의학박사 학위논문

Role of miRNAs in MAPK signaling and epithelial to mesenchymal transition

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Role of miRNAs in MAPK signaling and epithelial to mesenchymal transition

by

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ABSTRACT

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miRNA regulates cellular responses to ionizing radiation (IR) through translational control of target genes. In the first study I analyzed time-series changes in miRNA expression following irradiation in H1299 lung cancer cells and selected significantly changed miRNAs based on ANOVA analysis. Predicted target mRNAs were enriched in MAPK signaling, uncovering that the expression of miR-26b was down regulated, and its target ATF2 was up regulated in irradiated cells. When c-Jun N-terminal kinase activity was inhibited, expression of miR-26b was induced following irradiation. From these results, I concluded that IR-induced up-regulation of ATF2 was coordinately enhanced by suppression of miR-26b in lung cancer cells, which may enhance the effect of IR in the MAPK signaling pathway.

Epithelial-mesenchymal transition (EMT) is associated with increased aggressiveness, invasive and metastatic potential. In the second study enrichment analysis leads me to the selection of miR-506 which was significantly related to breast cancer patient survival (>98% patients) and targeting several EMT marker genes. Overexpression of miR-506 could suppress the expression of VIM1, SNAI2 and CD151 in MB231 human breast cancer cell lines. NF-κB binds to the upstream promoter region of miR-506 to suppress the transcription. Over expression of miR-506 inhibited TGFβ-induced mesenchymal transition even in presence of NF-κB, and suppressed cell adhesion, invasion and migration of MB231 cells. From these results, I conclude that miR-506 play a key role in the process of NF-κB- and TGFβ-mediated EMT through the posttranslational control of EMT-related genes.

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miRNA에 의한 MAPK 세포신호전달과 상피세포의 간엽세포화 조절에 대한 연구

miRNA는 이온화 방사선 (IR, ionizing radiation)에 대한 세포 반응을 표적 유전자의 번역단계에서 조절한다. 본 연구에서는 miRNA에 의한 암발생조절을 연구하기 위하여 방사선치료저항성 및 상피세포의 간엽세포화 조절에 관련되는 miRNA를 발굴하고, 그 기능을 분석하였다. 방사선치료저항성에 관련된 miRNA 연구를 위하여 H1299 폐암 세포에 이온화방사선을 처리한 뒤, microarray를 이용하여 전체 miRNA 발현의 변화를 시계열분석 (time series anaylsis) 으로 알아보기 위한 연구를 수행하였다. Variance analysis에 근거하여 IR에 의해 유의하게 변하는 miRNA들을 선택하였고, 이러한 miRNA의 예상되는 표적 mRNA들중에 MAPK (mitogen-activated protein kinase) 신호전달에 관련된 유전자가 유의미한 수준으로 많이 존재하였다. H1299 세포에 SP600125를 사용하여 JNK (c-Jun N-terminal kinases) 활성을 저해한 후에, 이온화방사선을 조사하면 miR-26b의 발현이 유도되었다. 이를 통해 이온화방사선에 의해 유도된 ATF2의 증가는 miR-26b의 억제로 인하여 협동적으로 일어나는 것이며, 결국 MAPK 신호전달 경로에서 이온화방사선의 효과를 증진시킬 것이라는 것을 알 수 있었다.

상피세포의 간엽세포화 (EMT, epithelial-mesenchymal transition)는 암세포의 이동, 침습 및 전이와 관련있다. EMT와 관련된 12개의 유전자의 번역을 억제할 것으로 예측되는 34개의

miRNA 중에서 miR-506 가 많은 EMT 유전자들을 동시에 억제할 수 있었다. 특히 유방암에 대한 miRNA 발현분석 데이터 (GSE22216)에 대한 메타 분석(>98% patients, n = 202)에서 miR-506 의 발현이 유방암 환자의 생존과 유의미하게 연관되어 있었다. miR-506 의 발현이 증가한 경우 vimentin (VIM), snail homolog 2 (SNAI2), cluster of differentiation 151 (CD151)과 같은 간엽세포 관련 유전자들의 발현이 감소하였다. 유방암에서 EMT 를 유발하는 것으로 알려져 있는 nuclear factor-kappa B (NF-κB)가 miR-506 의 promoter region 에 결합하여 miR-506 의 발현을 억제하였다. miR-506 의 과발현은 NF-κB 이 존재함에도 불구하고 정상 유방상피세포주인 MCF10A cells 의 TGFβ 에 의한 간엽세포화를 억제하였다. 또한 miR-506 의 과발현이 MB231 유방암세포의 adhesion, invasion, migration 을 억제할 수 있었다는 것을 확인하였다. 이러한 결과로부터 NF-κB 가 miR-506 의 발현을 억제함으로써 EMT 관련 유전자의 발현이 증가한다는 것을 증명하였다. 이를 통하여 miR-506 억제는 TGFβ 또는 NF-κB 에 의해 유도되는 EMT 과정에 반드시 필요한 단계임을 알 수 있었다.

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Chapter I

Coordinated regulation of ATF2 by miR-26b in γ-irradiated lung cancer cells

ABSTRACT

MiRNAs are short 20–25 nucleotide RNA molecules that are transcribed by RNA polymerase II and negatively regulate gene expression by targeting the 3'UTRs of mRNAs. MiRNA regulates cellular responses to ionizing radiation (IR) through translational control of target genes. In this study I analyzed time-series changes in miRNA expression following γ-irradiation in H1299 lung cancer cells using microarray analysis. Significantly changed IR-responsive miRNAs were selected based on analysis of variance analysis, and predicted target mRNAs were enriched in mitogen-activated protein kinase (MAPK) signaling. Concurrent analysis of time-series mRNA and miRNA profiles uncovered that expression of miR-26b was down regulated, and its target activating transcription factor 2 (ATF2) mRNA was up regulated in γ-irradiated H1299 cells. IR treatment in miR-26b over-expressed H1299 cells could not induce expression of ATF2. When c-Jun N-terminal kinase activity was inhibited using SP600125, expression of miR-26b was induced following γ-irradiation in H1299 cells. In conclusion I showed that IR-induced up-regulation of ATF2 was coordinately enhanced by suppression of miR-26b in lung cancer cells, which may enhance the effect of IR in the MAPK signaling pathway.

Keywords: ionizing radiation; MAPK signaling; concurrent analysis; ATF2; miR-26b

INTRODUCTION

MiRNAs are short 20–25 nucleotide RNA molecules which are transcribed by RNA polymerase II and bind to the 3′ untranslated region (UTR) of their targets mRNA to suppress their translation (Bartel 2009). At the post-transcriptional level, miRNAs are involved in many biological processes, including development (Marson, Levine et al. 2008), proliferation, cell death (Brennecke, Hipfner et al. 2003), and tumorigenesis (Kent and Mendell 2006). Many studies have analyzed the transcriptional regulation of mRNAs and miRNAs in γ-irradiated cells to understand responses at cellular level towards ionizing radiation (IR) (Weidhaas, Babar et al. 2007; Jeong, Wu et al. 2009; Arora, Qureshi et al. 2011).

DNA damage response, which is induced as a result of ionizing radiations exposure, plays crucial roles in genome maintenance through cell cycle arrest followed by DNA repair and/or apoptosis (Ljungman and Lane, 2004). Cellular responses to DNA damage, induced by IR exposure are tightly controlled by cell cycle checkpoints such that, cells are arrested at the transition from G1 to S phase (G1/S) or G2 to M phase (G2/M) of the cell cycle to allow DNA repair and cell survival. However, when damage is either nonrepairable or misrepaired, apoptosis is induced through both mitotic and apoptotic processes (Brown and Benchimol 2006). Ataxia-telangiectasia mutated (ATM) is a high molecular weight protein serine/threonine kinase that plays a crucial role in the maintenance of genomic integrity by activating cell cycle checkpoints and promoting repair of DNA double-strand breaks.

In the process of DNA damage response induced by ionizing radiations, it has been reported that ATM plays a central role in the activation of IKK and TAB2. TAB2 activation requires ELKS which is further involved in the activation of NFkB. NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells) belongs to the category of "rapid-acting" primary transcription factors and is involved in cellular responses to stimuli such as stress (Sarada, Himadri et al. 2008).

Mitogen-activated protein kinase (MAPK) signaling pathway plays an important role in various biological processes, such as apoptosis, proliferation, differentiation, WNT signaling, and p53 signaling. MAPK signaling is often deregulated in human cancers, leading to uncontrolled cell proliferation and survival (Brown and Benchimol 2006). Ionizing radiations can induce activation of MAPK pathways to control cell survival in a cell type-dependent manner (Tombes, Auer et al. 1998). The IR responsive activation of MAPK signaling pathway (Wang, McGowan et al. 2000) is related to cell proliferation.

Cellular signaling pathways can be regulated by transcriptional and post-translational control of genes. The miRNAs miR-7, miR-4, miR-79, miR-2, and miR-11 are involved in Notch signaling pathways by targeting the regulatory sequence motifs in the 3' UTR of target genes (Lai, Tam et al. 2005). miR-15 and miR-16 are involved in the Nodal signaling pathway (Martello, Zacchigna et al. 2007). Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, a DNA damage-signaling mediator, is regulated by miR-9 and let-7g in response to IR in lung cancer cell lines(Arora, Qureshi et al. 2011).

In the present study, I examined the time-series expression profile of miRNAs in γ-irradiated lung cancer cell lines. I tried to identify IR-responsive miRNAs that regulate expression of MAPK signaling genes through concurrent analysis of miRNA and mRNA profiles. I demonstrated the coordinated regulation of activating transcription factor 2 (ATF2), which is encoded by a MAPK signaling gene, by miR-26b in response to IR.

MATERIALS AND METHODS

Cell culture

H1299 human lung cancer cells were maintained in RPMI 1640 and A549 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Sigma Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine [both cell lines were purchased from ATCC]. The cultured cells were either exposed to 2 Gy of radiation using a 4-MV linear accelerator (Clinac 4/100; Varian, Palo Alto, CA, USA) or left unirradiated as a negative control. The specific JNK inhibitor SP600125 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). H1299 cells were incubated with 10 μM SP600125 for 30 min, and then exposed to IR (2 Gy) followed by total RNA isolation at indicated times.

MiRNA microarray

MiRNA from each cell line was extracted using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's protocols. Purified miRNAs were labeled using the mirVana miRNA Array Labeling Kit and coupled to the Cy5 Post-Labeling Reactive Dye (Amersham, GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The labeled samples were washed and hybridized in duplicate to mirVana miRNA Bioarrays (Ambion) using the mirVana miRNA Bioarray Essentials Kit. Fluorescence intensities were processed and measured using the GeneChip scanner 3000 7G (Agilent Technologies, Santa Clara, CA, USA). The levels of miRNA

hybridization were determined using GenePix Pro 6.0 software as recommended by the manufacturer. The background-adjusted intensity for each miRNA was subjected to a global variance stabilization normalization procedure (Huber, von Heydebreck et al. 2002). All data is MIAME compliant and the raw data has been deposited in a MIAME compliant database (GEO).

Statistical and bioinformatics analysis

To identify miRNAs for which expression levels changed significantly throughout the time-course, I used one-way ANOVA analysis. Considering the correlation structure of within-array replicates (Smyth, Michaud et al. 2005) in mirVana miRNA Bioarrays, I performed one-way ANOVA analysis on 328 human miRNAs. DIANA (http://diana.cslab.ece.ntua.gr/), which integrates human and mouse miRNAs into pathways to predict miRNA targets (Alexiou, Maragkakis et al. 2009), was performed initially to identify pathways.

RNA preparation and quantitative real-time PCR

Total RNA was extracted from cell lines using the TRIzol method, and then reverse transcribed to complementary DNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo-(dT)12–18 primers according to the manufacturer's protocol. The quantitative RT-PCR for indicated genes was performed in a reaction mixture containing SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan). Quantitation of miRNAs was performed using TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Samples were analyzed using the ABI PRISM 7000 sequence detection system (Applied BioSystems). All PCRs were

performed in triplicate, and the specificity of the reaction was determined by melting curve analysis at the dissociation stage. I synthesized specific primers for ATF2 (forward: 5'-AGATTTATTAATTTTTCTGTGCTCAA-3'; reverse: 5' ACACCCCCATTTATTAAAACA CC-3'), FOS1 (forward: 5'-TGTGTTCCTGGCAATAGTGTG-3'; reverse: 5'-CAATGAACA TTGATGTTGAAGAAA-3'), MAP3K5 (forward: 5'-GCAGCAGCTATTGCACTTCA-3'; reverse: 5'-TGGTCACATTTTGGTTTTGTTC-3') and PPARG (forward: 5'-CCTGCAGG AGATCTACAAGGA-3'; reverse: 5'-GGTGTCAGATTTTCCCTCAGA-3'). The relative quantitative method was used for the quantitative analysis. The calibrator was the averaged ΔCt from the untreated cells. The endogenous control was glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for genes and U6B for miRNAs.

Western blotting

Cells were harvested and lysed in NP-40 buffer containing phenylmethylsulfonyl fluoride and Protease Inhibitor Cocktail (Sigma, St. Louis, MO, USA). Protein extracts were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). Membranes were incubated with an ATF2 antibody (1:1000; Santa Cruz Biotechnology Inc.) in Tris-buffered saline Tween 20 buffer with non-fat dry milk, and then incubated with horseradish peroxidase-conjugated secondary antibody (dilution 1:5000; Bio-Rad). Immunoreactive bands were visualized using the West-Q-

Chemiluminescent Substrate Kit Plus (BIOTANG, Waltham, MA, USA).

Constructs, transfection, and luciferase assay

The precursor of miR-26b was cloned into pcDNA3 (Invitrogen) by genomic DNA PCR with primers (forward: 5′-CCGGAATTCCGGATGGGAATTGGATACAT-3′; reverse: 5′-ATTGCGGCCGCAGCTACCCTGACCACTGCTGC-3′). The 3′ UTRs of ATF2 were cloned downstream of the Renilla luciferase gene in the psiCHECK2vector (Promega, Fitchburg, WI, USA). The construct was transfected using FuGENE HD reagent (Roche, Basel, Switzerland) for real-time RT-PCR, Western blotting, and luciferase assays. Luciferase assays were performed using the Dual-Luciferase assay kit (Promega). Normalization of Renilla expression was performed using firefly luciferase present in the psiCHECK2 vector.

RESULTS

Study of differentially regulated genes and miRNAs

In order to understand the post-transcriptional control of cellular responses to ionizing radiations by miRNAs, I analyzed the genome-wide expression profile of miRNA in H1299 human lung cancer cells at 0, 4, 8, 12, and 24 hours after exposure with 2 Gy of γ-radiation. I analyzed the miRNA expression profile by one-way analysis of variance (ANOVA) to select IR-responsive miRNAs. Among 328 human miRNAs on the microarray, I found that the expression of 56 (17.1%: 30 up-regulated and 26 down-regulated) was significantly changed in H1299 cells ($p<0.05$; Figure 1 and Table 1). Interestingly, I found prominent changes at 8 hours after irradiation in most of the IR-responsive miRNAs.

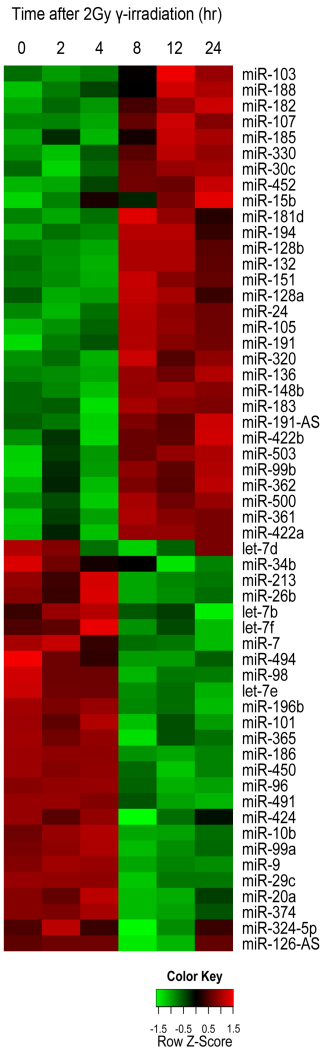


Figure 1. Heatmap illustrating expression of miRNAs in response to γ-irradiation in H1299 cells. Reverse transcribed small RNAs from each time point were labeled with Cy5. The color code represents the relative expression of indicated miRNAs for each time point. A list of all miRNAs is available in Table 2.

Table 1. List of miRNAs significantly changed in their expressions upon ionizing radiation.

miRNA		Log2[Expression level]	Log2[Activation Fold Change]	ANOVA -ln(p value)
Up-regulated miRNA	miR-183	5.6	1.5	17.07
	miR-191*	3.1	1.5	2.04
	miR-128b	8.4	1.2	17.83
	miR-188	4.8	1.2	3.60
	miR-503	5.1	1.2	6.92
	miR-362	6.9	1.1	10.59
	miR-15b	11.3	0.8	2.31
	miR-24	11.5	0.8	3.71
	miR-422a	7.9	0.8	9.33
	miR-128a	8.2	0.7	9.50
	miR-148b	6.7	0.7	8.89
	miR-151	7.6	0.7	8.19
	miR-181d	8.6	0.7	5.73
	miR-185	7.1	0.7	7.67
	miR-191	10.7	0.7	4.19
	miR-361	7.8	0.7	7.07
	miR-99b	7.4	0.7	4.21
	miR-105	5.3	0.6	4.53
	miR-107	11.1	0.6	5.07
	miR-132	6.0	0.6	10.50
	miR-136	6.1	0.6	6.50
	miR-182	8.7	0.6	4.69
	miR-194	5.3	0.6	4.74
	miR-500	5.4	0.6	5.22
	miR-320	9.2	0.5	6.10
	miR-330	5.7	0.5	2.72
	miR-422b	8.7	0.5	4.57
	miR-30c	10.0	0.4	6.64
	miR-452	5.7	0.4	2.78
	miR-103	11.0	0.3	1.33
Down-regulated miRN	miR-494	8.8	-4.1	12.29
	miR-374	6.6	-3.2	13.13
	miR-450	3.9	-2.8	6.94
	miR-98	6.5	-2.5	7.97
	miR-34b	4.0	-2.3	3.95
	miR-29c	8.4	-2.1	16.27
	miR-424	6.3	-2.1	8.55
	miR-365	5.1	-1.8	7.74
	miR-126*	3.2	-1.7	4.05
	miR-324_5p	3.8	-1.5	4.60
	miR-9	6.0	-1.5	15.23
	miR-491	8.4	-1.4	9.29
	miR-213	3.2	-1.3	2.47
	miR-101	5.2	-1.1	4.53
	miR-10b	8.6	-1.0	13.51
	miR-186	7.2	-1.0	12.56
	let-7e	10.4	-0.9	7.45
	let-7f	12.0	-0.9	7.34
	miR-7	8.0	-0.9	8.20
	let-7d	11.7	-0.8	2.69
	let-7b	10.3	-0.7	3.07
	miR-196b	5.7	-0.7	6.80
	miR-20a	9.8	-0.6	5.58
	miR-26b	9.9	-0.5	6.18
	miR-96	5.6	-0.4	2.45
	miR-99a	10.8	-0.4	5.11

Pathway selection

In order to explore the physiological meaning of miRNAs responding to ionizing radiations, I listed the predicted target mRNAs of these miRNAs and selected the enriched signaling pathway based on enrichment and statistical analysis of predicted target mRNA by DIANA-microT-3.0. Among the listed signaling pathways, I focused on the top 10 pathways based on the statistical significance (Table 2). I especially chose the MAPK signaling pathway for further analysis because this signaling pathway is essential for survival in response to DNA damage (Cannell, Kong et al. 2010).

Table 2. Enrichment analysis for signaling pathways on target mRNAs of IR-responsive miRNAs

KEGG pathways	-ln[*p* value]*
Ribosome	25.8
MAPK signaling pathway	23.5
Axon guidance	22.8
Focal adhesion	20.7
Oxidative phosphorylation	20.1
Ubiquitin mediated proteolysis	17.9
TGF-beta signaling pathway	17.7
Adherens junction	17.2
Wnt signaling pathway	16.9
Regulation of actin cytoskeleton	16.2

* *p* value based on DIANA analysis

Enrichment Analysis

To validate regulation of the MAPK signaling pathway by IR-responsive miRNAs, I meta-analyzed mRNA expression profiles of the same γ-irradiated H1299 cells from my lab's previously published datasets (Jeong, Wu et al. 2009). In concurrent analysis of target mRNA and IR-responsive miRNA, I applied two criteria: 1) statistically significant changes ($p<0.05$) in mRNA expression upon irradiation by ANOVA analysis and 2) the high inverse correlation value ($r<-0.4$) between mRNA and miRNA expression. As summarized in Figure 2 and Table 3, I identified 35 pairs of IR-responsive miRNAs and target mRNAs, including 19 miRNAs and 23 non-overlapping mRNAs for MAPK signaling pathway genes in H1299 cells.

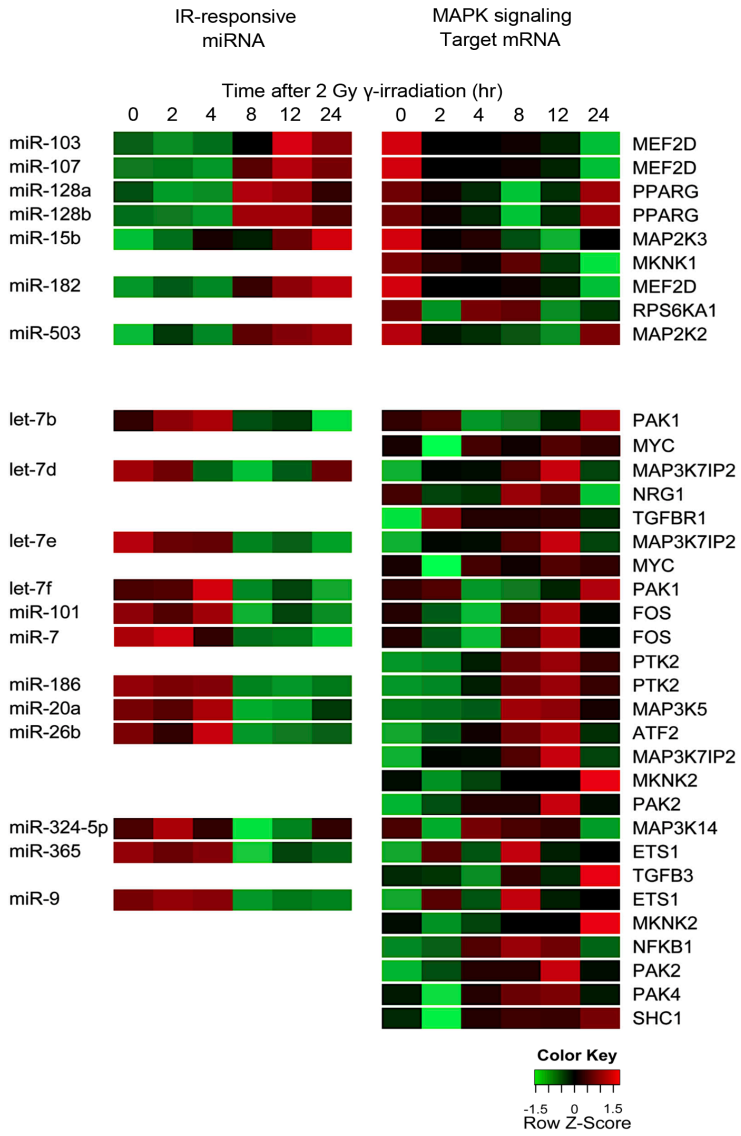


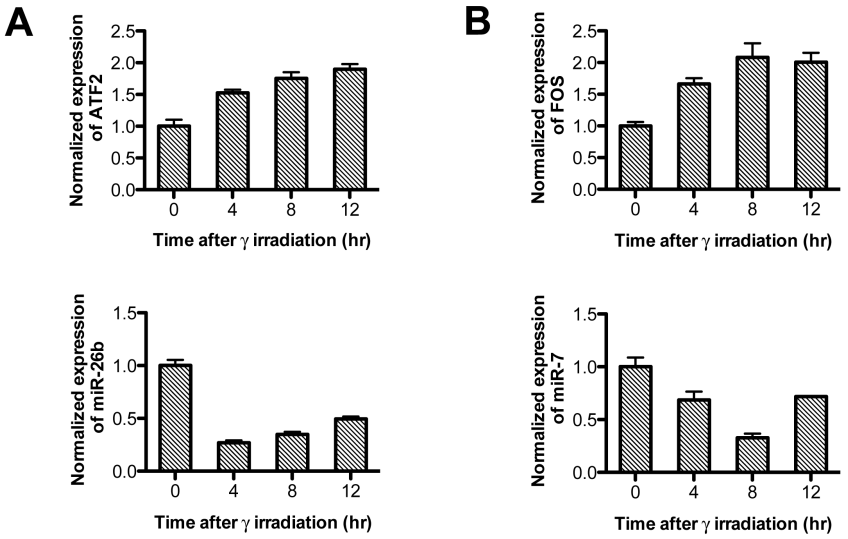
Figure 2. **Heatmap illustrating the pairs of miRNAs and target** mRNAs for the MAPK signaling pathway in response to γ-irradiation in H1299 cells.

Table 3. List of IR-responsive miRNAs and their target mRNAs in MAPK signaling pathway.

miRNA		ANOVA -ln(p-value)	Target mRNA	ANOVA -ln(p-value)	Pearson correlation (r^2)
Up-regulated miRNA	miR-103	1.33	MEF2D	4.25	-0.718
	miR-107	2.03	MEF2D	4.25	-0.617
	miR-128a	2.70	PPARG	2.41	-0.452
	miR-128b	3.14	PPARG	2.41	-0.397
	miR-15b	2.31	MAP2K3	4.99	-0.479
			MKNK1	2.44	-0.948
	miR-182	2.84	MEF2D	4.25	-0.774
			RPS6KA1	2.17	-0.420
	miR-503	2.37	MAP2K2	4.58	-0.411
Down-regulated miRNA	let-7b	3.07	PAK1	2.74	-0.434
			MYC	8.18	-0.405
	let-7d	2.69	MAP3K7IP2	2.36	-0.837
			NRG1	2.08	-0.528
			TGFBR1	1.91	-0.535
	let-7e	3.44	MAP3K7IP2	2.36	-0.592
			MYC	8.18	-0.394
	let-7f	3.57	PAK1	2.74	-0.420
	miR-101	2.09	FOS	2.13	-0.569
	miR-7	3.65	FOS	2.13	-0.491
			PTK2	5.26	-0.941
	miR-186	4.55	PTK2	5.26	-0.941
	miR-20a	3.17	MAP3K5	4.62	-0.984
	miR-26b	1.57	ATF2	7.71	-0.575
			MAP3K7IP2	2.36	-0.593
			MKNK2	5.49	-0.567
			PAK2	1.69	-0.542
	miR-324	1.81	MAP3K14	4.75	-0.552
	miR-365	3.31	ETS1	3.40	-0.590
			TGFB3	1.48	-0.650
	miR-9	3.69	ETS1	3.40	-0.461
			MKNK2	5.49	-0.670
			NFKB1	2.52	-0.473
			PAK2	1.69	-0.594
			PAK4	2.49	-0.640
			SHC1	2.80	-0.705

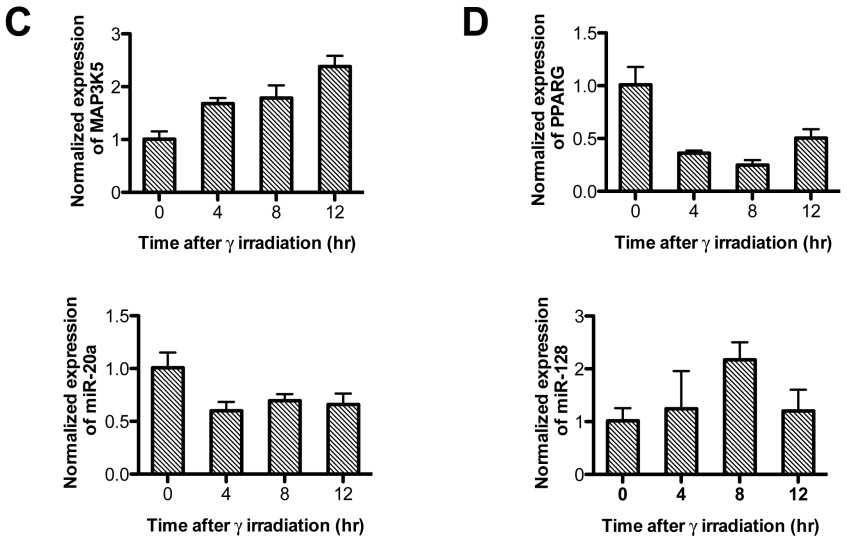
Analysis Validation

I validated the expression patterns of IR-responsive miRNAs and target mRNAs for the MAPK signaling pathway. Among 35 pairs, I selected and analyzed four (miR-26b: ATF2, miR-7: FOS, miR-20a: MAP3K5, and miR-128:PPARG) pairs by reverse transcription-polymerase chain reaction (RT-PCR; Figure 3A,B,C and D). As detected in microarray datasets (Figure 2), I found that ATF2, FOS, and MAP3K5 were up-regulated and PPARG was down-regulated upon IR exposure. MiRNAs such as miR-26b, miR-7, and miR-20a were down-regulated, and miR-128 was up-regulated upon IR exposure. By real-time RT-PCR, I demonstrated that the expression patterns of selected IR-responsive miRNAs and target mRNAs were well matched with those of the microarray expression data.




```
    3' ugGAUAGGACUUAAUGAACUu 5' miR-26b
        :| |::|   ||||||||
100:5' aaUUUUUUUUUUUUACUUGAa 3' ATF2
```

```
    3' ugUUGUUUUAGUGAUCAGAAGGu 5' miR-7
        |: |:|| |||   ||||||
65:5' agAGGAGAAACAC--AUCUUCCc 3' FOS
```




```
   3' gauggacgugauauuCGUGAAAu 5' miR-20a
                      |||||||
83:5' gaggagaaaggcgcuGCACUUUa 3' MAP3K5
```

```
    3' uuucucuggccAAGUGACACu 5' miR-128
                       ||:||||||
68:5' caccuaagaaaUUUACUGUGa 3' PPARG
```

Figure 3. Expression patterns of IR-responsive miRNAs and MAPK signaling target mRNAs in irradiated H1299 cells. The expression of four pairs of miRNA and target mRNA such as (A) miR-26b:ATF2, (B) miR-7:FOS, (C) miR-20a:MAP3K5, and (D) miR-128:PPARG) were quantitated using real-time reverse transcription-polymerase chain reaction (RT-PCR) at the indicated time. The values were normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA for target mRNAs and U6B small RNA for miRNAs. All values are presented as means ± standard deviation (SD) from triplicate experiments.

Relation between miRNA and mRNA

Down-regulated IR-responsive miRNAs may augment the function of target mRNAs. To test the relationship between down-regulated IR-responsive miRNAs and target mRNAs, I selected the pair of ATF2 and miR-26b among 35 pairs to demonstrate coordinated regulation between miRNAs and target mRNAs upon IR exposure. One predicted target was identified for miR-26b at position 112–118 of the ATF2 3' UTR, as shown in Figure 3A. I tested and found that overexpression of miR-26b in H1299 cells could inhibit translation of target mRNAs (Figure 4A). In luciferase assays, miR-26b suppressed the translation of luciferase in constructs with the 3′ UTR of ATF2, but not those without the 3′UTR (Figure 4B). The suppressive effect of miR-26b on ATF2 was also observed in -irradiated H1299 cells (Figure 4C), which was sustained until 12 hours after IR exposure.

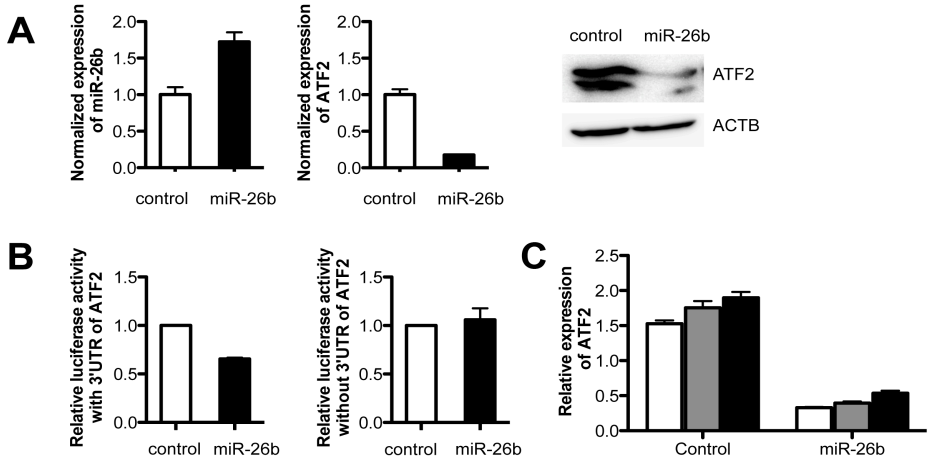


Figure 4. Suppression of activating transcription factor 2 (ATF2) by miR-26b. (A) In miR-26b transfected H1299 cells, the expression of miRNA was confirmed by real-time RT-PCR. The expression of ATF2 mRNA in miR-26b transfected cells was measured by real-time RT-PCR. The relative ATF2 expression levels were normalized against GAPDH and presented as mean ± SD from triplicate experiments. The protein level of ATF2 was also examined by western blot in miRNA-transfected cells. (B) Cells were transfected with the empty renilla luciferase reporter gene (psiCHECK2) or the reporter gene fused to the ATF2 3' UTR. In addition, the cells were co-transfected with miR-26b or without miR-26b; Results are expressed as relative light units (RLU) and were normalized with the luciferase activity expressed constitutively by the psiCHECK2 vector. (C) The relative expression of ATF2 in miR-26b transfected and IR exposed cells at 4 (white), 8 (grey) and 12 (black) hours respectively.

miRNA-mRNA coordination in response to MAPK Signaling Inhibition

Next, I wanted to confirm the effect of MAPK signaling on down-regulation of miR-26b in γ-irradiated cells. I inhibited the MAPK signaling pathway using SP600125, a c-Jun N-terminal kinase (JNK) inhibitor, in γ-irradiated H1299 cells. Treatment with SP600125 did not change the basal expression level of ATF2; however, induction of ATF2 upon -irradiation was markedly blocked in SP600125-treated H1299 cells until 12 hours after IR exposure (Figure 5A). Expression of ATF2 requires activation of MAPK signaling, which was inhibited at JNK by the chemical inhibitor. Conversely, expression of miR-26b was induced by treatment with SP600125 in H1299 lung cancer cells (Figure 5B). Additionally, I confirmed the effect of SP600125 in the A549 lung cancer cell line.

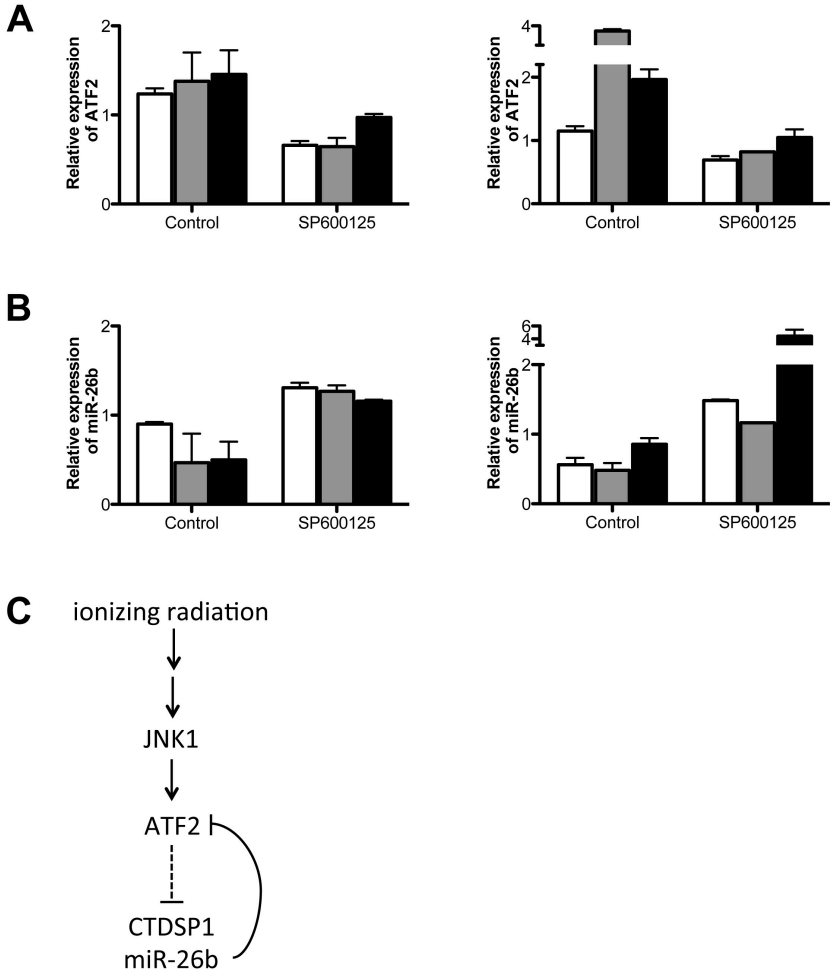

A
Relative expression of ATF2
Control
SP600125
B
Relative expression of miR-26b
C
ionizing radiation
JNK1
ATF2
CTDSP1
miR-26b

Figure 5. Effect JNK inhibitor on the expression of miR-26b in response to ionizing radiation (IR). (A) H1299 and A549 cells were treated with 10 μM SP600125 for 30 minutes, and then exposed to IR. The relative expression of ATF2 mRNA in cells treated with SP600125 at 4 (white), 8 (grey) and 12 (black) hours respectively. (B) Relative expression of miR-26b in IR-exposed H1299 and A549 cells with SP600125 treatment. (C) Ionizing radiation induced the expression of ATF2, which down-regulated the expression of miR-26b in γ-irradiated lung cancer cells.

DISCUSSION

Cellular responses to exogenous stimulation can be monitored by alterations in gene expression, including expression of miRNAs. IR can induce progressive changes in cell survival, growth, and proliferation by affecting gene expression. Previous reports have suggested that radiation can change the expression pattern of genes(Park, Hwang et al. 2002; Smirnov, Morley et al. 2009). I analyzed miRNA profiles to understand the mechanism of miRNA-mediated cellular responses to IR, and to identify regulation of the MAPK signaling pathway by IR-responsive miRNAs. In the present study, I have elucidated the JNK-mediated transcriptional suppression of miR-26b in γ-irradiated cells, for which miRNA can suppress the translation of target ATF2 mRNA, a member of the MAPK signaling pathway. From these findings, I suggest that the cellular response to IR is coordinately regulated by the interaction between the MAPK signaling pathway and miRNA.

ATF2 is a cAMP-response element-binding (CREB) protein with a basic leucine zipper (bZIP) domain, through which ATF2 interacts with other bZIP proteins such as JUN, FOS, CREB, and ATF1 (Maekawa, Sakura et al. 1989; Kerppola and Curran 1993). DNA damage and pro-inflammatory cytokines can induce activation of ATF2 transcriptional activity by JNK (Buschmann, Yin et al. 2000). The role of diverse signaling in activation of ATF2 is also illustrated by hetero-dimeric partners of ATF2, which are also activated in a stimulus-specific manner. Thus, a particular stimulus can lead to different ATF2

complexes, thereby activating or repressing distinct sub-sets of target genes (van Dam and Castellazzi 2001).

miR-26b is an intronic miRNA residing in intron IV of CTDSP1, C-terminal domain small phosphatase 1. The transcriptional control of host CTDSP1 mRNA is not fully understood, but many putative binding sites exist for transcription factors such as CREB in ENCODE Transcription Factor Binding Analysis (Euskirchen, Rozowsky et al. 2007). ATF2 could suppress transcription of target genes through dimerization with other bZIP transcription factors. Over-expression of bZIP proteins such as ATF2 and CREB altered the gene expression in human myometrial cells (Bailey, Tyson-Capper et al. 2005). Meta-analysis on this microarray datasets in GEO (GSE1059) revealed down-regulation of CTDSP1 in ATF2 over-expressed cells. I need further study regarding the transcriptional control of miR-26b by ATF2 in lung cancer cells; however, JNK activity and expression of ATF2 repressed expression of miR-26b is performed in the current study.

Deregulation of the MAPK signaling pathway can be induced by IR-induced DNA damage (Dent, Yacoub et al. 2003). In the present study, it was found that MAPK signaling is induced in -irradiated H1299 cells, which might mediate the survival of H1299 lung cancer cells upon IR exposure. Furthermore, activation of MAPK signaling led to down-regulation of miR-26b, which supported the maintenance of ATF2 activity in turn. From these results, I could demonstrate that exposure of H1299 lung cancer cells to IR induced MAPK signaling followed by suppression of miR-26b expression, which led to the

escape of ATF2 mRNA from post-translational suppression by miR-26b. I propose that miR-26b mediates coordinate regulation of ATF2 and the MAPK signaling pathway in response to IR.

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CHAPTER II

miR-506 regulates TGFβ and NF-κB mediated epithelial mesenchymal transition in breast cancer

ABSTRACT

Epithelial-mesenchymal transition (EMT) is associated with increased aggressiveness, invasive and metastatic potential. Among thirty four miRNAs predicted to target twelve EMT-related genes, I found that miR-506 is predicted to target almost all the EMT genes, also it is significantly related to breast cancer patient survival in meta- analysis (>98% patients, n = 202) of breast cancer miRNA profiling data (GSE22216). Apart from miR-506, I found that, eight miRNAs (miR-135a, miR-139-5p, miR-30c, miR-29a, miR-29c, miR-128a, miR-101, miR-34c) were significantly related to breast cancer patient survival. When overexpressed, miR-506 suppressed the expression of mesenchymal genes such as VIM1, snail homolog 2 (SNAI2), cluster of differentiation 151 (CD151), and induced the expression of epithelial gene E-cadherin (CDH2) in MB231 human breast cancer cell lines. Next, I confirmed through luciferase assay using reporter vector with wild type UTR, or mutated UTR, that miR-506 targets the 3'UTR of VIM, SNAI2 and CD151. Upon sequence analysis, I found that there is a Nuclear factor-kappa B (NF-κB) (known inducer of EMT in breast cancer) binding site approximately 1Kb upstream of promoter region of miR-506. Through Chip assay and promoter activity assay I confirmed that NF-κB binds upstream of miR-506 promoter and suppresses it.

Further, I confirmed that miR-506 suppression is an indispensible step for the NF-κB induced EMT by showing that overexpression of miR-506 inhibited TGFβ-induced mesenchymal transition of MCF10A cells even in presence of NF-κB. Apart from this

when I co-transfected both miR-506 and NF-κB in MB231 cells, I found that CD151 expression, which is positively correlated with NF-κB in several breast cancer cell lines and in patient samples ($p<0.05$, n = 133, 70.4% of total patients), is suppressed. In functional study of miR-506, I found that ectopic expression of miR-506 could suppress adhesion, invasion and migration of MB231 cells.

From all the results, I concluded a novel molecular mechanism in which NFkB binds upstream of the promoter region of miR-506 and leads to its suppression in order to induce the EMT marker genes which are targeted by miR-506. Hence miR-506 suppression is a indispensible step in the progression of TGFβ and NF-κB induced EMT in breast cancer cells.

Keywords: Epithelial mesenchymal transition, miR-506, NF-κB, TGFβ, Breast cancer.

INTRODUCTION

Epithelial to mesenchymal transition (EMT) is a process which enables the cancer cells to lose their cell-cell and cell-matrix contact, gain the migratory properties and become motile mesenchymal cells (Thiery 2002). The transcriptional reprogramming processes allow epithelial tumor cells to lose cell polarity and cell-junction proteins and acquire signal transduction activities associated with mesenchymal cells facilitating migration and survival in an anchorage-independent environment (Evdokimova, Tognon et al. 2009). The gain of mesenchymal cell markers such as VIM1, snail homolog 2 (SNAI2), and fibronectin (FN1) has been observed in tumor progression (Emadi Baygi, Soheili et al. 2010; Wang, Kuiatse et al. 2010). Cellular changes resulting in a more mesenchymal-like state has been associated with poor prognosis. Mesenchymal-like tumor cells gain migratory capacity through abnormal survival signals via receptors such as fibroblast growth factor receptor (FGFR), hepatocyte growth factor receptor (MET), transforming growth factor-beta receptors (TGFβR), insulin-like growth factor 1 receptor (IGF1R), platelet derived growth factor receptor (PDGFR) and regulatory kinases such as phosphoinositide-3-kinase (PI3K), v-akt murine thymoma viral oncogene homolog 1 (AKT) and mechanistic target of rapamycin (MTOR) (Rosenfeldt, Hobson et al. 2001; De Wever and Mareel 2003; Surmacz and Bartucci 2004; Harvey and Lonial 2007; Ridolfi, Matteucci et al. 2008; Wesche, Haglund et al. 2011).

Up-regulation of NF-κB was found in human breast tumor cell lines, carcinogen-transformed mammary epithelial cells and the majority of primary human and rodent breast tumor tissue samples (Min, Eddy et al. 2008). NF-κB has been reported as a central mediator of EMT in a mouse model of breast cancer progression (Huber, Azoitei et al. 2004; Huber, Beug et al. 2004). More recently, NF-κB is identified up-stream of SNAI2 expression during EMT of human mammary epithelial MCF10A cells over-expressing a constitutively active IGF1R (Kim, Litzenburger et al. 2007). SNAI2 can also repress endogenous CDH1 gene expression. In breast cancer cell lines, SNAI2 levels were shown to correlate with loss of E-cadherin transport (Dubois-Marshall, Thomas et al. 2011).

There are 33 tetraspanin family proteins forming complex with the integrins, which are functionally relevant in cell-cell adhesion in a cadherin-independent manner. CD151 is the first member of the tetraspanin family to be associated as a promoter of human tumor metastasis (Ang, Lijovic et al. 2004). During cancer, CD151 regulates adhesion-dependent signaling and post-adhesion events including cell migration (Chometon, Zhang et al. 2006; Yamada, Tamura et al. 2008). Also CD151 role in EMT induction and overall survival of cancer patients is well known (Ang, Lijovic et al. 2004; Ke, Shi et al. 2011). TGFβ has been postulated as a pro-oncogenic factor in the late step of the tumor progression. In transformed cells, TGFβ enhances the ability to degrade the extracellular matrix, cell invasiveness and EMT, which are crucial steps for metastasis (Tobar, Villar et al. 2010). It is reported

that NF-κB and CD151 induction are indispensible steps for TGFβ mediated response (Sadej, Romanska et al. 2010; Tobar, Villar et al. 2010).

From the meta-analysis on miRNA profiles of breast cancer, I selected miRNAs related to patient survival. Especially miR-506 could suppress the translation of EMT-related genes such as VIM, SNAI2 and CD151. TGFβ- and NF-κB-mediated EMT could be blocked by miR-506, which provides the possibility of therapeutic target for EMT.

MATERIAL AND METHODS

Cell culture, cloning

MB231, MB157, and MB468 human breast cancer cell lines, were maintained in Dulbecco's Modified Eagle's medium (DMEM, Sigma Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin**.** MB436 cells were maintained in Leibovitz's L-15 medium supplemented with 10% FBS and 10µg/ml insulin. MCF10A, normal mammary epithelial cells were grown in the base medium (MEBM) along with the additives MEGM (Lonza Clonetics, Walkersville, MD). MCF10A cells were treated with 20ng/mlTGF–β1 (R&D System Inc. Minneapolis, MN) for the indicated periods to induce EMT.

Human pre-miR-506 was amplified using genomic DNA of MB231and cloned into pcDNA3 (Invitrogen, CA). NF-κB was amplified using cDNA of MB-231 and cloned into pcDNA3 (Invitrogen, Carlsbad, CA) using gene specific primer (Table 1).

Table 1. List of Primer sequences used in this study

CONDITION	NAME	FORWARD	REVERSE
3' UTR Cloning	CD151	AATTCTCGAGCACTACTGACCCTGCCTTGG	AATTGCGGCCGCCAGGCAGAGAGTGTGTCTGG
	SNAI2	AATTCTCGAGTTTGCTGATGGCTAGATTGAGA	AATTGCGGCCGCTTCAACAATGGCAACCAGAC
	CD151 mutant	GAGGCAGCTTCAAGCATCTTTTGCTGCGCAC	GTGCGCAGCAAAAGATGCTTGAAGCTGCCTC
	SNA I2 mutant	GAGATCTGCCAGACGCGAATGAAGGTGCCTTAAAAAGTATTC	GAATACTTTTTAAGGCACCTTCATTCGCGTCTGGCAGATCTC
	VIM mutant	GAAACAGCTTTCAAGCATCTTTCTGCAGTTTTTCAGG	CCTGAAAAACTGCAGAAAGATGCTTGAAAGCTGTTTC
QRT PCR	CD151	AACCTCAGAGGCAGCTTCAA	GACCACCAGGCAGAGAGTGT
	SNAI2	CAAGAACAAAACACAGGAGAATG	GCCAGGAATGTTCAAAGCTAA
	VIM	TTCTCAGCATCACGATGACC	TCTTGCGCTCCTGAAAAACT
Promoter Assay	miR-506 promoter	AATTCTCGAGTCAAATCAGGGATTTAGTATTTTCA	AATTAAGCTTCATCTTAATAGTAACACGGGGAAA
	miR-506 mutant promoter	CGTCCATCACTTCTTTGTGGAGCGAACATTCAAAACCCTCTC	GAGAGGGTTTTGAATGTTCGCTCCACAAAGAAGTGATGGACG
CHIP	miR-506	TCAAATCAGGGATTTAGTATTTTCA	TTTATGGGTACAGAGTCACAGTTAGG
Expression Vectors	miR-506	CCGGAATTCTTGCACCTTTTGGAGTGAAA	ATTGCGGCCGCACCTGGAAATGGCTCATCAC
	NF-κB	CCGGAATTCCATATTTGGGAAGGCCTGAA	ATTGCGGCCGCTCGGAGCTCGTCTATTTGCT

RNA preparation and quantitative real-time PCR

Total RNA was extracted from cell lines using the TRIzol method, and then reverse transcribed to complementary DNA using

Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo-(dT)12–18 primers according to the manufacturer's protocol. The quantitative RT-PCR for indicated genes was performed in a reaction mixture containing SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan). Quantitation of miRNAs was performed using TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Samples were analyzed using the ABI PRISM 7000 sequence detection system (Applied BioSystems). All PCRs were performed in triplicate, the relative quantitative method was used for the quantitative analysis. The calibrator was the averaged ΔCt from the untreated cells. The endogenous control was glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for genes and U6B for miRNAs.

Luciferase assay

The 3' UTRs of CD151, VIM1 and SNAI2 were fused to the renilla gene using the XhoI/NotI restriction sites of the psiCHECK2vector (Promega Fitchburg, WI). Mutation in miR-506 target site in these UTRs were generated using the Quik Change Multi site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primer used to amplify WT and mutant (MUT)3'UTR are listed in Table 1. Luciferase assays were performed using the Dual-Luciferase assay (Promega). Normalization of the Renilla expression was performed using the luciferase gene present on the psiCHECK2 vector.

Western blotting

Cells were harvested and lysed in RIPA buffer and Protease Inhibitor Cocktail (Sigma, St. Louis, MO). Protein extracts were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA). Membranes were incubated with an anti-CD151 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), E-cadherin/CDH1 (Abcam,Cambridge,UK), SNAI2 (Cell Signaling Technology, Danvers, MA), and Vimentin (DAKO, Glostrup, Denmark) respectively, in Tris-buffered saline Tween 20 buffer with non-fat dry milk, and then incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA). Immunoreactive bands were visualized using the Chemiluminescent Substrate Kit (Thermo Scientific, Rockford, IL).

miR-506 promoter analysis

To obtain a luciferase construct p.miR-506-luc derived fragment from the miR-506 promoter, was inserted into pGL3 Basic plasmid (Promega). Cells were transfected with reporter plasmid and TK-Renilla (Promega) for normalization. The transfected cells were harvested after 48 hours and dual luciferase assay was performed according to the manufacturer instructions (Promega).

Adhesion Assay

Adhesion assays were performed after 48hrs post-transfection of miR-506, using a Cytoselect 48-well cell adhesion assay [extracellular matrix (ECM) array; Cells Biolabs, catalogue no CBA-070] in accordance with the manufacturer's protocol. Briefly cells were counted and plated at a density of 2*10^6 cells/ml, incubated in a tissue culture incubator for 90 minutes at 37C. After incubation, adherent cells were stained and quantified at OD 560nm after extraction.

Migration Assay

For migration assays, 48hrs after transfection the cells were seeded into upper chamber of Transwell insert (pore size, 8um; Costar) in serum free medium. The lower chambers were filled with medium containing 10% serum. The non-migratory cells were removed from the upper chamber by scraping with a cotton bud. The cells remaining on the lower surface of the insert were fixed with 4% formaldehyde and stained with crystal voilet.

Invasion Assay

For invasion assays, cells were seeded in a Matrigel BD (Bio-Coat) coated chamber, the lower chambers were filled with medium containing 10% serum and were incubated at 37c overnight. The non-invasive cells were removed from the upper chamber by scraping with a cotton bud. The cells remaining on the lower surface of the insert were fixed with 4% formaldehyde and stained with crystal voilet.

ChIP assay

Chromatin immunoprecipitation experiment was modified from the EZ-CHIP (Upstate, Tenecula, CA) protocol using anti-NF-kB antibody (SantaCruz Biotechnology, Santa Cruz, CA) (primers provided in Table 1)

Bioinformatic and statistical analysis

Quantitative results were expressed as mean ± s.e.m. Independent student's t test or the Wilcoxon signed-rank test was performed to analyze gene and miRNA expression levels and data. The log-rank test was used to test for differences in survival in univariate analysis.

The normalized mRNA and miRNA expression data were obtained from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) using series matrix files of datasets GSE2990, GSE32474 (mRNA) and GSE26375 (miRNA). Correlation between groups was assessed by Pearson correlation [r], with $p < 0.05$ being considered significant.

Kaplan-Meier plots were used to estimate distant metastasis-free survival in the GEO dataset GSE22216, in which retrospective clinical study is performed to identify breast cancer prognostic markers and associated pathways. Early primary breast cancers were considered in this study, those had complete 10-years follow-up, clinical and demographics information. Samples were stratified into high and low miRNA expression groups. High expression was defined as tumors expressing the miRNA within the upper median expression range ($p <$

0.05). All other tumors were considered to have low miRNA expression ($p < 0.05$). Significance of survival differences between groups was assessed by log-rank test, with $p < 0.05$ being considered significant.

RESULTS

Selection of miRNAs regulating EMT and breast cancer patient survival

In order to study the function of miRNA in epithelial to mesenchymal transition, I designed a model (Figure 1A) in which I considered 12 well known EMT-related genes SNAI2 (Ryu, Song et al. 2011), VIM1 (Kokkinos, Wafai et al. 2007), N-cadherin (CDH2)(Strizzi, Bianco et al. 2004), E-cadherin (CDH1) (Lombaerts, van Wezel et al. 2006), Sox-9(Pritchett, Athwal et al. 2011), Rock-1(Peng, Zhang et al. 2012), Rock-2(Rodrigues-Diez, Carvajal-Gonzalez et al. 2008), CD151(Ke, Shi et al. 2011), FOXQ-1(Qiao, Jiang et al. 2011), ITGA-7(Vasko, Espinosa et al. 2007), ITGB-1(Jenndahl, Taylor-Papadimitriou et al. 2006), and Rho-1(Zondag, Evers et al. 2000; Cho and Yoo 2007)). Using TARGETSCAN, 34 miRNA were predicted to target 12 EMT-related genes under a criterion of total context score (< -0.20). Interestingly I found that miR-506/124 (targeting the same seed sequences) are predicted to target most of the EMT related genes considered in comparison with the other candidate miRNAs (Table 2), hence I enriched these miRNAs (miR-506/124) by retrospective analysis on a series of breast cancer miRNA expression profiling in GEO dataset GSE22216. I divided the patient samples into high and low miRNA expression based on the median value ($P < 0.05$, n=206). Interestingly I found that miR-506 showed a significant association with DRFS in more than 98% of patients (Fig. 1B)(P=0.0458). MiRNA, miR-124 showed no significant impact on

DRFS in these patients (Figure 1C), thereby leading me to consider miR-506 for the present study. I also analyzed the effects of other miRNAs on distant relapse free survival (DRFS) in same patients (n=202) and classified miRNAs into three categories; 1) tumor suppressive miRNAs (miR-135a, miR-139-5p, miR-30c, miR-29a, miR-29c, miR-128a, miR-101, miR-34c), 2) oncogenic miRNAs (miR-128a, miR-101, miR-34c) and 3) unrelated miRNAs (miR-507, miR-138, miR-190, miR-133a, miR-142-3p, miR-30d, miR-128b, miR-135b, miR-153, miR-133b, miR-145, miR-124, miR-449a, miR-138, miR-96, miR-144, miR-152, miR-449b, miR-30b, miR-34a, miR-183, miR-190, miR-148a, miR-148b, miR-384, miR-29b) (Table 3) (Figure 2).

I also analyzed the relationship of the expression of miR-506 and lymph node metastasis. The involvement of lymph nodes in breast cancer is less when miR-506 expression is high (Figure 3A). I checked the expression of miR-506 in six breast cancer cell lines MB436, MB157, MB468, BT20 and MB231 respectively by quantitative RT-PCR, and found that the expression level of miR-506 in breast cancer cell lines was less than that of normal breast cell line MCF10A (Figure 3B).

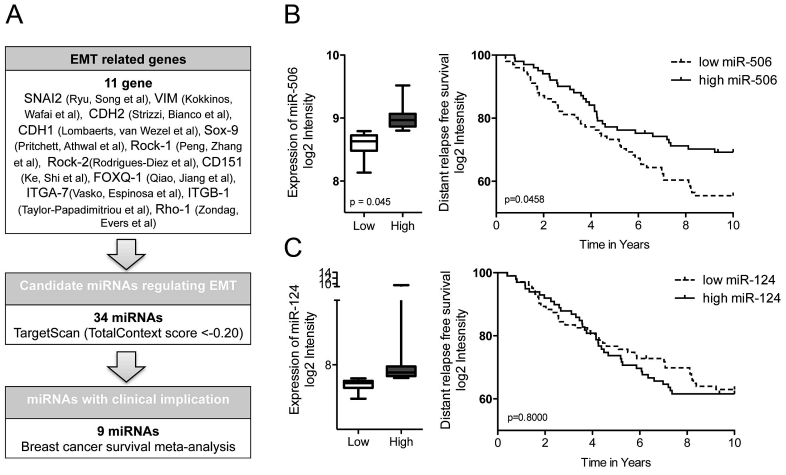
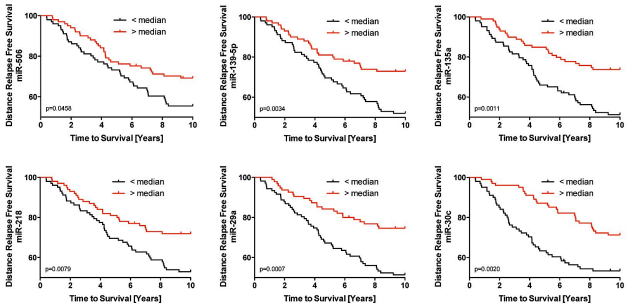
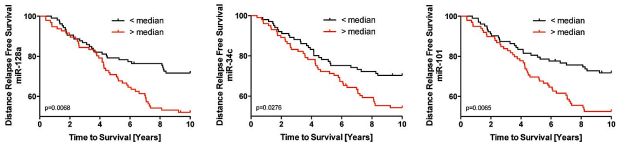


Figure 1.**Model for miRNA study in breast cancer** – A) Previously reported 12 EMT related genes were considered and miRNA targeting these genes were identified using TargetScan, further filtering is done considering a total context score of < -0.20 as a result I obtained 34 miRNAs. Next clinical significance of miRNAs is studied considering GEO dataset GSE22216 and as a result of analyses miRNAs are categorized into tumor suppressor, oncogenic or non-responsive as mentioned in Table 3. B) In GEO dataset GSE22216, patients were divided into two sets based on the miR-506 expression (greater or less than the median, $p < 0.05$) and I found that expression of miR-506 affected distant relapse free survival in more than 98% ($n=202$, $p < 0.05$). C) Similar to miR-506, I analyzed the impact of miR-124, which shared same seed region as that of miR-506, in same breast cancer patients and found that miR-124 did not affected the DRFS in these patients.

Tumor Suppressor miRNAs



Oncogenic miRNAs



Non-responsive miRNAs

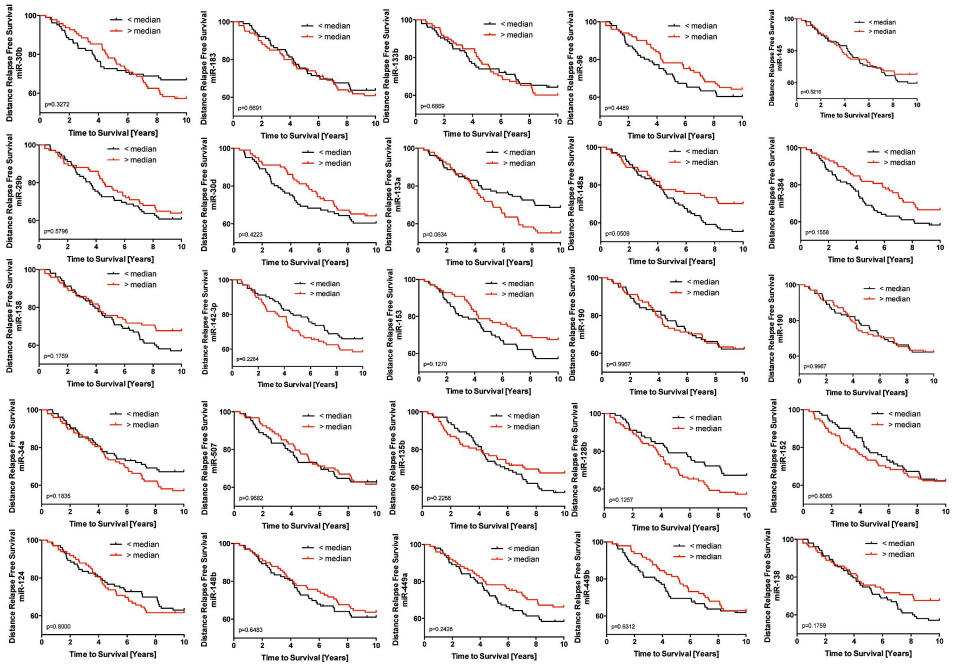


Figure 2.Impact of miRNAs on distant relapse free survival of breast cancer patients. I analyzed the impact of remaining 32 miRNAs selected by model, on DRFS of breast cancer patients and classified miRNAs into three categories; 1) tumor suppressive miRNAs, 2) oncogenic miRNAs and 3) unrelated miRNAs respectively.

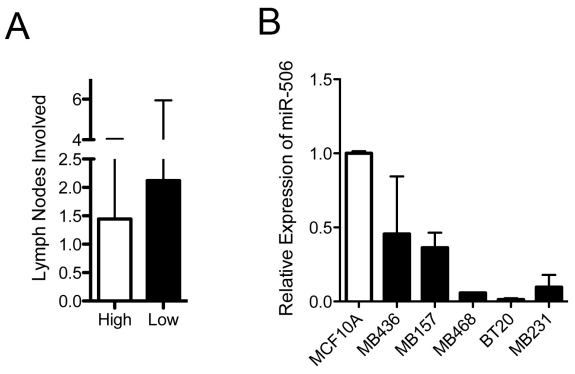


Figure 3. Relation of miR-506 on lymph nodes involved and its expression in breast cancer cell lines. A) Total number of lymph nodes was analyzed in miR-506 over or under expressing patients. I found that numbers of lymph nodes involved are less in patients in which expression of miR-506 was high. B) I analyzed the expression of miR-506 in six breast cancer cell lines MB436, MB157, MB468, BT20 and MB231 respectively by quantitative RT-PCR, and found that the expression level of miR-506 in breast cancer cell lines was less than that of normal breast cell line MCF10A.

Table 2. MiRNAs targeting EMT related genes, which have been considered.

		Conserved Sites				
Genes	miRNA	Total	8mer	7mer-m8	7mer-1A	Total Context score
SNAI2	**miR-124/124ab/506**	3	1	2	0	-0.71
FOXQ1	miR-133abc	1	1	0	0	-0.42
	miR-124/124ab/506	1	1	0	0	-0.4
	miR-96/507/1271	1	1	0	0	-0.4
	miR-34ac/34bc-5p/449abc/449c-5p	1	0	1	0	-0.23
	miR-128/128ab	1	0	1	0	-0.2
Vim	**miR-124/124ab/506**	1	0	1	0	-0.26
N-CADH /CDH2	**miR-124/124ab/506**	1	0	1	0	-0.28
	miR-145	1	0	1	0	-0.22
ITGA7	**miR-124/124ab/506**	1	1	0	0	-0.32
ITGB1	miR-183	1	0	1	0	-0.62
	miR-124/124ab/506	2	0	2	0	-0.56
	miR-29abcd	1	0	1	0	-0.24
ROCK1	miR-148ab-3p/152	1	0	1	0	-0.38
	miR-144	1	1	0	0	-0.36
	miR-124/124ab/506	1	1	0	0	-0.32
	miR-190/190ab	1	0	1	0	-0.27
	miR-153	1	0	1	0	-0.21
ROCK2	miR-142-3p	1	1	0	0	-0.41
	miR-138/138ab	1	1	0	0	-0.4
	miR-135ab/135a-5p	1	1	0	0	-0.28
	miR-153	1	0	1	0	-0.25
	miR-139-5p	1	1	0	0	-0.23
SOX9	miR-145	1	1	0	0	-0.41
	miR-30abcdef/30abe-5p/384-5p	1	1	0	0	-0.4
	miR-138/138ab	1	1	0	0	-0.24
	miR-101/101ab	1	0	1	0	-0.22
CD151	**miR-124/124ab/506**	1	0	1	0	-0.21

Table 3. Categorical representation of miRNAs based on their impact on distant relapse free survival of breast cancer patients.

Property	miRNA	Log-rank (Mantel-Cox) Test		Gehan-Breslow-Wilcoxon Test		Targeting Genes
		Chi square	P value	Chi square	P value	
Tumor Suppressor miRNAs	miR-135a	10.680	0.001	10.280	0.001	ROCK1
	miR-506	3.988	0.046	3.858	0.050	SNAI2, FOXQ1, VIM1, N-CADH, ITGA7, ITGB1, ROCK1, CD151
	miR-139-5p	8.587	0.003	7.626	0.006	ROCK2
	miR-30c	9.545	0.002	11.920	0.001	SOX9
	miR-29a	11.480	0.001	11.090	0.001	ITGB1
	miR-29c	12.040	0.001	13.340	0.000	ITGB1
OncomiRs	miR-128a	7.327	0.007	6.188	0.013	FOXQ1
	miR-101	7.415	0.007	6.592	0.010	SOX9
	miR-34c	4.850	0.028	4.152	0.042	FOXQ1
Non-Responsive miRNAs	miR-507	miR-138	miR-190	miR-133a	miR-142-3p	miR-30d
	miR-128b	miR-135b	miR-153	miR-133b	miR-145	
	miR-124	miR-449a	miR-138	miR-96	miR-144	
	miR-152	miR-449b	miR-30b	miR-34a	miR-183	
	miR-190	miR-148a	miR-148b	miR-384	miR-29b	

Regulation of EMT-related genes by miR-506

miR-506 is predicted to target the 3'UTRs of CD151, VIM1, and Snai2. I overexpressed precursor miR-506 in MB231 human breast cancer and checked the expression of these target genes and CDH1. The mRNA and protein expression level of CD151, VIM1, and SNAI2 is suppressed by miR-506, while that of CDH1 was up regulated (Figure 4A and 4B). I cloned miR-506-binding sequences in 3'UTR of CD151, VIM1, and SNAI2 for luciferase assay. Overexpression of miR-506 resulted in a significant decrease in luciferase activity with wild type 3'UTR of CD151, VIM1 and SNAI2, but not with mutant sequences (Figure 5). Taken together, CD151, VIM1 and SNAI2 were direct targets of miR-506.

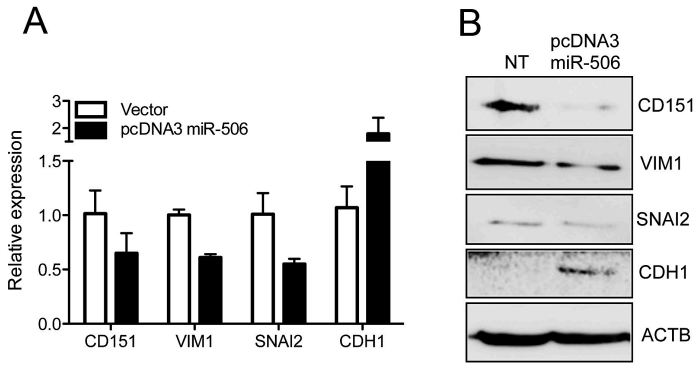


Figure 4. Impact of miR-506 over-expression on EMT marker genes. A) miR-506 was over-expressed in MB231 breast cancer cell line and its impact on EMT marker genes like CD151, VIM1 and SNAI2 is checked by quantitative real time PCR and B) western blotting.

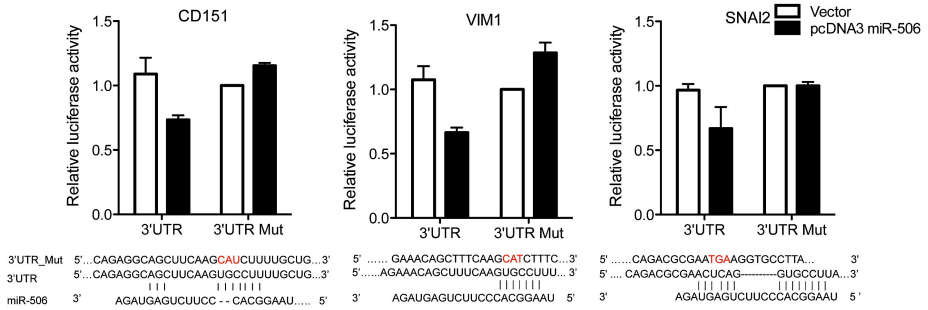


Figure 5. miR-506 targets the 3'UTR of EMT market genes. The wild type or mutant type 3' UTRs of CD151, VIM1, and SNAI2 were fused to the renilla gene using the XhoI/NotI restriction sites of the psiCHECK2vector. Luciferase assays were performed. I confirmed that miR-506 targets the 3'UTR of these EMT marker genes. Normalization of the Renilla expression was performed using the luciferase gene present on the psiCHECK2 vector.

NF-κB-mediated regulation of miR-506

In promoter sequence analysis, I found a putative binding site for NF-κB at -1013 bp of precursor miR-506. I confirmed binding of NF-κB to the upstream of miR-506 promoter by chromatin immuno-precipitation (ChIP) assay (Figure 6A). NFkB suppression leads to the induction of promoter activity of miR-506. Next, in order to check the specificity of impact of NF-κB on promoter activity, I mutated the NF-κB binding region and then transfected the cells with siNF-κB, there was hardly any change when the NF-κB binding site was mutated (Figure 6B)

I checked the correlation of mRNA expressions of NF-κB and miR-506 in MB436, MB231, MB468 and MB157 cell lines respectively, together with MCF10A normal breast epithelial cells. The expression of NF-κB was consistently high in four cancer cell lines and miR-506 level was low. On the other hand, the expression of NF-κB mRNA was low and miR-506 level was high in MCF10A cells (Figure 7A).

To test whether NF-κB suppressed miR-506, I knock-downed NF-κB in MB231 using siNF-κB and examined the expression of miR-506. As shown in Figure 7B, the expression of miR-506 was induced by the suppression of NF-κB. On the other hand, when I overexpressed miR-506 in MB231 cells, the expression of NF-κB was not changed at all (Figure 7C).

These results revealed that NF-κB could bind to the promoter region of miR-506 to suppress the transcription.

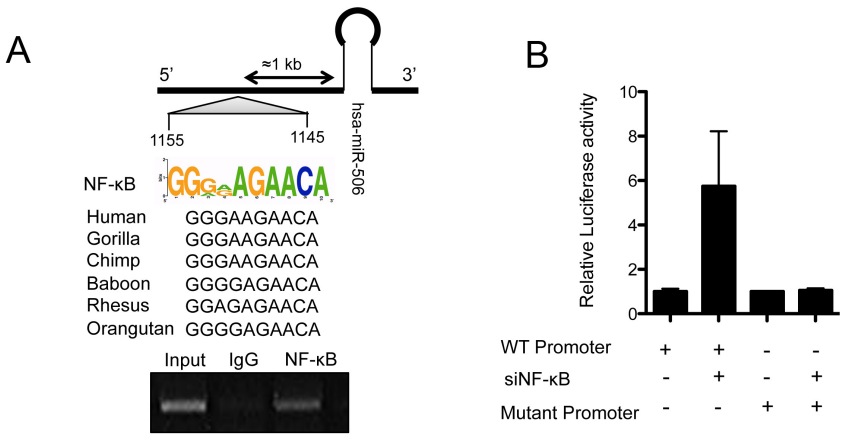


Figure 6. NF-κB binds upstream of promoter region of miR-506 and suppresses its promoter activity. A) About 1kb upstream of promoter region of miR-506 is NF-κB binding site, which was confirmed by chromatin immunoprecipitation (ChIP) assay. B) In order to obtain a luciferase construct p.miR-506-luc derived fragment from the miR-506 promoter, was inserted into pGL3 Basic plasmid. Cells were transfected with reporter plasmid. The transfected cells were harvested after 48 hours and dual luciferase assay was performed which revealed that NF-κB suppression could induce the promoter activity of miR-506, whereas upon mutation of NF-κB binding site, the promoter activity of miR-506 remained unchanged upon NF-κB suppression.

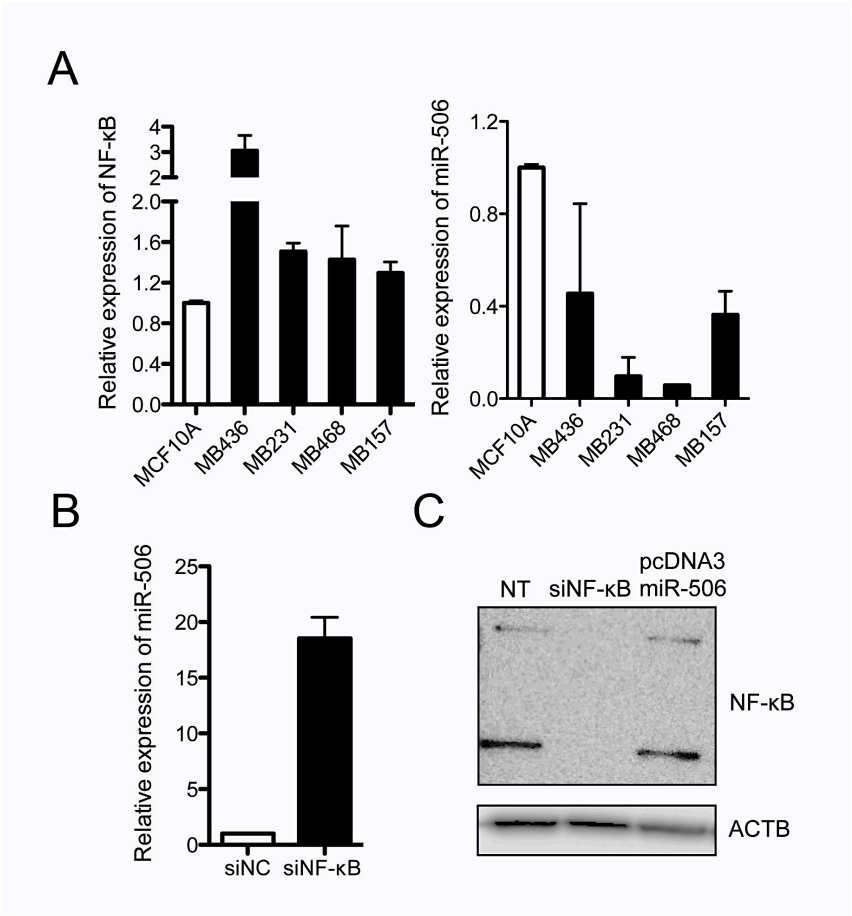


Figure 7. miR-506 is negatively correlated and downstream to NF-κB. A) NF-κB is induced in MB436, MB231, MB468 and MB157 and miR-506 is down regulated in these cell lines as compared to MCF10A normal breast cell line. B) Over-expression of miR-506 when MB231 cells are transfected with siNF-κB. C) NFkB remained unaffected when miR-506 is transfected in MB231 cells (western blot).

Relationship between NF-κB, miR-506, and EMT

Till now, I had found that NF-κB leads to the inhibition of miR-506 expression by affecting its promoter activity, also miR-506 targets to 3'UTR of EMT marker genes like VIM1, SNAI2 and CD151. It is well known that NF-κB induces EMT(Borthwick, Gardner et al. 2012), so I asked if NF-κB induces EMT by inducing the expression of genes which are targets of miR-506 ? Secondly, if miR-506 down regulation via NF-κB is dispensable or indispensible for EMT induction?

As the first step, I analyzed the GEO dataset GSE2990 that comprised of gene expression studies in 189 breast cancer patient tissues. It has been reported previously that CD151 is upstream of various EMT genes (Ke, Shi et al. 2011). I also found that in 133 patients (70.4%), mRNA expression level of CD151 was positively correlated with various EMT markers, importantly with NF-κB (Figure 8A). Apart from this, I analyzed NCI60 database (datasets GSE32474 and GSE26375 respectively), and found that, CD151 mRNA level in 60 human cancer cell lines was significantly and inversely correlated with miR-506 expression ($R=-0.4259$, $p=0.0008$)(Figure 8B).

In order to check the impact of NF-κB on CD151 expression, I transfected the MB231 cells with siNF-κB, as a result the expression of CD151 is suppressed (Figure 8C). When MB231 cells were transfected with siCD151, the expression of NF-κB remained unchanged thereby indicating that CD151 is downstream of NF-κB. When both NF-κB and siCD151 are co-transfected then the expression of CD151 is induced as

compared to that of siCD151 transfection alone (Figure 8D) thereby suggesting that NF-κB is inducing the expression of CD151.

Next in order to understand if miR-506 inhibition is indispensible for NF-κB induced EMT, first, I tested the role of miR-506 in NF-κB-mediated up-regulation of CD151. When I introduced miR-506 and NF-κB expression vector together in MB231 cells, the expression level of CD151 was suppressed as much as that in miR-506 alone (Figure 9A and 9B). Next I studied the impact on miR-506 on EMT transition.

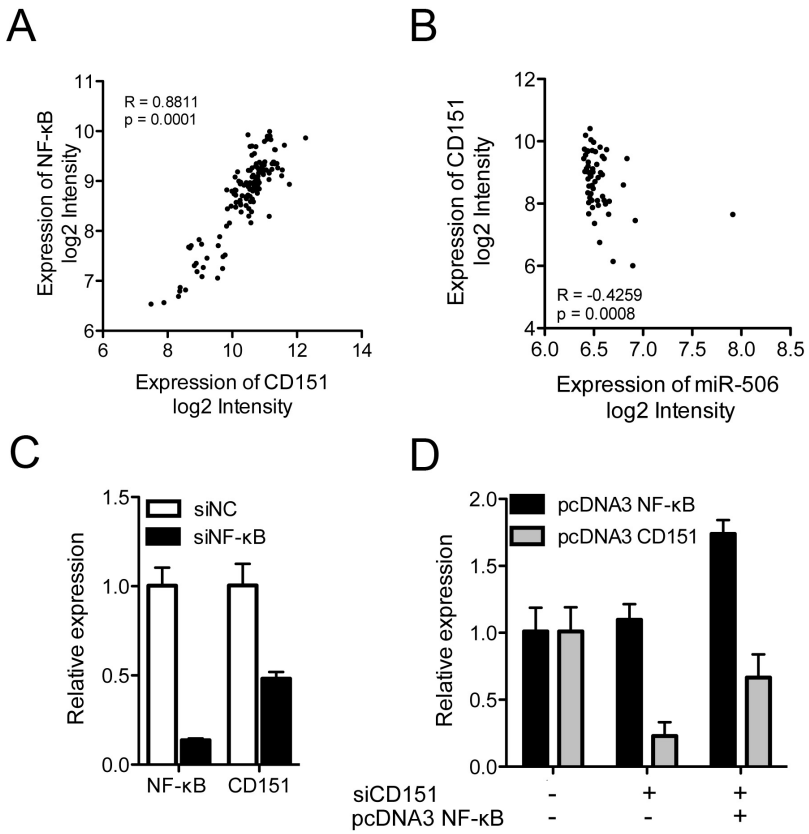


Figure 8. NF-κB mediated epithelial to mesenchymal transition. A) Breast cancer patient gene expression analysis considering GEO dataset GSE2990 revealed that there exists a positive correlation between NF-κB and CD151 in 70.4% of patients (n=133). B) I analyzed NCI60 database (GSE32474 and GSE26375 respectively), and found that, CD151 mRNA level is inversely correlated with miR-506 expression (R=-0.4259, p=0.0008). C) I found that when NF-κB is suppressed in MB231 cells it leaded to the inhibition of CD151 expression. D) When CD151 is inhibited, the expression of NF-κB remained unchanged, and

when both siCD151 and NF-κB are transfected the NF-κB over-expression leads to the induction of CD151 expression as compared to siCD151 transfection alone.

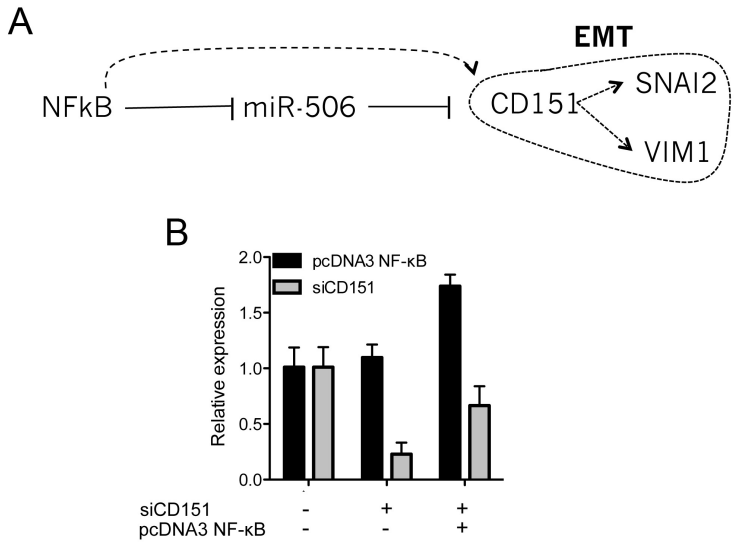


Figure 9. miR-506 suppression is indispensible for NF-κB induced expression of EMT markers. A) Cartoon representing the probable regulatory mechanism of EMT regulation by NF-κB via miR-506. B) Considering CD151 as a marker for EMT induction, I co-transfected both miR-506 and NF-κB in MB231 cells and checked the expression of CD151 at level which was found to be suppressed.

Suppression of TGFβ-induced EMT by miR-506

TGFβ is a well-known inducer of epithelial to mesenchymal transition(Cufi, Vazquez-Martin et al. 2010; Yilmaz, Maass et al. 2011), and the depletion of CD151 specifically attenuates TGFβ-induced scattering and proliferation of breast cancer cells(Sadej, Romanska et al. 2010). Therefore I checked if the overexpression of miR-506 could inhibit TGFβ-induced EMT of MCF10A cells. TGFβ treatment changed the MCF10A cell morphology from round epithelial to elongated mesenchymal form. Without TGFβ treatment, the overexpression of miR-506 did not affect the epithelial characteristics of MCF10A cells. But miR-506 over-expression followed by TGFβ treatment in MCF10A cells prevented the morphological change induction via TGFβ (Figure 10A). To validate the effect of miR-506 in EMT at molecular level, I checked the expression of EMT-related genes such as SNAI2, CD151 and CDH1 (Figure 10B), which clearly demonstrated the suppressive function of miR-506 in TGFβ-induced EMT. Interestingly, the expression of NF-κB remained unaffected in miR-506 transfected cells, induced by TGFβ (Gingery, Bradley et al. 2008), and remained induced in TGFβ and miR-506 transfected cells as compared to the control condition, thereby strongly indicating that miR-506 is down-stream of NF-κB and in presence of miR-506, NF-κB could not induce epithelial to mesenchymal transition (Figure 10B). From the above two experiments I concluded that miR-506 inhibition is a indispensible step in the TGFβ and NF-κB induced epithelial to mesenchymal transition in breast cancer.

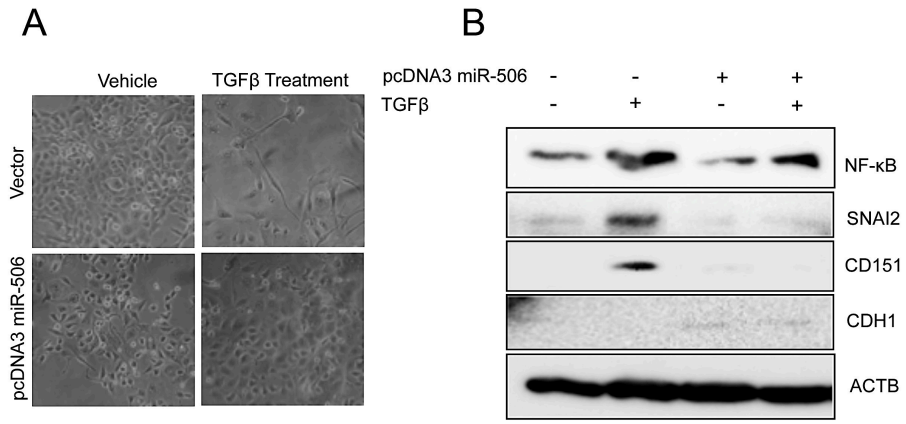


Figure 10. Role of miR-506 in the regulation of epithelial to mesenchymal transition. A) MCF10A normal breast cells are treated with TGFβ in the first condition transfected with miR-506 in the second condition and miR-506 transfected cells are treated with TGFβ in the third condition. I found that miR-506 prevented TGFβ induced morphological transition of MCF10A cells from epithelial to mesenchymal state. B) Impact of miR-506 is studied on TGFβ induced EMT at molecular level by studying the protein level expression of EMT markers NF-κB, SNAI2, CD151, and CDH1 respectively. TGFβ alone, induced EMT, but in presence of miR-506, TGFβ could only induce the expression of NF-κB, all the other EMT marker genes were remained unaltered.

Functional Study of miR-506

miR-506 over-expression reduces adherence of breast cancer cells to extracellular matrix

CD151 is a well-known Tetraspanin family member, which is known to play role in regulating cell-cell adhesion hence I studied the role of miR-506 in the regulation of cell adhesion via in-vitro adhesion assay. It was observed that miR-506 over-expression leads to decreased ability of MB231 cells to attach a range of ECM components (Fibronectin, Collagen1, Collagen 4, Laminin1, Fibrinogen) as compared with control cells (Figure 11A). However, adhesion to the negative control bovine serum albumin (BSA) did not differ between miR-506 - over-expressing cells and control cells.

miR-506 over-expression suppresses the Invasive & Migratory property of MB231 breast cancer cells.

Next, I checked the impact of miR-506 on the migratory and invasive properties of breast cancer cells via in-vitro assays. The capacity of MB231 cells to invade through the matrigel (artificial basement membrane matrix) was suppressed by the miR-506 transfection as compared to that of control cells (Figure 11B).

I also observed a decrease in the migration of MB231 cells transfected with miR-506 as compared to that of empty vector transfected cells via wound healing assay (Figure 11C) as well as Boyden chamber assay (Figure 11D) respectively.

NF-κB is known to induce migration (Schmidmaier and Baumann 2008), in order to check the impact of miR-506 on NF-κB mediated migration, I co-transfected NF-κB with miR-506 in MB231 cells, miR-506 could suppress NF-κB-induced cell migration potential (Figure 12). These data further suggests that miR-506 suppresses EMT in breast cancer cells.

In conclusion, in this study I have shown the role of miR-506 in the regulation of TGFβ and NF-κB mediated epithelial mesenchymal transition in breast cancer (Figure 13).

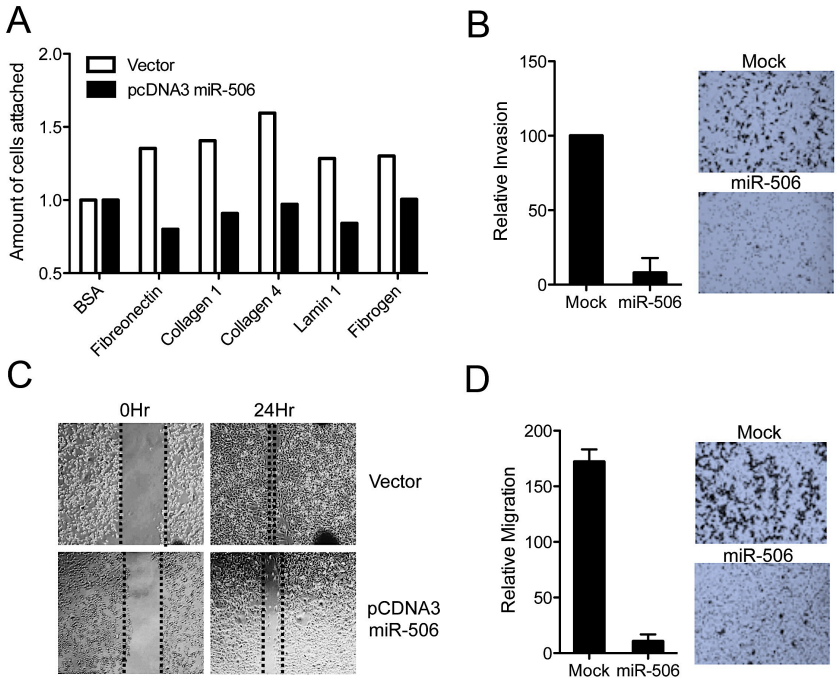


Figure 11. Functional Study of miR-506 – A) Impact of miR-506 on the various components responsible for cell adhesion (ECM Array) like Fibronectin, Collagen 1, Collagen 4, Lamini1, and Fibrogen. BSA is used as a control. B) Impact of miR-506 over-expression on the regulation of overall invasive capacity of MB231 cells [Matrigen Invasion Assay]. C) Shows the impact of miR-506 over-expression on the regulation of overall migratory property via Wound healing assay [2D Migration assay] and D) Boyden Chamber Assay [3D migration assay]. D)

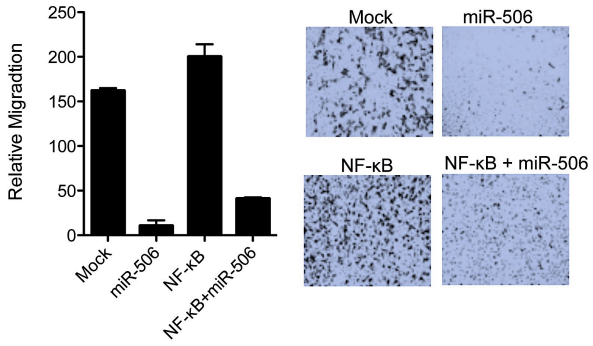


Figure 12. Impact of miR-506 on NF-κB induced migration of breast cancer cells. MB231 cells are transfected with NF-κB alone, and NF-κB and miR-506 in combination and then overall migration is studied via Boyden chamber assay. I found that miR-506 over-expression remarkably suppressed the NF-κB induced migratory properties of MB231 breast cancer cells.

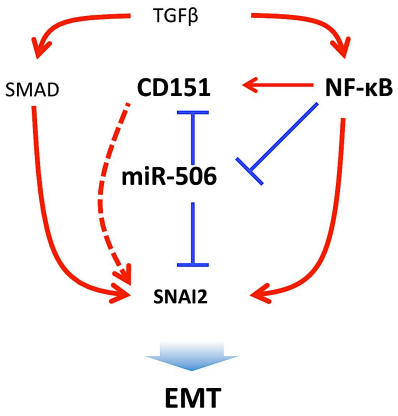


Figure 13. Conclusion. In conclusion I have shown a novel molecular mechanism showing the role of miR-506 in the regulation of TGFβ and NF-κB mediated epithelial mesenchymal transition in breast cancer.

DISCUSSION

miRNAs are one of the most important regulators of gene expression. miRNA can affect fundamental cellular processes such as differentiation, proliferation, cell cycle, and apoptosis (Bushati and Cohen 2007; Skaftnesmo, Prestegarden et al. 2007; Bueno and Malumbres 2011). In addition, miRNAs can provide another viewpoints in cancer research, including tumorigenesis, invasion and recurrence (Bushati and Cohen 2007; Skaftnesmo, Prestegarden et al. 2007; Lynam-Lennon, Reynolds et al. 2010; Bueno and Malumbres 2011; Koturbash, Zemp et al. 2011). Recent approaches on miRNA research have been accomplished through various, high-throughput biochemical screenings that unveiled of over 1,000 human miRNAs in human cancer. Several hundreds of miRNAs were mapped to altered regions in cancer genome. Either overexpression or deletion of miRNAs can drive the initiation and the progression of human cancer. miRNAs play critical roles in normal stem cell functions during development, have also emerged as important regulators of CSCs as well (Liu and Tang 2011). Reports demonstrated that modulation of specific miRNA alterations in cancer cells using miRNA replacement or anti-miRNA technologies can restore miRNA activities and repair gene regulatory networks affecting apoptotic signaling pathways or drug sensitivity, and improve the outcome of treatment (Wu 2010). In my study, I observed that miR-506 played a role as a master suppressor of EMT in breast cancer through direct targeting CD151, VIM1 and SNAI2.

The role of NF-κB in normal mammary development (Brantley, Chen et al. 2001; Connelly, Robinson-Benion et al. 2007) and its constitutive activation in breast cancer has been well established(Connelly, Robinson-Benion et al. 2007; Liu, Tan et al. 2008). NF-κB is suggested to play a role in either the initiation or in the growth of cancer cells at sites of metastasis. Growing evidence indicates that NF-κB plays a central role especially in epithelial to mesenchymal transition(Maier, Schmidt-Strassburger et al. 2010; Li, Chen et al. 2011). As a transcription factor, NF-κB can lead to the expression of EMT-related genes, and I added CD151, a key regulator of TGFβ-induced EMT, as a downstream target of NF-κB (Fig X). There is a NF-κB binding site in the promoter region of miR-506, and I confirmed the suppressive function of NF-κB on miR-506 expression. NF-κB might have many transcription target genes to potentiate the transcriptional control. In this sense, the suppression of miR-506 might be required for the progression of EMT by NF-κB.

miR-506 is also predicted to regulate many of the known transcription activators of CD151 such as ZDHHC2, ITGA3, ZDHHC17 and ZDHHC3 (Sharma, Yang et al. 2008). In this sense, miR-506 might suppress EMT regulated by CD151 systemically at various levels. CD151 has a positive role in breast tumor cell growth in vivo, whereas its down-regulation causes an inhibition of tumor cell growth, diminished experimental lung metastasis(Sadej, Romanska et al. 2010). CD151 expression is elevated in breast cancer, with even more up-regulation in high-grade and oestrogen negative subtypes including basal-like breast cancer(Yang, Richardson et al. 2008).

CD151 over-expression was shown to correlate with decreased survival of patients with breast cancer when assessed in 56 cases (Sadej, Romanska et al. 2009). Loss of CD151 decreased the integrin-mediated cell migration, spreading, invasion, and signaling through FAK, Rac1, and Lck with the alterations in the subcellular localization of α6 integrins (Chien, Lin et al. 2008; Kwon, Park et al. 2012).

I have shown the involvement of miR-506 in the regulation of CD151; hence I studied the functional relevance of miR-506 with respect to invasion, migration, and adhesion and showed that miR-506 if over-expressed in breast cancer cells could act as a tumor suppressor miRNA. Certain studies also supported the relevance of high-CD151 expression to a higher lymph node involvement and thereby advanced stage of invasive breast cancer in this study(Kwon, Park et al. 2012) . Combination of these promoting effects of CD151 expression on breast cancer progression including tumor size and lymph node involvement may be responsible for a poor prognosis of breast cancer patients with high CD151expression(Sadej, Romanska et al. 2010). In order to analyze the relation of miR-506 with involvement of lymph nodes, I considered GEO dataset and found that in patient with high expression of miR-506, lower number of lymph nodes are involved as compared to the patients with low miR-506 expression, thereby further adding to the clinical importance of miR-506.

Cytokines of TGFβ super families are implicated in a variety of normal and pathologic phenomena. It is well known that TGFβ leads to the induction of epithelial to mesenchymal transition(Cufi, Vazquez-Martin et al. 2010; Yilmaz, Maass et al. 2011). The depletion of CD151

attenuates pulmonary metastasis of breast cancer cells by regulating TGFβ signaling(Sadej, Romanska et al. 2010). CD151 is positively correlated with TGFβ, hence in-order to check the involvement of miR-506 in the regulation of epithelial to mesenchymal transition, I treated the MCF10A normal breast cancer cell with the TGFβ and found that the EMT markers were induced but when miR-506 was overexpressed along with the TGFβ the EMT marker could not be induced. On the other hand TGFβ leaded to the induction of NF-κB, which remained induced even in presence of miR-506 but it was not able to induce the expression of EMT markers in the presence of miR-506. This experiments suggested that miR-506 inhibit the TGFβ and NF-κB induced epithelial to mesenchymal transition.

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