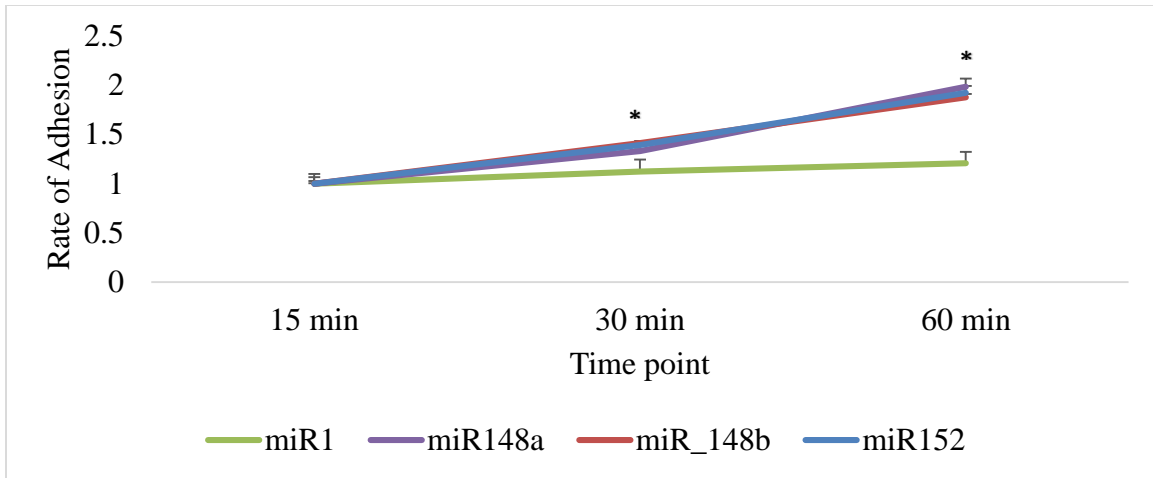


Figure 3.4 miR148/152 family increases the adhesion of DU145 cells. Adhered cells are stained with crystal violet and the intensity of dye dissolved in SDS is measured using a plate reader. Data is shown relative to 15 minute time point with SEM (N=3) * indicates significant increase (P<0.05) of adhesion in miRNA overexpressing cell lines when compared to the control cell line.

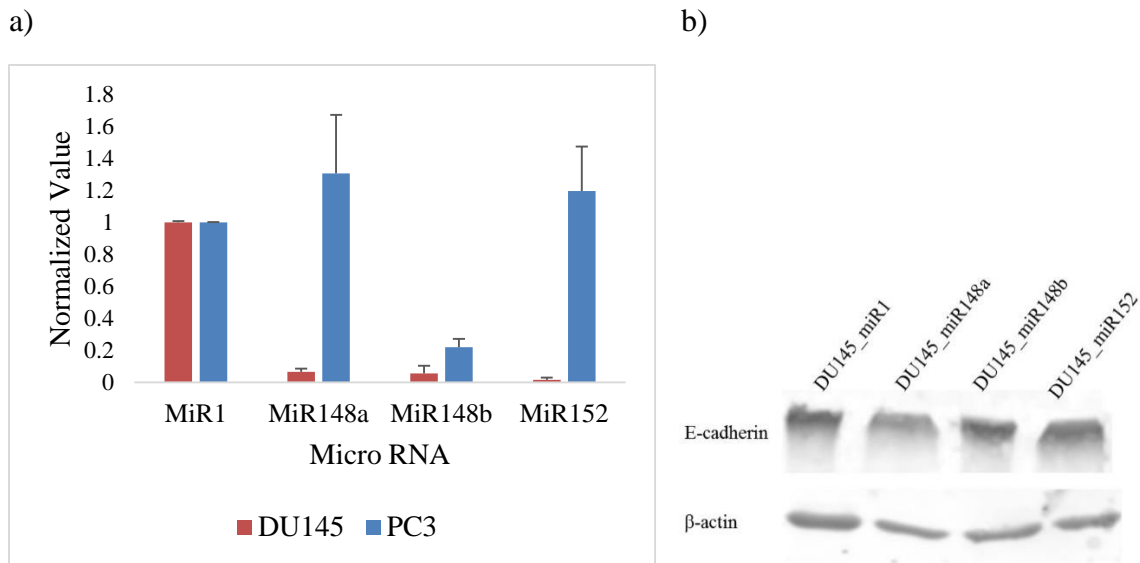


Figure 3.5 Effect of miR148/152 family on E-cadherin expression. Though qPCR suggests decreased E-cadherin expression in DU145 cell line (a), there is no detectable change in the protein levels as shown by western blot. a) RPL13A is used for normalization and data is shown relative to control cell line.

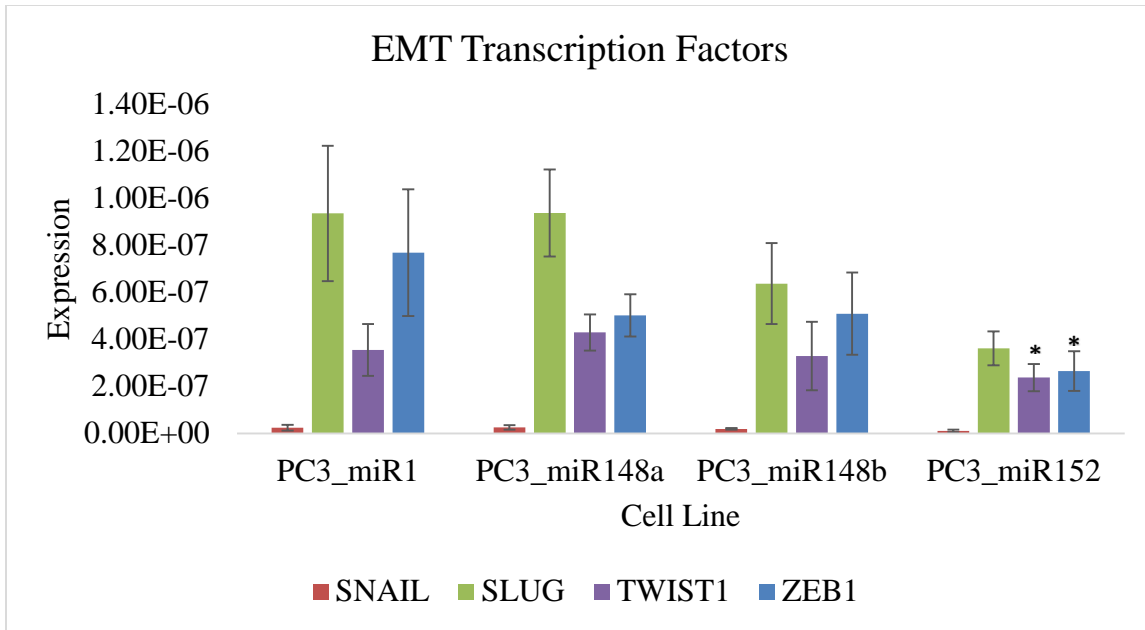


Figure 3.6 miR152 downregulated the expression of SLUG and ZEB1 in PC3 cell lines. Expression of Transcription factors in stable cell lines is verified by qRT-PCR. RPL13A was used for normalization of qPCR data. * indicates significant downregulation ($P < 0.05$) of transcription factors in miRNA overexpressing cell lines when compared to the control cell line.

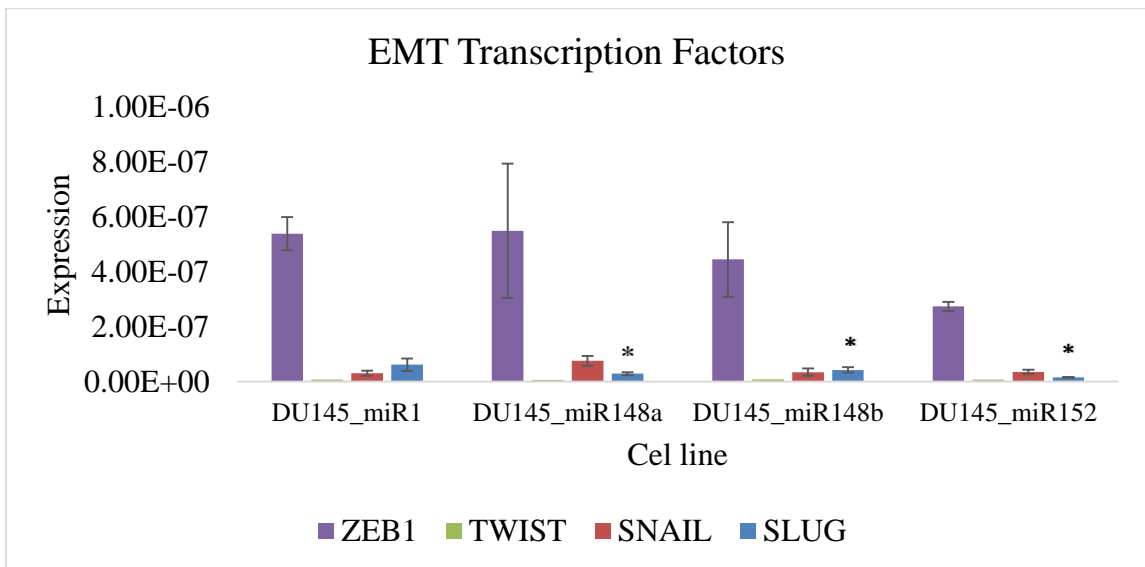
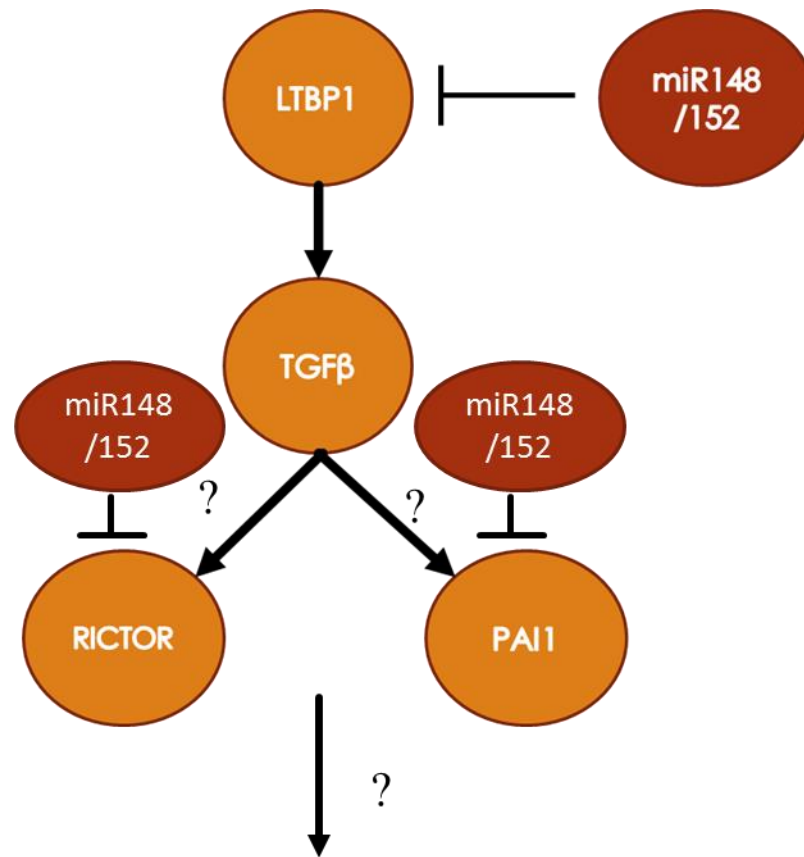


Figure 3.7 Mir148a/152 family downregulated the expression of SLUG in DU145 cell lines. Expression Of the transcription factors in stable cell lines is verified by qRT-PCR. RPL13A was used for normalization of qPCR data. * indicates significant downregulation ($P < 0.05$) of transcription factors in miRNA overexpressing cell lines when compared to the control cell line.



- Decreased migration and invasion
- Increased adhesion

Figure 3.8 Probable model for the effect of miR148/152 family on cancer progression. miR152 family impedes TGFβ mediated EMT. By downregulating one or more genes associated with TGFβ, directly or indirectly, miR148/152 family causes decrease in the metastatic/mesenchymal characteristics like migration and invasion and increase in the epithelial characteristics like adhesion.

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APPENDIX A

POSSIBLE ROLE OF TGF β

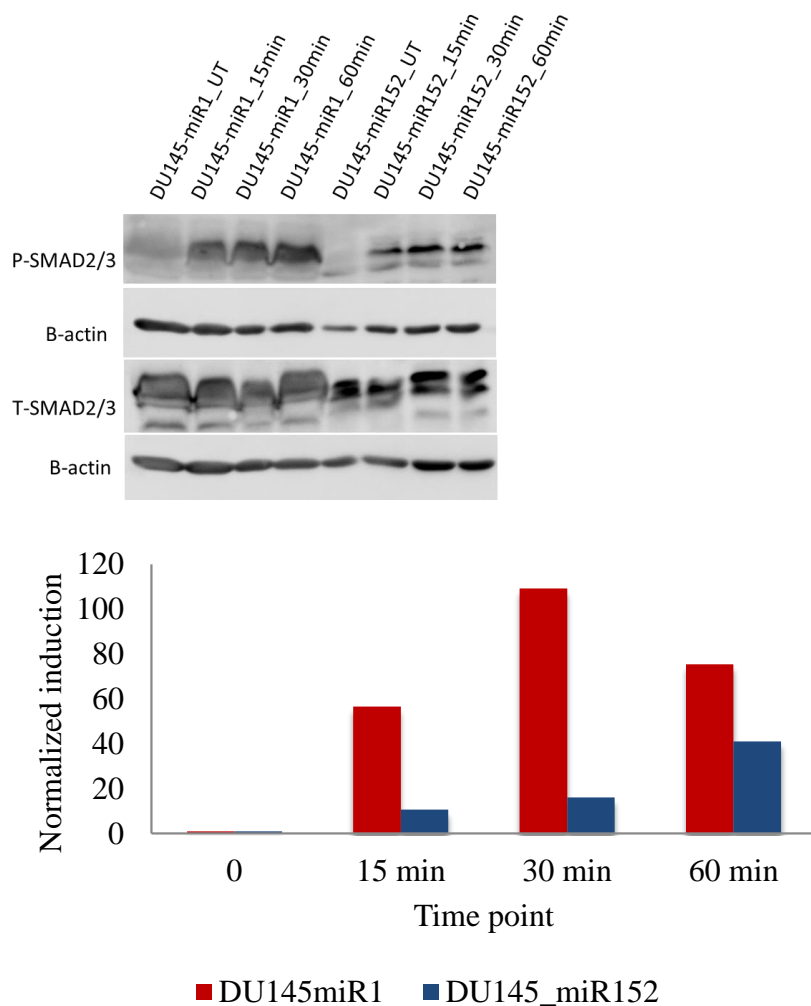


Figure A.1. Effect of miR152 on TGF β signaling. Cells were serum starved overnight and treated with 100pm TGF β for indicated time points. Increase in the phosphorylated SMAD2/3 is a direct result of exogenous TGF β signaling. Phospho-SMAD2/3 and total-SMAD 2/3 levels from the western blot are were normalized to β -actin levels. Then, the resultant levels of p-smad2/3 was normalized to t-SMAD2/3. MiR152 decreases the effect of TGF β induced pSMAD2/3 signaling.