STUDY OF HSA-miR-20a MEDIATED REGULATION OF MMP-2 IN BREAST CANCER CELL LINE

Project Thesis

Submitted By

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INTRODUCTION

MicroRNAs (miRNAs) are21-to22-nucleotide molecules that regulate cellular phenotype through altering the stability or translational efficiency of targeted mRNAs. Important associations between miRNA gene expression and human cancer have implicated miRNA in human tumorigenesis.(1)

The epithelial to mesenchymal transition (EMT) is a powerful process in tumor invasion, metastasis, and tumorigenesis and describes the molecular reprogramming and phenotypic changes that are characterized by a transition from polarized immotile epithelial cells to motile mesenchymal cells.

It has been observed and demonstrated that microRNA-20a (mir-20a) regulates cancer and metastasis. Reduced migratory and invasive property, altered cellular morphology along with reduced capability for attachment to basement membrane was acquired by overexpression of miRNA-20a in invasive MDA-MB-231 cells initially expressing low levels of this miRNA, indicating that there is a direct correlation between abundance of mir-20a and metastatic property. (2)

The molecular mechanisms governing the mir-20a mediated Mesenchymal to Epithelial transition (MET) are of great importance since they provide the future prospect of an effective therapeutic strategy against breast cancer.

1.1 WHAT IS MICRO-RNA?

MicroRNA (miRNA) are a class of recently discovered 21-25 nucleotide RNA molecules, which bind with imperfect complementarity to their target mRNAs, generally within the 3' UTR (untranslated region). MiRNA are found in plants, animals, and some viruses, and their main function is in RNA silencing and post-transcriptional regulation of gene expression. This results, in the messenger-RNA (mRNA) molecules being silenced, by one or more of the following processes

a. Cleavage of the mRNA strand into two pieces
b. Destabilization of the mRNA through shortening of its poly(A)tail
c. Less efficient translation of the mRNA into proteins by ribosomes

miRNAs resemble the small interfering RNAs (siRNAs) of the RNA interference (RNAi) pathway, except miRNAs derive from regions of RNA transcripts that fold back on themselves to form short hairpins, whereas siRNAs derive from longer regions of double-stranded RNA. The human genome may encode over 1000 miRNAs, although more recent analysis indicates that the number is closer to 600.

MiRNAs are abundant in many mammalian cell types and appear to target about 60% of the genes of humans and other mammals. Many miRNAs are evolutionarily conserved, which implies that they have important biological functions. For example, 90 families of miRNAs have been conserved since at least the common ancestor of mammals and fish, and most of these

Conserved miRNAs have important functions, as shown by studies in which genes for one or more members of a family have been knocked out in mouse. (3)

1.2 microRNA Biogenesis and Maturation

The biosynthesis of miRNAs is a tightly regulated multistep process that starts in the nucleus of the cell, following transcription, and continues through the cytoplasm where finally the mature miRNA molecule exerts its main function. Each one of the multiple steps that compose miRNA biosynthesis are very well coordinated. It starts with Drosha initiate the processing by specific cropping of the stem- loop precursor in the nucleus. The resulting structure, is the precursor miRNA (pre- miRNA), which is a signature motif for all dsRNAs that are involved in small-RNA pathways. Exportin-5 recognizes this signature motif and exports pre- miRNAs to the cytoplasm through nuclear pores on a GTP–GDP gradient. Following export, the pre-miRNA is handed over to another RNase III enzyme, Dicer, that dices the pre-miRNA into a miRNA duplex that is further unwinded giving rise to the mature functional miRNA molecule.(4)

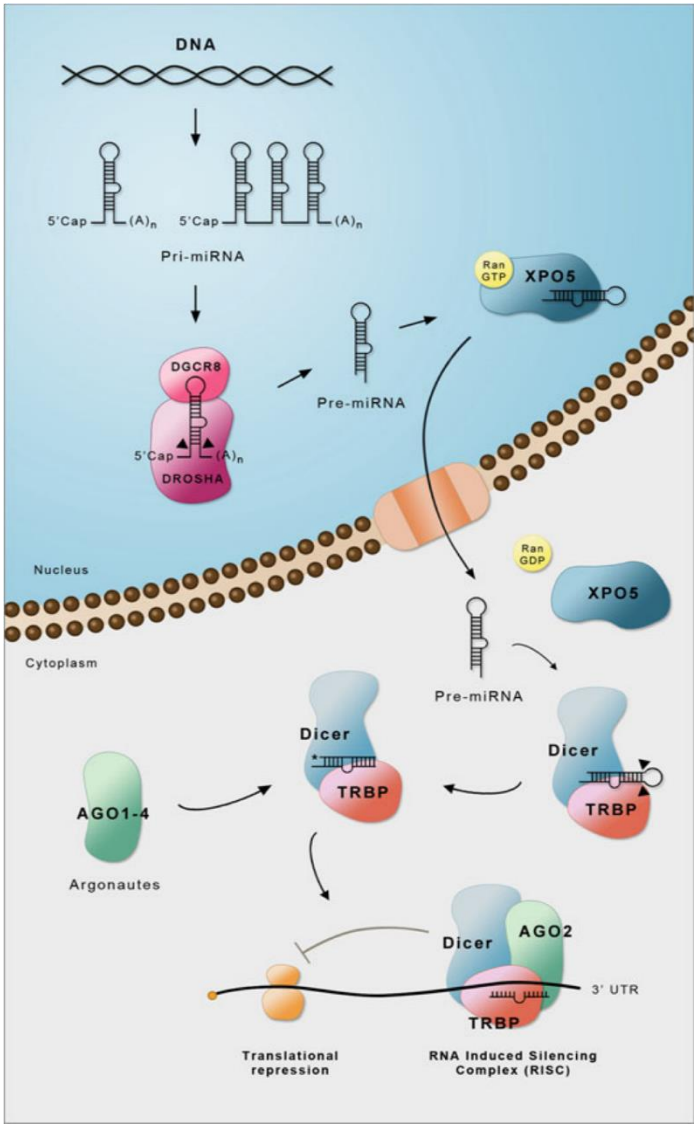


Fig A: MicroRNA biogenesis pathway. Long primary transcripts (pri-miRNAs) containing one or several miRNAs are transcribed by RNA polymerase II and cleaved by the microprocessor complex, containing at least Drosha (RNAase III endonuclease) and DGCR8 in humans (a double- stranded RNA-binding protein). This complex recognizes the double-stranded RNA structure of the pri-miRNA and specifically cleaves at the base of the stem–loop, hence releasing a 60- to 70-nucleotide precursor (pre)-miRNA. This pre-miRNA is then exported through the exportin-5 pathway into the cytoplasm where it is further processed into a mature miR/miR* duplex by DICER1, a second RNase III endonuclease together with its catalytic partner TAR-binding protein (TRBP). The miR/miR* duplex is then loaded into a multicomponent complex, the RNA-induced silencing complex (RISC), constituted of at least TRBP, DICER1, and one Argonaute (Ago2 in human). The miR serves as a guide for target recognition while the miR* passenger strand is cleaved by Ago2. Most of animal miRNAs harbor an imperfect homology with their targets and, therefore, inhibit translation by an RISC-dependent mechanism. (4)

1.3 MicroRNAs and Posttranscriptional Regulation of Gene Expression

The small size of miRNAs with a limited amount of sequence information is the reason for its non-specificity. Since Partial base-pairing between a miRNA and its target mRNA, is sufficient for repression and/or degradation of the target; a wide net of mRNAs can be regulated by the same miRNA or vice versa. MiRNAs select mRNA targets for downregulation through the association with a large, multi-protein complex, the RISC. This selection requires the presence of sequences within the target mRNA which are imperfectly complementary to the miRNA sequence. MiRNA-binding sites commonly occur within the 3′-untranslated region (3′-UTR) of the mRNA, but functional miRNA binding sites can also occur with the 5′UTR or coding region. (5)

The 3′-UTRs of messenger RNAs serve as docking platforms for miRNAs and RNA-binding proteins, which control mRNA stability, localization, and translation. The most common feature is perfect base-pairing between nucleotides 2 and 7 at the 5′ end of the miRNA, which is called the "seed" sequence, and the target site. There is still a huge amount of unknown factors regarding the exact composition of the miRNA complex as well as the mechanisms used to control target gene expression, although it is known is that miRISC inhibits the expression of mRNAs basically in one of the two ways depending on the degree of complementarity between miRNA and the target. There are mismatches and bulges in most miRNA target sites; therefore the degree of complementarity between the miRNA and the target, is thought to be a major determinant in distinguishing the two mechanisms of posttranscriptional silencing: a) translation inhibition or b) mRNA degradation. Several other mechanisms that have been documented, include translational inhibition at the level of initiation and elongation, rapid degradation of the nascent peptide, and mRNA degradation. The core component of the mRNP complex is the Argonaute protein and in mammals one of the four Argonaute proteins (AGO1-4) is recruited into the complex. AGO2 is the only Argonaute with "slicer" activity and is responsible for the cleavage of the mRNA target midway into the complementary region. Other proteins are also essential for this silencing complex to work, such as the RNA-binding protein fragile-X-mental-retardation protein (FMRP), the p-body marker GW182, and the decapping activator RCK/p54, dictate how the silencing of the mRNA target will occur.(6)

1.4 MicroRNA Biogenesis and Cancer

1.4.1 Oncogenic or Tumor-Suppressor MicroRNAs

In correspondence with various broad effects, miRNAs have been proposed to function as oncogenes or tumor-suppressor genes due to the function of a number of inhibitory tumor-suppressive and oncogenic mRNAs, respectively. Overexpressed miRNAs in cancer, such as miR-17-92, may function as oncogenes and promote cancer development by negatively

regulating tumor-suppressor genes and/or genes that control cell differentiation or apoptosis. Non-expressed or low-expressed miRNAs in cancer, such as let-7, function as tumor-suppressor genes and may inhibit cancers by regulating oncogenes. First, oncogenic miRNAs can undergo a gain of function in tumors. This has been more clearly demonstrated for the miR-17 ~ 92 cluster, whose amplification in B-cell lymphomas promotes their development, potentially through its control of B-cell differentiation. Furthermore, tumor-suppressive miRNAs could undergo loss of function in tumors. This has been shown for several miRNAs, including the let-7 family, whose expression can limit tumorigenesis through inhibition of oncogenes like the RAS family and HMGA2. In particular, let-7 family members are in sites frequently deleted in human tumors, and their processing is inhibited by the oncogenic Lin-28 proteins. Finally, oncogenes can acquire mutations to remove miRNA-binding sites in tumors. This has been described for HMGA2, whose translocation promotes lipoma development by releasing the transcript from let-7-mediated tumor suppression. (7)

2.1 miR-17-92 family and its role in cancer

MicroRNAs (miRNAs) are an abundant class of small non-coding RNAs (ncRNAs) and they function to regulate gene expression at the post-transcriptional level. Although their functions were originally described during normal development, miRNAs have emerged as integral components of the oncogenic and tumor suppressor network, regulating nearly all cellular processes altered during tumor formation. In particular, mir-17-92, a miRNA polycistron also known as oncomir-1, is among the most potent oncogenic miRNAs. Genomic amplification and elevated expression of mir-17-92 were both found in several human B-cell lymphomas, and its increased expression exhibits strong tumorigenic activity in multiple mouse tumor models. MiR-17-92 carries out pleiotropic functions during both normal development and malignant transformation, as it acts to promote proliferation, inhibit differentiation, increase angiogenesis, and sustain cell survival. Unlike most protein coding genes, mir-17-92 is a polycistronic miRNA cluster that contains multiple miRNA components, each of which has a potential to regulate hundreds of target mRNAs. This unique gene structure of mir-17-92 may underlie the molecular basis for its pleiotropic functions in a cell type- and context-dependent manner. (8)

2.1.1 Main Targets of the miR-17/92 Cluster

Phosphatase and tensin homolog (*PTEN*) and *E2Fs* were among the first validated miR-17/92 targets. Reporter assays revealed targets for miR-19a and miR-19b-1 in *PTEN's* 3′UTR, and the introduction of miR-19a and miR-19b-1, or of the full cluster, in miR-17/92-deficient cells sufficed to restore *PTEN* expression levels. In addition, miR-17 and miR-20a modulate the expression of *E2F1*. Lastly, miR-20a targets the 3′UTRs of both *E2F2* and *E2F3*.
The ability of the cluster's members to cooperate is evident in the context of TGF-*β* signaling. In particular, miR-17 and miR-20a directly target the *TGF-β* receptor II *(TGFBRII)*, whereas miR-18a targets Smad2 and Smad4, two members of the TGF-*β* signaling pathway. *TGF-β* activation exerts an effect mediated in part by the cyclin-dependent kinase inhibitor (p21) and the apoptosis facilitator BCL2L11 (BIM), both of which are targeted by miR-17/92.In

addition, *BCL2L11* is targeted by miR-20a, miR-92, miR-19a and miR-19b-1and also by miR-106b/25.During the endoplasmic reticulum related stress, unfolded protein response TFs, activating TFs, activating transcription factor 4 (Atf4) and nuclear factor-erythroid-2-related factor 2 (Nrf2) are activated and downregulate *Mcm7*, the host gene for the miR-106a/25 cluster. Downregulation of miR-106b/25 and repression of *BCL2L11*consequently trigger apoptosis.
Lastly, miR-18a and miR-19 directly repress the anti-angiogenic factors thrombospondin-1 (*TSP-1*) and connective tissue growth factor (*CTGF*).In addition, miR-17 and miR-20a participate in the regulation of the insulin gene enhancer protein (*Isl-1*) and the T-box 1 protein (*Tbx1*). (9)

3.1 Mesenchymal to Epithelial Transition (MET)

Epithelial to mesenchymal transition (EMT) is an important process in tumor invasion and metastasis that involves characteristic phenotypic changes through a transition from polarized immotile epithelial cells to motile mesenchymal cells. Conversely, mesenchymal to epithelial transition (MET) is the reverse procedure in the final stage of metastasis, where extravasated mesenchymal cells at the secondary site revert back to epithelial cells. The process of MET or vice versa involves changes in cellular morphology and migratory properties, which are governed by a number of factors. Increase in epithelial properties is accompanied by increased expression of epithelial markers like E-cadherin, claudin etc. and decrease in the expression of mesenchymal markers, like N-cadherin, fibronectin, vimentin and matrix metalloproteinase (MMPs) etc. The expressions of these key players are regulated by a number of transcription factors, like Snail, Slug, ZEB1, ZEB2, Twist etc. Often the progression of cancer through EMT is significantly induced by the interaction of multifunctional cytokines, e.g., Transforming Growth Factor-β (TGF-β) with its receptors, e.g., TGFBR2 and thereby activating the TGF-β pathway. The overall pathway leading to metastasis involves fine orchestration and stringent controls of expression of all these factors. (10)

3.1.1 MiR-20a and MET

The effect of miR-20a in MET were tested upon MDA-MB 231 cells and the following was found

a) MiR-20a overexpression reduces MDA-MB231 cell migration and invasion viz, the most prominent feature of MET which involves loss of motility and lesser ability to invade.

b) mRNA levels of metastasis-related transcription factors were altered by hsa-miR-20a – The mRNA levels of transcription factors measured by qRT-PCR shows decrease from the initial levels of snail, slug, ZEB 1, and mostly in TWIST 1 (49% decrease).

c) Differential expression of epithelial and mesenchymal marker proteins in MDA-miR-20a-S cell-line -
TWIST 1 is an activator and repressor of metastasis related gene. MiR-20a overexpression shows a decrease in TWIST 1 which results in subsequent change in the epithelial and mesenchymal markers. The levels of this markers (N-cadherin, claudin and E-cadherin, fibronectin) evaluated by qPCR and Western Blot reveals that the mRNA and protein levels of epithelial markers were elevated while those of Mesenchymal markers were attenuated.

d) EMT-inducer TGF-β was suppressed by the over-expression of hsa-miR20a -
TGF-β (Transformation growth Factor β) is an EMT-inducer. The effect of miR-20a on the mRNA level of TGF-β and its receptor (TGFBR2) were evaluated in MDA-miR-20a cells by qRT-PCR. The result show a decrease in the mRNA level of both TGF-β and TGFBR2. (11)

4.1 MATRIX METALLOPROTEINASE (MMP)

MMPs are a large family of zinc dependent endoproteinases, whose function is degrading all extracellular components.
MMPs influence essential cellular processes like cell proliferation, migration, and adhesion along with fundamental processes like tissue remodeling, angiogenesis, wound healing etc.
But the importance of this proteases are of high relevance due to their upregulation in cancer and several diseases.
It is of utmost importance, the key factor responsible for the overexpression of these family of proteases, as it may give an effective therapeutic strategy against fighting cancer in the future.

4.1.1 MMP-2 (MATRIX METALLOPROTEINASE-2)

According to domain structure, MMPs are classified into Collagenases, Gelatinases, Stromelysins, Membrane type and others
The (Gelatinase-A) MMP-2 is constitutively expressed in several cells including Fibroblasts, keratinocytes, Endothelial cells, Chondrocytes, Monocytes and have been particularly implicated in tumor invasion and Metastasis formation. The primary substrate for these enzymes is type IV collagen, a major component of the basement membrane, which represents a substantial barrier for tumor cell metastasis. (12)
The reason for the overexpression of MMP-2 in several cancer types may be due to a mutation that causes a gain of function of the MMP gene or due to a change in its post transcriptional regulation.

METHODS AND TECHNIQUES

5.1 BUFFER COMPOSITIONS

a. Propagation media: DMEM Media, 10% FBS, 100 u/ml Penicillin, 0.1 mg/ml Streptomycin, 1.25 µg/ml Amphotericin B.
b. TAE Buffer (Tris-Acetate EDTA): 40mM Tris Base, 1mM EDTA, 0.114 % Glacial Acetic Acid, pH adjusted to 8.5.
c. TBE Buffer (Tris Borate EDTA): 89mM Tris Base, 89mM Boric Acid, 2mM EDTA (pH 8.0), final pH adjusted to 8.3.
d. Tris EDTA (TE Buffer): 10mM Tris-HCl pH-8.0, 1mM EDTA pH-8.0.
e. 6X Loading Dye: 0.25% Bromophenol Blue (w/v). 50% Glycerol, 6X TAE Buffer.
f. Phosphate Buffered Saline (PBS): 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4 (pH adjusted to 7.4).
g. Tris – Glycine: 25mM Tris, 250mM Glycine (pH - 8.3), 0.1% SDS.
h. 5X Sample Buffer For Protein Loading: 250mM Tris-HCl (pH – 6.8), β-Mercapto Ethanol, 4% SDS, 50% Glycerol, 20mg Bromophenol Blue.
i. Transfer Buffer: 25M Tris Base, 192mM Glycine, 20% Methanol (to be added freshly).
j. Tris Buffered Saline (TBS): 10mM Tris-HCl (pH - 8), 150mM NaCl.
k. Blocking Buffer TBST Buffer: 0.05% Tween 20 in 1X TBS.
l. Blocking Buffer: 5% Skimmed Milk in 1X TBST.
m. 10X HBS Solution (Transfection): Hepes 100mg, NaCl 160mg, KCl 7.4mg, Dextrose 20mg, Na_2HPO_4 (anhydrous – 1.45mM) 2.06 mg with H_2O, volume makeup to 2 ml.
n. Plasmid Isolation (Large Scale using $LiCl_2$ & PEG)
    - Solution 1: 50mM Glucose, 25mM Tris-HCl, pH – 8; 10mM EDTA.
    - Solution 2: 1% SDS, 0.2 N NaOH.
    - Solution 3: 3M Potassium Acetate.

5.2 LARGE SCALE PLASMID ISOLATION BY PEG AND LiCl METHOD

PcDNA 3.1 (+), PCmiR20a, PEGFPC1, pEMMP2 were isolated by the following protocol

1. Plasmids were inoculated to (5ml LB Broth + 5 µl Antibiotic marker) from glycerol stock and grown overnight.
2. This culture (500 µl) was inoculated to (200ml+ 200 µl antibiotic) & grown overnight.
3. Centrifuged at 7000 rpm for 20 minutes and supernatant decanted.
4. 5 ml Solution 1 was added and suspended well. The dissolved pellet was transferred to a medium sized plasmid isolation tube & incubated on ice for 15 minutes.
5. 5 ml solution 2 was added & mixed vigorously by inverting the tubes. Incubated at room temperature till solution became clear (15 mins).
6. 5 ml solution 3 was added & mixed vigorously by inverting the tubes. Incubated on ice for 15 minutes.
7. Centrifuged at 8000 rpm for 30 minutes at 4º C.
8. The supernatant was taken into another centrifuge tube and equal volume of isopropanol added. Incubated at - 20º C for 30 minutes.
9. Centrifuged at 8000 rpm for 30 minutes at 4 º C.
10. The supernatant was discarded and the pellet was dissolved in 800 µl of TE.
11. Now equal volume (800 µl) of ice-chilled 5M LiCl was added and mixed vigorously.
12. Incubated at -20 º C for 20 minutes.
13. Centrifuged at 12000 rcf for 10 minutes at 4 º C.
14. The supernatant was taken in two 2 ml microcentrifuge tubes.
15. 1 ml 18% PEG (in 1.6M NaCl) was added to supernatant and stored at -20 º C overnight.
16. Centrifuged at 13000 rcf for 20 minutes at 4 º C.
17. The supernatant was very carefully pipetted because the pellet remains adherent to the wall of the tube.
18. The damp pellet of nucleic acid was dissolved in 400 µl of TE (pH -8) and RNase A (2 µl) and incubated at 37 º C for 2 hours.
19. Phenol (pH-8): Chloroform (1:1) was added & mixed. Followed by a spin at 10,000 rpm for 5 minutes at room temperature.
20. The aqueous phase very carefully pipetted. Then equal volume of chloroform was added and mixed. Centrifuged at 10,000 rpm for 5 minutes at 4 room temperature.
21. The aqueous phase was pipetted and the volume measured.

22. Now 1/10th volume of 5M Ammonium acetate and 3 volumes of 100% Ethanol was added.
23. Incubated at -20º C for 30 minutes and centrifuged at 14000 rcf for 20 minutes at 4º C.
24. The supernatant was discarded and the pellet washed in 70% ethanol, followed by a spin at 14000 rcf for 1 minute at 4 º C. Finally the pellet was air dried under light & dissolve in TE.
25. Finally the absorbance (O.D) was measured and the plasmid was run on Agarose gel to check the conformation.

5.3 COMPOSITION OF SDS - POLYACRYLAMIDE GEL ELECTROPHORESIS

Western blot were performed on SDS PAGE, gels were prepared according to the following protocol

COMPONENTS	10% RESOLVING GEL (10 ml)	12% RESOLVING GEL (10 ml)	5% STACKING GEL (5 ml)
D_2 H_2O	4 ml	3.3 ml	3.4 ml
30% ACRYLAMIDE	3.3 ml	4 ml	830 µl
1.5M Tris-HCl (pH - 8.8)	2.5 ml	2.5 ml	630 µl (Tris 1M pH - 6.8)
10% SDS	100 µl	100 µl	50 µl
10% APS	100 µl	100 µl	50 µl
TEMED	8 µl	8 µl	6 µl

5.4 COMPOSITION OF NATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS

PCR products were visualized by running 25µl of PCR products with 5µl of 6X gel loading dye on native polyacrylamide gel, prepared using the following protocol

COMPONENTS	6% NATIVE PAGE (15ml)
D_2 H_2O	10.3375 ml
5X TBE	1.5 ml
30% ACRYLAMIDE	3 ml
10% APS	150 µl
TEMED	12.5 µl

5.5 COMPOSITION OF AGAROSE GEL ELECTROPHORESIS

Isolated plasmids were run on agarose gels for checking their quality and size

COMPONENTS	1% AGAROSE GEL
AGAROSE	1 gm
1X TE	100ml

5.5 BRADFORD ASSAY

SAMPLE	VOLUME OF BSA FROM 0.5mg/ml (µl)	CONC. OF BSA (µg/ µl)	VOLUME OF 0.5M NaCl (µl)	VOLUME OF BRADFORD REAGENT ADDED (µl)	$O.D_{595}$	MEAN O.D	FINAL O.D
1A 1A	_	_	200	1800			
2A 2B	10	2.5	190	1800			
3A 3B	20	5	180	1800			
4A 4B	30	7.5	170	1800			
5A 5B	40	10	160	1800			
SAMPLE	_	_	199	1800			

A Standard curve was drawn taking the concentration of BSA (µg/µl) in the X-axis and the absorbance value in the Y-axis.
From the curve the unknown concentration of the sample was calculated solving the equation.

5.6 MDA-MB-231 CELLS

MDA-MB-231 cells are a group of invasive, triple negative breast cancer cells that are obtained from pleural effusion. In simpler terms it means that this cells lack estrogen receptor (ER), progesterone receptor (PR) expression as well as human epidermal growth factor receptor 2 (HER2).

Having this mutations causes this cells to become immune towards most drug based therapies, as a result most of the treatment relies upon chemotherapy.
A plus side towards this is that owing to another mutation in the BRCA1 gene (DNA repair gene) this cells are highly sensitive towards cytotoxic agents which opens several possibilities of treatment.

Triple negative breast cancer cells (TNBCs) constitute 10-20% of all breast cancers, more frequently affect younger patients, and are more prevalent in African-American women (13). TNBC tumors are generally larger in size, high grade, have lymph node involved at diagnosis, and are biologically more aggressive (14). Less than 30% of women with metastatic TNBC survive 5 years, and almost all die of their disease despite adjuvant chemotherapy, which is the mainstay of treatment (15).

It is clear that there should be a higher concern towards further research on TNBC in order to better understand the molecular basis of TNBC and to develop effective treatment against this aggressive type of breast cancer.

5.7 TRANSFECTION

1. Cell Seeding: 10 µl of cell suspension was taken and in it 10µl of Trypan Blue was added and mixed. From this mixture 10 µl of solution was taken in each chamber of the Hemocytometer and observed under Olympus phase contrast microscope (20X).
2. Cell counting: From the average counting number multiplied with the dilution factor and the volume of the hemocytometer (10^4), the total number of cell present per ml of cell suspension was calculated.
3. For RNA isolation, 0.2 *10^6 cells in 2ml media was seeded and for protein isolation 0.4 * 10^6 cells in 4 ml media was seeded.
4. A transfection media change was done (DMEM + 10% FBS without Antibiotic), prior to the addition of the transfection mix.

5. TRANSFECTION MIX:

For 35mm plate (RNA)

2X HBS	110 µl
TE	85 µl
DNA(µg/µl)	15 µl
$CaCl_2$	20 µl

For 60mm plate (Protein)

2X HBS	220 µl
TE	170 µl
DNA(µg/µl)	30 µl
$CaCl_2$	40 µl

6. After incubating the transfection mix at room temperature for 10 minutes it was added dropwise.
7. After 6 hours of transfection a media change was done (DMEM + 10% FBS with antibiotic).
8. 48 hours after transfection total RNA and whole protein were isolated.

5.8 TOTAL PROTEIN ISOLATION

I. The media was decanted from the plates and 1ml 1X PBS (chilled) was added.
II. The cells were then scraped using a cell scraper and taken in a Microcentrifuge tube.
III. The solution were centrifuged at 3000 rpm for 2 minutes at room temperature.
IV. The supernatant was decanted and 1ml 1X PBS was added to the pellet and mixed.
V. The solution was then taken in a Microcentrifuge tube and centrifuged at 3000 rpm for 2 minutes at room temperature.
VI. The supernatant was decanted and the pellet can be stored at -80º C for later use.
VII. Lysis buffer was added to the pellet and incubated at 4 º C for 30 minutes.

COMPOSITION WHOLE CELL LYSIS BUFFER

TRIS – Cl (pH - 8)	50mM
NaCl	150mM
SDS	0.1%
NP – 40 (TRITON X 100)	1%
SODIUM DEOXYCHOLATE	0.5%
PIC (100 X) PROTEASE COCKTAIL INHIBITOR	1X
NaF	50mM

VIII. After completion of incubation, the cells were centrifuged at 4 ºC for 20 minutes and the supernatant was collected. With 1µl of supernatant Bradford assay was done and the remaining was used for western blotting

5.9 WESTERN BLOTTING

1. PREPARATION OF SAMPLE MIXTURE: The volume of the cell extract was measured and 1/4th volume of 5X sample buffer was added and boiled.

2. CALCULATION OF FINAL CONCENTRATION:

$$\frac{\text{Volume of cell extract} \times \text{concentration of protein from Bradford assay}}{\text{Total sample volume (vol. of cell extract + 5X sample buffer)}}$$

EQUAL VOLUME AND EQUAL AMOUNT OF PROTEIN WAS LOADED INTO EACH WELL (FOR MMP-2 AND β-actin 40µg OF PROTEIN WAS LOADED).

3. The transfer apparatus was setup during this time, with the blotting paper was allowed to soak in the transfer buffer at 4º C.
4. After the gel electrophoresis was complete, a nitrocellulose membrane was prepared in size with the gel and was placed within the transfer apparatus in proper arrangement for the transfer to occur. In order for the transfer (100V, 0.3 A) was maintained for 2.5 hours at 4º C.
5. After the transfer was complete, the membrane was kept in blocking buffer (in rocking condition) for 1 hour at room temperature.
6. After that the membrane was washed with a 1X TBST solution for 5 times in 5 minute interval.
7. Next a primary antibody (1000 times diluted in 1x TBST) was added to the membrane and kept in the rocker for overnight incubation.
8. Next day the primary antibody was stored away and the membrane was again washed with a 1X TBST solution 5 times in 5 minutes interval.
9. Then a secondary antibody (conjugated with HRP) was added (5000 times diluted in blocking buffer) to the membrane and allowed to incubate for 2.5 hours.
10. Then the membrane with the secondary antibody was washed with 1X TBST solution (3 times) & 1X TBS solution (2 times) in a 5 minute interval.
11. Finally the membrane was ready to be developed in X-Ray film for analysis.

12. PREPARATION OF THE DEVELOPING MIXTURE: 5ml 100mM Tris - HCl (pH – 8) + 1.5µl H_2O_2 + 25µl luminol + 12.5µl coumaric acid).
13. The mixture was added to the membrane in a dark room and then it was introduced in a clean wrap. Membranes were then exposed to X- Ray films.
14. Finally the film were developed with a developer and fixer solution, later to be observed under light.

- [Antibody of MMP-2 was obtained from R & D Biosystems.]
- [Antibody of β – actin was obtained from Santa Cruz Biotechnology.]

6.0 TOTAL RNA ISOLATION

MDA-MB-231 cells transfected/co-transfected with pEGFPC1 + pCDNA, pEFFPC1 + pCmiR20a, pEMMP2 + pCmiR20A, pEMMP2 + pCmiR20a were harvested, after 48h incubation (post transfection), in 700µl Trizol reagent. The samples were initially stored at -80ºC and RNA was isolated from it according to the following protocol:

1. 200µl of chloroform was added to each of the sample and incubated for 3 minutes at room temperature.
2. Then the samples were centrifuged at 12000 rcf for 15 minutes at 4º C.
3. The aqueous phase were carefully taken in a microcentrifuge tube and 500 µl of isopropyl alcohol added and incubated for 15 minutes at room temperature.
4. This solution was centrifuged at 12000 rcf for 10 minutes at 4º C.
5. The supernatant was discarded and the pellet was washed with 80% Ethanol, followed by another spin at 8000 rcf for 8 minutes at 4º C.
6. The supernatant was again discarded using pipette man and the pellet was air-dried.
7. Next the pellet was dissolved in 15µl of nuclease free water and incubated for 10 minutes at 55º C.
8. Form this solution 5µl was taken and absorbance measured (260/280 nm) for checking purity and calculating the concentration.
9. The remaining solution (10µl) was added to a DNase mixture (which was prepared prior to the addition) and incubated for 1.5 hours at 37º C.

PREPARATION OF DNase MIXTURE:

COMPONENTS	FOR INDIVIDUAL	FOR 6 SETS
DNASE 1	1 µl	6 µl
BUFFER	1.25 µl	7.5 µl
NUCLEASE FREE WATER	0.25 µl	1.5 µl
RNA	10 µl	

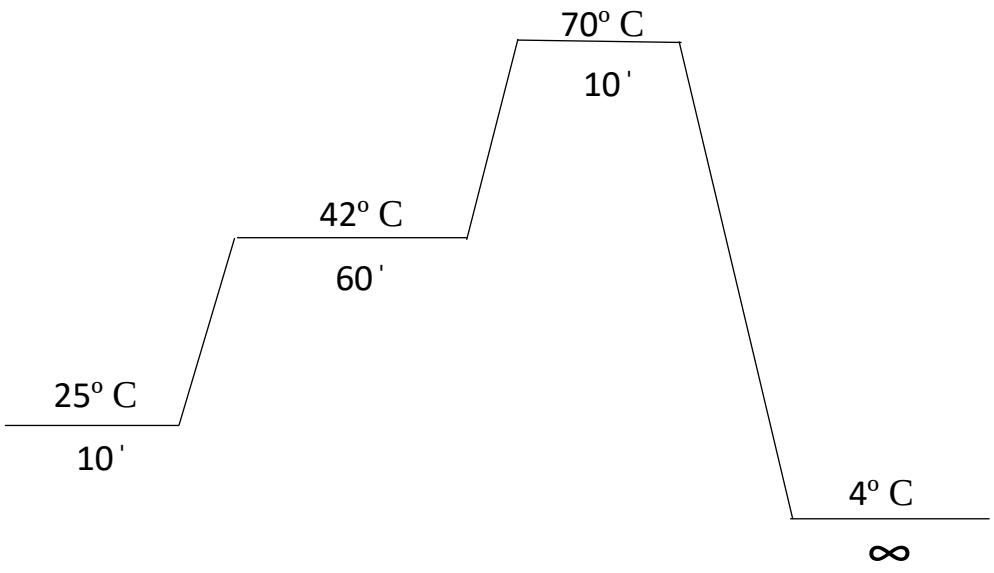
(EACH SAMPLE GOT 2.5µl OF DNase MIX)

10. 1.3µl of 50mM EDTA ($1/10_{th}$ volume) was added and incubated for 10 min.at 65º C.

PREPARATION OF THE REVERSE TRANSCRIPTION MIXTURE

SAMPLE	O.D 260	O.D 280	RATIO (260/280)	CONC (µg/ µl)	FINAL CONC. (µg/ µl)	VOLUME FOR 4 µg RNA	RANDOM HEXAMER (1:1)	D_2 H_2O	TOTAL VOLU--ME
pEGFPC1+ pCDNA							2 µl		13 µl
pEFFPC1 + pC20a							2 µl		13 µl
pEMMP2 + pC20a							2 µl		13 µl
pEMMP2 + pC20a							2 µl		13 µl

11. The reverse transcription mix was added by the above protocol and allowed to undergo in a PCR machine following the below chart.



12. After the cDNA preparation, to check the integrity of the isolated RNA a semi quantative PCR was done for GFP, NEOMYCIN, MMP-2 and β – actin using the following primer

GFP_{FWD} = 5 ' - CCGGGGTGGTGCCCATCCTGGTC - 3 '
GFP_{REV} = 5 ' - CCCTTCAGCTCGATGCGGTTCACC -3 '

NEO_{FWD} = 5 ' - TGCCCTGAATGAACTGCAGG - 3 '
NEO_{REV} = 5 ' - TGATCGACAAGACCGGCTTC - 3 '

Actin $_{FWD}$ = 5 ' - CTTCCTTCCTGGGCAT - 3 '
Actin $_{REV}$ = 5 ' - CAGGGTACATGGTGGTG - 3 '

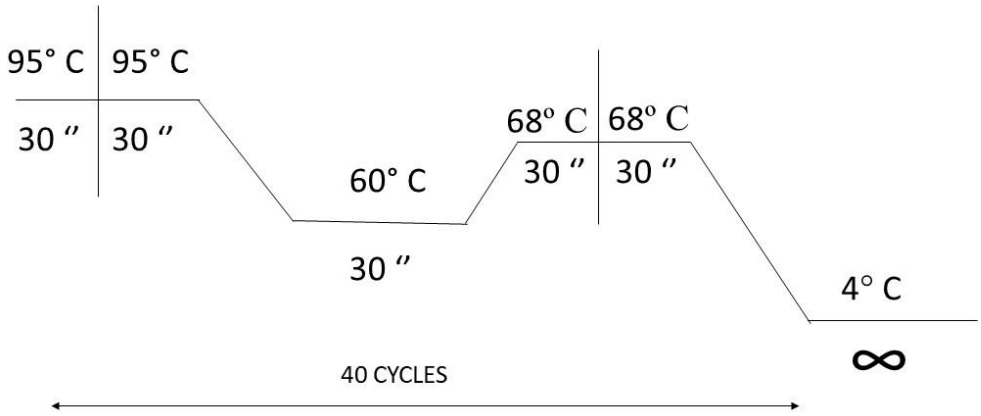
$MMP\text{-}2_{FWD}$ = 5 ' - GGGAATTCGCAGTGGGACAG - 3 '
$MMP\text{-}2_{REV}$ = 5 ' - GGGGATCCACTGTGCAGTGACTT - 3 '

13. PREPARATION OF THE PCR MIXTURE

COMPONENTS	FOR INDIVIDUAL (25 µl)
10X PCR BUFFER	2.5 µl
10mM dNTP	0.5 µl
FORWARD PRIMER	0.5 µl
REVERSE PRIMER	0.5 µl
TAQ POLYMERASE	0.1 µl
$MgCl_2$	0.5 µl
TEMPLATE (DNA)	6 µl
WATER	14.4 µl

Gnerally a mixture for the total number of samples are prepared in a single 0.5 ml PCR tube, and from their 19 µl of mixture are allocated into separate PCR tubes and finally 6 µl of template are added.

14. PROTOCOL FOR THE THERMAL CYCLER



Following the above protocol the PCR was completed.

6.1 REAL-TIME POLYMERASE CHAIN REACTION (PCR)

Since a normal PCR result would only amount to a qualitative approach, it was necessary to do a real-time pCR , which as the name suggests measures the amount of PCR products after each amplification cycle (i.e., in real-time).

1. cDNA prepared from the total cellular RNA of transfected cell previously, was used as template and specific primers were used. pcDNA 3.1 (+) and β-actin were used as transfection control and internal control. The relative gene expression was measured by ΔΔct method.

2. For reporter assay, gfp mRNA levels were quantified by qRT-PCR. In this case Neomycin resistant gene and GFP were used as transfection control and internal control.

I. PREPARATION OF THE qPCR MIXTURE

COMPONENTS	FOR INDIVIDUAL
10µM FORWARD PRIMER	0.2 µl
10µM REVERSE PRIMER	0.2 µl
TEMPLATE	2 µl
WATER	2.6 µl
2X MASTER MIXTURE	5 µl

Like the PCR here also a total mixture excluding the template for the required number of samples were prepared in a single 0.5 ml PCR tube and later allocated into separate qPCR tubes.
The 2X master mix is light sensitive as it contains SYBR GREEN, so its addition was done in absence of light.

- [2X mastermix (EvaGreen qPCR MasterMix-ROX) required for real-time PCR was obtained from abm.]

OBSERVATION AND RESULTS

6.2 PLASMID ISOLATION AND GEL ELECTROPHORESIS

The following plasmids were isolated
pEGFPC1, pCmiR20a, Pcdna 3.1 (+), pEMMP2 by large scale plasmid isolation technique and run on 1% agarose gel to check conformation.
The samples were 10 times diluted with water and along side them a 1Kb ladder was run simultaneously to check the size of the plasmids.

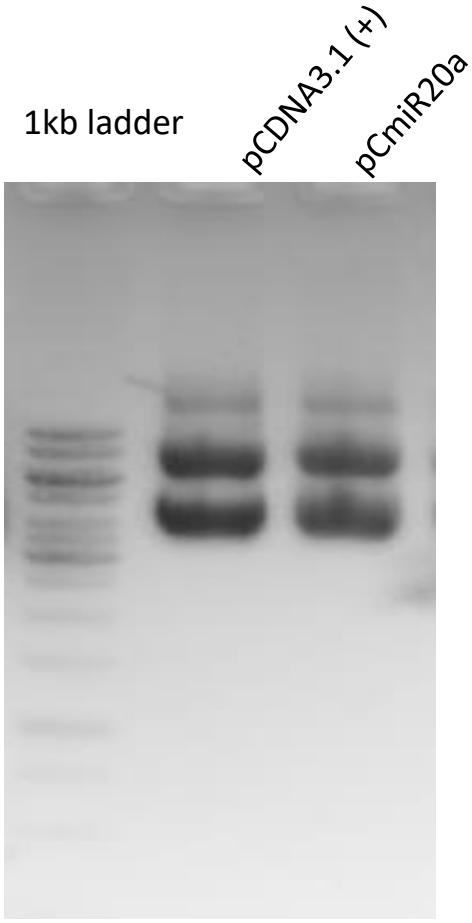


Fig B: pCDNA 3.1 (+) and pCmiR20a run on 1% agar--ose gel.

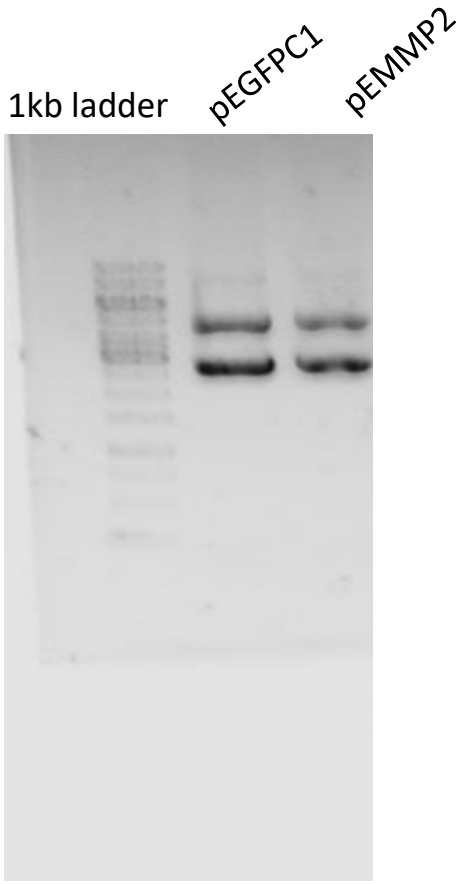


Fig C: pEGFPC1 and pEMMP2 run on 1% agarose gel.

6.3 A SEMI-QUANTATIVE PCR SHOWING THE BAND INTENSITY OF MMP-2 (m-RNA) UPON OVEREXPRESSION OF miR20a ON MDA-MB-231 CELLS

A PCR was done showing the effects of miR20a overexpresion on MMP-2 m-RNA levels using the C-DNA as template. A positive control (pC DNA 3.1 +) empty vector was also transfected into the MDA-MB-231 cells to compare the intensity of the of MMP-2 bands.
The following primer were used

$MMP\text{-}2_{FWD}$ = 5 ' - GGGAATTCGCAGTGGGACAG - 3 '
$MMP\text{-}2_{REV}$ = 5 ' - GGGGATCCACTGTGCAGTGACTT - 3 '

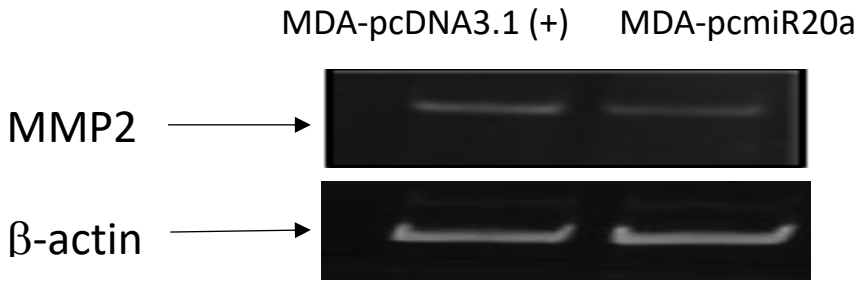


Fig D: A semi quantative PCR showing the band intensity of MMP-2 and β – actin on pcDNA 3.1 (+) and pcmiR20a transfected cell.

From the above picture it is clearly visible that the band intensity of MMP-2 on pcmiR20a transfected cell is much low compared to the pcDNA3.1 (+) transfected cell.
The band intensity of β – actin (housekeeping gene) seems to be same in regards to both the transfected cell, which clears away any suspicions regarding the integrity of both the cDNA.

This clearly indicates and gives a qualitative support to the claim that miR20a plays an important role in cancer metastasis and is an important regulator of MET.

6.4 OVEREXPRSSION OF miR20a IN MDA-MB-231 CELLS RESULTS IN DECREASE OF MMP-2 RNA LEVEL BY REAL-TIME PCR TECHNIQUE

A real-time PCR technique gives a quantitative result in regards to normal PCR technique which gives a qualitative idea regarding the subject. In order to prove and verify the theory regarding miR20a's effect on MMP-2 m-RNA level a real-time PCR was done using the same template and primers used in the normal PCR method.

The data obtained from the experiment was calculated and represented in the form of a Bar graph where the X-axis represents the MMP-2 level of the pcmiR20a transfected cell and pcDNA3.1 (+) transfected cell and the Y-axis represents the fold change ($2^{-\Delta\Delta ct}$).

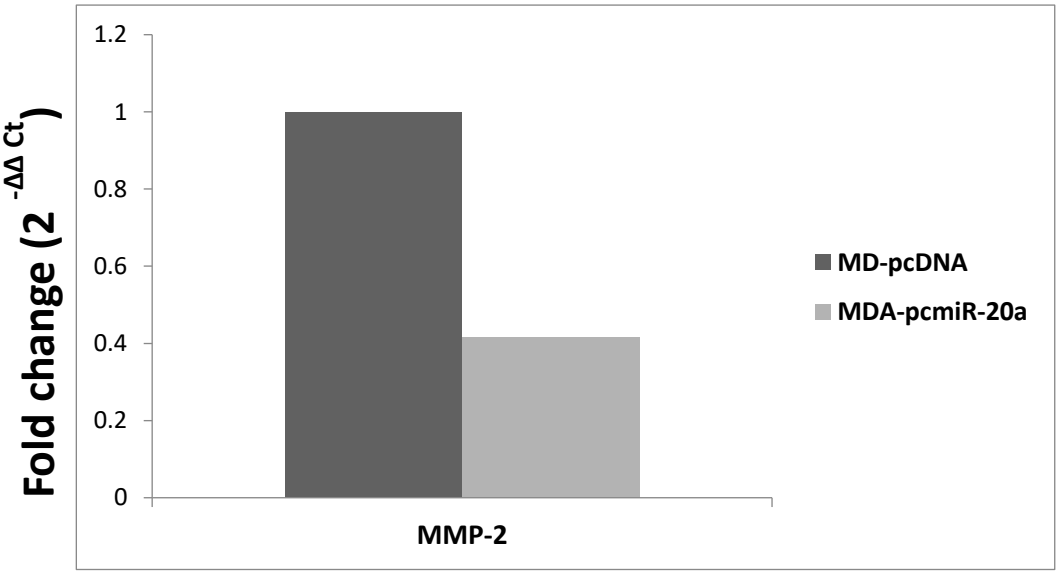


Fig E: A graphical representation depicting the decrease in the MMP-2 m-RNA level upon transfection with pcmiR20a and pcDNA. Tha data was obtained from real-time PCR technique.

The result obtained from the real-time PCR matches with the data from the normal PCR proving miR20a to actively take part in regulating MMP-2 levels of MDA-MB-231 cells.

6.5 OVEREXPRESSION OF miR20a IN MDA-MB-231 CELLS RESULTS IN DECREASE OF MMP-2 PROTEIN LEVEL

From the pcmiR20a transfected and pcDNA3.1 (+) transfected cell, a whole cell protein extract was prepared and was run on a SDS-PAGE, from there a western blot was performed.
Since miR20a decreases the mRNA level of MMP-2, there should be an overall decrease in the amount of MMP-2 protein translated in the transfected cell. This can be understood by a qualitative analysis of the band intensity of the MMP-2 protein by comparing between pcmiR20a transfected and pcDNA3.1 (+) transfected cell.
The protein level of β- actin alongside MMP-2 as a control.

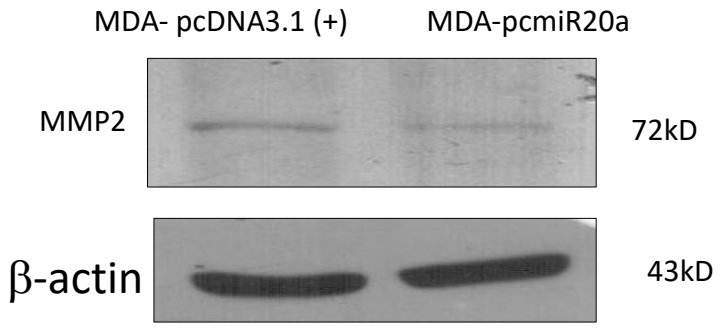


Fig F: Above is a western blot of MMP-2 showing a decrease in the band intensity of the pcmiR20a transfected cell in comparison to pcDNA transf--ected cell.

From the above picture it is clearly visible that the band intensity of MMP-2 on pcmiR20a transfected cell is much low compared to the pcDNA3.1 (+) transfected cell.
The band intensity of β – actin (housekeeping gene) seems to be same in regards to both the transfected cell, which clears away any doubt as to the integrity of the cells.

6.6 BINDING miR-20a DIRECTLY TO THE 3ˈ UTR OF MMP-2 REGULATES ITS EXPRESSION

A prior in silico target scan search revealed that miR-20a binds to the 3ˈ UTR of MMP-2. It was found that a sequence complementary to the seed sequence (GCACTTT) of miR-20a was present in MMP-2. So to verify the effect of miR-20a on MMP-2 mRNA expression a reporter assay was performed using recombinant pEGFPC1 vector where the 3ˈ UTR of MMP-2 gene was cloned at the 3ˈ- end of gfp gene to yield pEMMP2.
MDA-MB-231 cells were co-transfected with pEMMP2 and pcmiR20a and their gfp level measured.
Prior to comitting to the reporter assay by REAL-TIME PCR technique it was essential in verifying the stability of the c-DNA and proper primer functionality. As a result normal PCR was done with the c-DNA samples as templates using appropraiate primers.

A: PCR result using β-actin primer

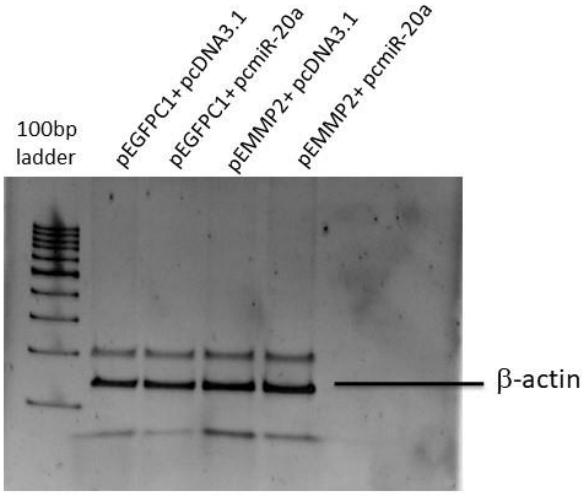


Fig G: PCR of the following samples pEGFPC1 + pCDNA, pEFFPC1 + pCmiR20a, pEMMP2 + pCmiR20A, pEMMP2 + pCmiR20a using β-actin primer.

B: PCR result using NEOMYSIN RESISTANT GENE primer

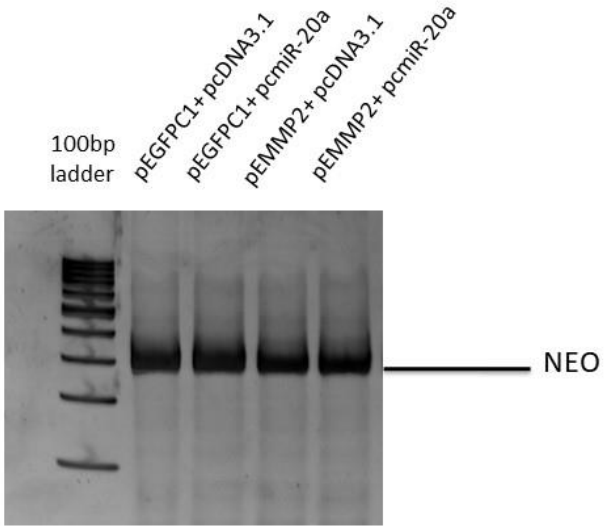


Fig H: PCR of the following samples pEGFPC1 + pCDNA, pEFFPC1 + pCmiR20a, pEMMP2 + pCmiR20A, pEMMP2 + pCmiR20a using NEOMYCIN resistant gene primer.

C: PCR result using GFP primer

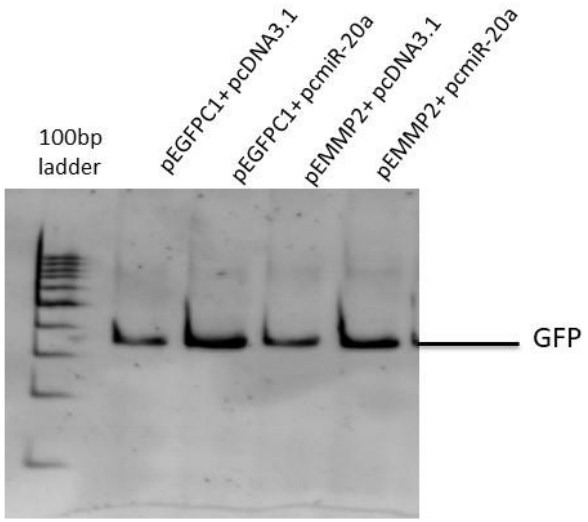


Fig I: PCR of the following samples pEGFPC1 + pCDNA, pEFFPC1 + pCmiR20a, pEMMP2 + pCmiR20A, pEMMP2 + pCmiR20a using GFP primer.

D: Result of the Reporter Assay

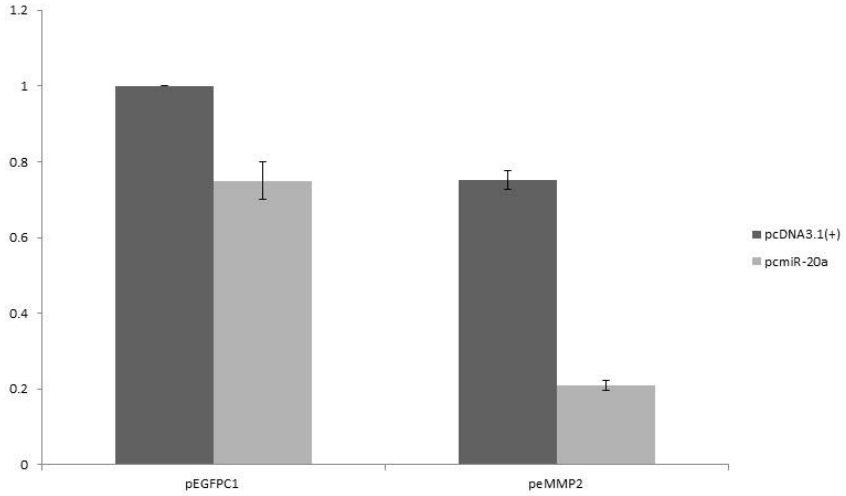


Fig J: Pegfpc1 and pEMMP2 mRNA expressions were measured by qRT-PCR using specific primer pairs. The relative expressions were measured by ΔΔct method and normalized. (Data are means of three independent experiments).

DISCUSSION

The epithelial to mesenchymal transition (EMT) is a powerful process in tumor invasion, metastasis, and tumorigenesis. It involves the molecular reprogramming and phenotypic changes that are characterized by a transition of polarized immotile epithelial cells to motile mesenchymal cells. (16)
Mesenchymal to epithelial transition (MET) is the reverse process of EMT and it occurs under certain physiological and pathological conditions. Among the several miRNAs that have been found to regulate the process of EMT, the most well-known are miR-200 and miR-17-92 family. It has already been established that miRNAs can interact with a wide range of target due to their non-specific nature and their role in alteration of genetic expression has a dramatic consequence for progression of tumorigenesis.

Previously the effect of miR-20a in promoting MET by downregulating twist-1 and TGFBR2 has been shown in MDA-MB-231 cells (17) & miR-20a was shown to have an inhibitory effect on the migratory and invasive property of MDA-MB-231 cells (18). This fact was intriguing to further explore the role of miR-20a on invasion related genes.

MMPs are zinc dependent endoproteinase, whose importance lies on the matter of their function in degrading the basement membrane, which represents a substantial barrier for tumor cell metastasis.
MMP-2 is one of the MMPs that was found to be highly effective in governing invasion in MDA-MB-231 cells.

MiR-20a was over-expressed inMDA-MB-231 cells and a significant decrease in MMP-2 MRNA level was observed in semi-quantitative PCR. Quantification of mRNA level after performing real-time PCR, also revealed a decrease level of MMP-2 on miR-20a overexpression.
This prompted us to know the effect of miR-20a on MMP-2 protein level. It was found that miR-20a downregulates the level of MMP-2.
In silico studies using Target scan revealed the presence of miR-20a seed sequence in the MMP-2 3ˈ UTR.

The decrease in the protein and RNA level expression led to us to find whether miR-20a binds directly to MMP-2 3ˈ UTR. For this purpose, reporter assay was performed, where pEMMP2 plasmids containing MMP-2 3ˈ UTR seed region cloned into a pEGFPC1 reporter vector was co-transfected with pcDNA.1 (+) or pcmiR-20a. The level of GFP in the presence of pcmiR-20a was highly reduced in pEMMP2 as measured normalized with necessary controls. The reporter assay result clearly elucidates that miR-20a directly binds to the 3ˈUTR of MMP-2 mRNA.

This result attest to the fact that miR-20a can be considered as a master regulator of invasion, since it can hybridize and consequently regulate the expression of the key invasion related MMP (MMP-2) leading to the suppression of the invasive potency of the cells.

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