

**COMBINATORIAL TARGETING OF PI3K AND MAPK PATHWAYS BY
MIR-564 TO INHIBIT PROLIFERATION AND INVASION IN BREAST
CANCER**

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF
MASTER OF SCIENCE
IN
MOLECULAR BIOLOGY AND GENETICS

By
Merve Mutlu
July 2016

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July, 2016

We certify that we have read this dissertation and that in our opinion it is fully adequate in scope and in quality, as a thesis for the degree of Master of Science.

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Abstract

COMBINATORIAL TARGETING OF PI3K AND MAPK PATHWAYS BY MIR-564 TO INHIBIT PROLIFERATION AND INVASION IN BREAST CANCER

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M.S. in Molecular Biology and Genetics

Supervisor: Özgür Şahin

July, 2016

Breast cancer is among most common malignant tumors worldwide and one of the deadliest cancer types among women. Like other cancer types, dysregulation of signaling pathways is the major cause of uncontrolled cell proliferation, inhibition of apoptosis and eventually metastasis of breast cancer. PI3K and MAPK signaling pathways are among top most deregulated pathways promoting proliferation and invasion in cancer. Clinically approved kinase inhibitors targeting main regulators of these pathways have limited success due to cross-talks between these cascades and creating potential bypass mechanisms. MiRNAs (miRNAs) are 18-22 nucleotides long small non-coding RNAs, functioning by targeting one or more genes simultaneously. The extensive studies on miRNAs showed that they are highly associated with the control of cancer-related processes such as proliferation, migration and invasion. In this thesis, my aim was to identify a potential miRNA functioning as a dual inhibitor of both PI3K and MAPK pathways regulating oncogenic processes in breast cancer. Our previous miRNA mimic screen with reverse phase protein array (RPPA) has been re-analyzed regarding PI3K, MAPK and cell cycle protein regulations. Among top 50 candidate miRNAs, miR-564 was shown to act as a dual inhibitor of PI3K and MAPK pathways in breast cancer cells and inhibiting proliferation through G1 cell cycle arrest. Furthermore, I showed that miR-564 reduces migration and invasion of aggressive breast cancer cells *via* blocking epithelial-mesenchymal transition (EMT). Direct targeting of AKT2, GNA12, GYS1 and SRF genes in combination may play role in miR-564 being a dual inhibitor of these pathways. Moreover, both high miR-564 expression and low expression of miR-564 target genes were shown to be associated with reduced invasiveness of tumors as well as distant relapse-free survival of breast cancer patients. Overall, I showed that, in addition to being a dual inhibitor of PI3K and MAPK pathways by combinatorial targeting network of genes, miR-564 is a prognostic marker for breast cancer and a promising druggable target.

Keywords: miR-564, PI3K pathway, MAPK pathway, AKT2, GNA12, GYS1, SRF, miRNA target network, breast cancer



Özet

MEME KANSERİNDE HÜCRE PROLİFERASYONUNU VE İNVAZYONUNU ÖNLEMEK İÇİN MİR-564 İLE PI3K VE MAPK SİNYAL İLETİM YOLAKLARININ AYNI ANDA HEDEFLENMESİ

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Temmuz, 2016

Meme kanseri dünya üzerinde en sık rastlanan malignant tümörler arasındadır ve kadınlar arasında en ölümcül olanıdır. Diğer kanser türlerinde olduğu gibi, sinyal yollarındaki düzensizlik meme kanserinde kontrolsüz hücre bölünmesine, apoptozun baskılanmasına ve metastaza sebep olmaktadır. PI3K ve MAPK sinyal yolları, kanserde hücrelerin çoğalmasına ve invazyonuna sebep olan en düzensiz sinyal yolları arasındadır. Bu yolların ana düzenleyici genlerine hedef alınan klinik olarak kanıtlanmış kinaz inhibitörleri, PI3K ve MAPK yolları arasındaki iletişim ve potansiyel çapraz aktivasyon mekanizmaları oluşumu nedeniyle sınırlı bir başarı göstermektedir. MikroRNAlar (miRNAlar) 18-22 nükleotid uzunluğunda, bir ya da daha fazla geni aynı anda hedefleyerek işlev gösteren, kısa protein-kodlamayan RNAlardır. Yapılan geniş çaplı araştırmalarda, miRNAların; proliferasyon, hücre göçü ve invazyon gibi kanserle ilişkili süreçlerde rol oynadığı gösterilmiştir. Bu tezde, meme kanserinde onkogenik süreci düzenleyici PI3K ve MAPK sinyal yollarının dual inhibitörü olabilecek potansiyel bir miRNA bulunması hedeflenmiştir. Sonuçlarının daha önceden yayınlanmış olan miRNA mimik taraması ile yapılan ters faz protein array (RPPA) verileri; PI3K, MAPK ve hücre döngüsü proteinleri ele alınacak şekilde tekrar analiz edilmiştir. Bu yollarla en fazla korelasyonu gösteren ilk 50 miRNA içerisinden, miR-564, potansiyel PI3K ve MAPK dual inhibitör olarak seçilmiş ve hücre büyümesini G1 fazında durdurduğu kanıtlanmıştır. Buna ek olarak, miR-564'ün epitel-mezenkimal geçişi (EMT) bloklayarak, agresif meme kanserlerinin hücre göçünü ve invazyonunu azalttığı gösterilmiştir. miR-564'ün her iki sinyal yolağını inhibisyonu AKT2, GNA12, GYS1 ve SRF genlerini direkt hedeflemesini içermektedir. Ayrıca, yüksek miR-564 seviyeleri ve düşük miR-564 hedef gen seviyeleri, tümörlerde düşük invazivlik ve meme kanseri hastalarında uzak nüksüz hayatta kalım süreleri ile ilişkilendirilmiştir. Sonuç olarak, PI3K ve MAPK sinyal yolları dual inhibitörü olmasına ek olarak, bir gen ağını birleşimsel

olarak hedefleyen miR-564, meme kanserinde belirteç olarak görev görmekte ve hedeflenebilecek bir ilaç olarak gelecek vadetmektedir.

Anahtar Kelimeler: miR-564, PI3K sinyal yolağı, MAPK sinyal yolağı, AKT2, GNA12, GYS1, SRF, miRNA hedef ağı, meme kanseri



Acknowledgements

My sincere thanks to Dr. Özgür Şahin for giving me this opportunity in his lab and sharing his valuable advices throughout my masters study.

I would like to acknowledge Özge Saatçi for her remarkable efforts on bioinformatic analysis and qPCR experiments of this project and most importantly her friendship and her support. I would like to acknowledge Suhail Ansari for his work on cell cycle analysis in this project.

Furthermore I would like to thank all present and former members of Şahin lab; Umar Raza, Emre Yurdusev, Erol Eyüpoğlu, Pelin Ersan, Hilal Bal and Selvi Durmuş for their cooperative work during my masters study.

I would like to thank Dr. Özlen Konu and Huma Shehwana from the Department of Molecular Biology and Genetics at Bilkent University for their help with the analysis of RPPA data.

I am deeply grateful to the Scientific and Technological Research Council of Turkey (TUBITAK) for kindly providing their financial support to this thesis.

Finally I would like to thank my beloved fiancée, Murat Uğur Kiraz, for his endless love and support throughout my study.

This thesis is funded by TUBITAK Project Number: 214S104.

Teşekkür

Tez çalışmam ve yüksek lisans eğitimim boyunca benimle değerli bilgi ve tecrübeleriyle bana yol gösteren, iyi bir bilim insanı olma yönünde beni yönlendiren Yrd. Doç. Dr. Özgür Şahin'e,

Tez çalışmamın biyoinformatik analizleri ve qPCR aşamalarında katkılarını esirgemeyen çalışma arkadaşım Özge Saatçi'ye,

Tez çalışmamın hücre döngüsü analizlerinde katkıda bulunan çalışma arkadaşım Suhail Ansari'ye,

Proje süresince yardımlarını ve arkadaşlıklarını her zaman hissettiğim Özgür Şahin laboratuvar üyeleri Umar Raza, Emre Yurdusev, Erol Eyüpoğlu, Pelin Ersan, Hilal Bal ve Selvi Durmuş'a,

RPPA veri analizinde yardımcı olan Yrd. Doç. Dr. Özlen Konu ve doktora öğrencisi Huma Shehwana'ya,

Yüksek lisans eğitimim boyunca beni finansal olarak destekleyip; çalışmalarına devam etmemde önemli bir katkı sağlayan, Türkiye Bilimsel ve Teknik Araştırma Kurumu'na (TÜBİTAK) ve değerli çalışanlarına,

Son olarak, bu süreçte sonsuz sevgisi ile beni yalnız bırakmayan nişanlım Murat Uğur Kiraz'a,

Sonsuz teşekkürlerimi sunarım.

Bu tez TÜBİTAK 214S104 numaralı proje bütçesi ile gerçekleştirilmiştir.

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Abbreviations

7-AAD	7-Aminoactinomycin D
ALL	Acute lymphoblastic leukemia
AKT1	V-akt murine thymoma viral oncogene homolog 1
AKT2	V-akt murine thymoma viral oncogene homolog 2
APS	Ammonium peroxodisulfate
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
BrdU	Bromodeoxyuridine
CDH1	E-cadherin
CDK2	Cyclin-dependent kinase 2
CDK4	Cyclin-dependent kinase 4
CREB5	cAMP responsive element binding protein 5
CycD1	Cyclin D1
CycD3	Cyclin D3
DAPI	4',6-diamidin-2-phenylindol
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	desoxynucleotide triphosphate
DUSP6	Dual specificity phosphatase 6
ECL	Enhanced chemiluminescence
EMT	Epithelial-mesenchymal transition
ERK1	Mitogen-activated protein kinase 3 (MAPK3)
ERK2	Mitogen-activated protein kinase 1 (MAPK1)
F-actin	filamentous actin
FBS	Fetal bovine serum
FN	Fibronectin
GEO	Gene Expression Omnibus
GNA12	Guanine nucleotide binding protein (G-protein) alpha 12
GYS1	Glycogen synthase 1
HP	High proliferative
HRP	Horseradish peroxidase
kDA	Kilo Dalton
KRAS	Kirsten rat sarcoma viral oncogene homolog
LP	Low proliferative

MIG6	ERBB receptor feedback inhibitor 1 (ERRFI1)
miRNA	miRNA
NCI60	National Cancer Institute 60 human tumor cell line anticancer drug screen
ORF	Open reading frame
PBS	Phosphate buffered saline
PI3KCA	Phosphatidylinositol-4,5-bisphosphate 3-Kinase Catalytic subunit alpha
PI3KCB	Phosphatidylinositol-4,5-bisphosphate 3-Kinase Catalytic subunit beta
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
PTEN	Phosphatase and tensin homolog
PTPN11	Protein tyrosine phosphatase, non-receptor type 11
p21	Cyclin-dependent kinase inhibitor 1A (CDKN1A)
p27/Kip1	Cyclin-dependent kinase inhibitor 1B (CDKN1B)
P/S	Penicillin/Streptomycin
qRT-PCR	Quantitative real time polymerase chain reaction
Rb	Retinoblastoma protein
RPPA	Reverse phase protein array
RTCA	Real-time cell analyzer
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SRF	Serum response factor
Ser	Serine
SNAI2	Snail family zinc finger 2
TAE	Tris-acetate EDTA
TBST	Tris buffer saline Tween20
TCGA	The Cancer Genome Atlas
Thr	Threonine
UTR	Untranslated region
ZEB1	Zinc finger e-box binding homeobox 1
ZEB2	Zinc finger e-box binding homeobox 2
ZO1	Zona occludens-1 (Tight junction protein 1, TJP1)

CHAPTER 1

Introduction

1.1. Breast cancer

Cancer, the plague of the modern age, is defined as the uncontrolled cell proliferation. More than 200 types of different cancer have been documented until now and top most deadly malignancies are lung, colorectal, breast and prostate cancers (Cancer Research UK, National Cancer Institute). According to National Cancer Institute, in the year 2016, with up to 246,600 estimated cases and 40,450 estimated deaths, breast cancer is the most common cancer in USA among women. Breast cancer is a heterogeneous disease showing different characteristics from patient to patient, and its subtypes have been well defined. It has been characterized to 5 different biological subtypes according to their gene expressions and 3 different clinical subtypes according to their clinico-pathological features (Figure 1.1)[1].

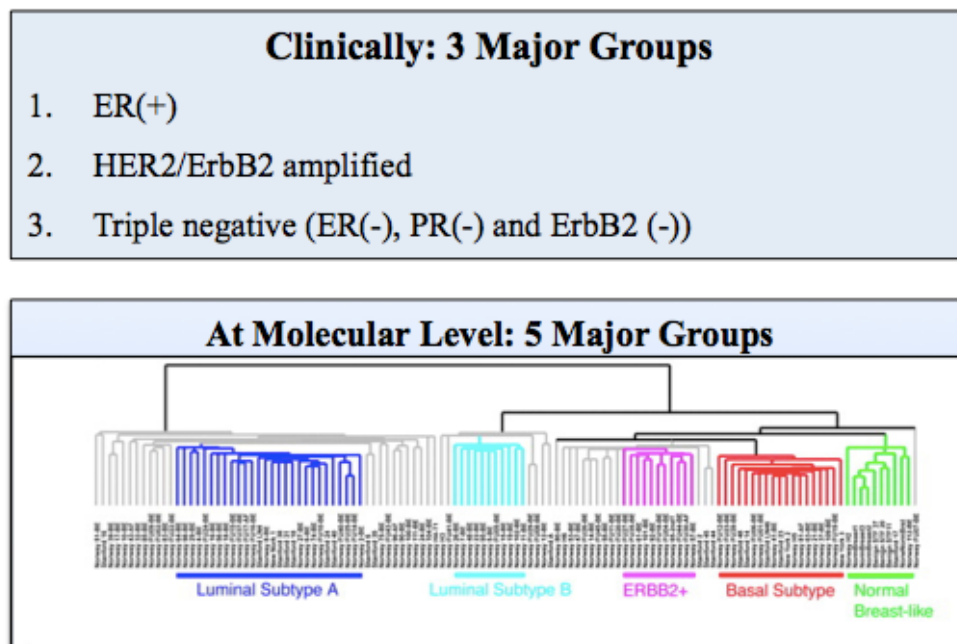


Figure 1.1. Clinical and molecular subtypes of breast cancer (Adapted with permission from: PNAS [1], Copyright (2003) National Academy of Sciences, U.S.A.).

Luminal A and luminal B biological classes corresponds to ER+ clinical subtypes of breast cancer regarding similarity in gene expression, 70% of basal subtype can be classified as TNBC[2, 3]. Even though each subtype contains particular properties, molecular

heterogeneity together with signaling pathway alterations are the major common ground in all subtypes, leading to proliferation, invasiveness and development of drug resistance in breast cancer[4].

1.2. PI3K and MAPK signaling pathways

Receptor tyrosine kinases (RTK) are cell membrane receptors that functions in cellular signal transduction. Direct binding of hormones, peptides or cytokines to RTKs initiates several cascades inside the cells. Insulin receptor, EGF receptor and VEGF receptors are among the famous members of RTK family. RTK protein members usually found as hetero or homo dimers on the cell membrane, and they are capable of trans-phosphorylation from tyrosine residues inside the cell.

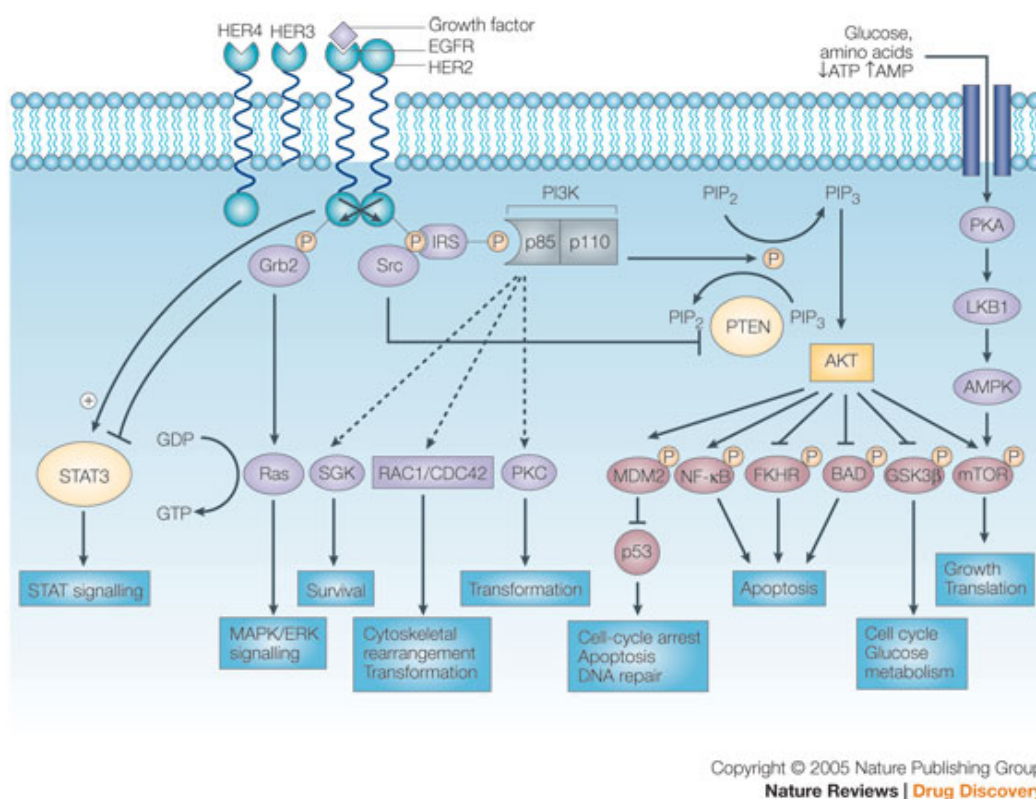


Figure 1.2. Schematic flow of PI3K and MAPK signaling pathways (Reprinted by permission from Nature Publishing Group: [Nature Reviews Drug Discovery][5], Copyright (2005).)

Among most important downstream signaling cascades of RTKs, PI3K/AKT and MAPK signaling pathways are among top most deregulated pathways in breast cancer. PI3K/AKT pathway has been shown to be associated with cell proliferation, survival and apoptosis[6]. The upstream member of the cascade, phospho inositol-3 kinase (PI3K) containing p110 catalytic subunit and p85 regulatory subunits, functions as a oncogene and shown to be mutated and dysregulated in several cancer types (Figure 1.2) [7]. Protein kinase B (PKB or

AKT) is located at the downstream of PI3K and being phosphorylated from its Ser473 and Thr308 residues leading to its activation. AKT protein family contains three members and only AKT1 and AKT2 are shown to be functioning in PI3K pathway [8]. PI3K/AKT signaling cascade starts with the phosphorylation of PI3K by activated EGFR, further triggers AKT1 and AKT2. JNK, p53 and NF- κ B are some of the transcription factors that are activated by this signaling pathway [9].

PTEN is a dual phosphatase tumor suppressor gene regulating, functioning as a switch on PI3K pathway by regulating cell cycle and metastasis [10]. Deletion, loss of homozygosity (LOH) or under expression *via* mutations in PTEN gene is a biomarker for several cancer types including breast cancer. Due to its important role in cell proliferation, apoptosis, and metastasis, kinase inhibitors such as PI3K inhibitors like LY294002 and wortmanin [11]; Akt inhibitors like perifosine [12], PIA [13] and triciribine [14] or PI3K/mTOR dual inhibitors like BEZ235 have been developed as targeted therapy for silencing of PI3K pathway [15].

Like PI3K/AKT pathway, Ras/Raf/MEK/ERK signaling pathway is controlled by the activation of EGFR receptor and has been intensively studied in cancer due to its role on cellular proliferation, survival and drug resistance. Activation of cascade starts with RAF protein phosphorylation followed by subsequent MEK and ERK phosphorylations. c-myc, c-jun and CREB are some of the important transcription factors that are located at the downstream of MAPK pathway (Figure 1.2)[16, 17]. Dual specific phosphatase 6 (DUSP6), ERBB receptor negative feedback regulator (MIG6) and tyrosine phosphatase (PTPN11) are the major negative regulators of this cascade [16]. Currently, RAF targeted agents and MEK inhibitors (PD98059) are used in clinics for targeted therapy of different tumors[18].

1.3. Breast cancer treatment *via* kinase inhibitors

Both PI3K and MAPK pathway targeting kinase inhibitors had limited capacity to inhibit cancer progression due to cross talk between these cascades [19, 20]. It has been shown that inhibition of PI3K pathway resulted in compensation *via* activation of MAPK pathway. For example ERK suppression by MEK inhibitors showed increased AKT activation *via* presence of EGF- stimulation[21, 22]. On the other hand, inhibition of MAPK pathway triggers PI3K pathway to overcome the inhibition of cancer progression. For example, in the presence of an mTOR inhibitor, RAD0001, activation of MAPK pathway has been occurred *via* S6K-PI3K-Ras feedback loop[23]. In another study, inhibition of ERK1/2 phosphorylation *via* targeting protein arginine methyl-transferase 5 (PRMT5) showed activation of PI3K pathway [24-26]. These evidences proving the cross-talks between PI3K and MAPK pathways indicates that combinatorial targeting of these pathways is essential, but the attempts for combinatorial targeting failed due to high toxicity concerns[27, 28]. For example, Our previous study

indicated that combination of lapatinib together with BEZ235 inhibitor, despite significant reduction of tumor mass, resulted in high toxicity in transgenic mouse models[29, 30]. Importantly, it has been shown that only the patients with *KRAS* or *BRAF* mutation together with PI3K pathway genetic alterations benefitted the dual inhibition approach with combination of kinase inhibitors, and in this regard, genetic profiling of each patient was necessary for suitable therapy approach [28, 31, 32].

Overall, these studies indicate that for more effective cancer treatment, a non-toxic dual inhibitor is necessary to overcome cancer progression, tumor growth and metastasis inhibition.

1.4. miRNAs and their role of post-transcriptional targeting

MiRNAs (miRNAs) are 18-25 nucleotide short non-coding RNAs and functions as post-transcriptional repressors *via* targeting 3'UTR of mRNAs. It has been shown that they contain specific mechanism for either block transcription or translation depending on the complementarity between seed sequence of miRNA and the 3'UTR sequence of the gene.

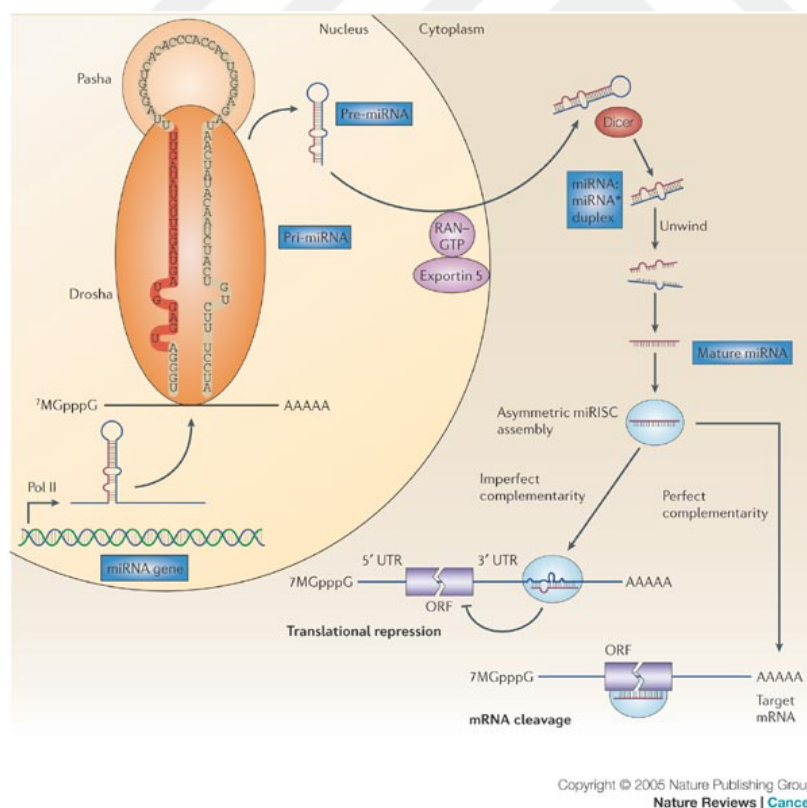


Figure 1.3. Schematic demonstration of miRNA formation and function (Reprinted by permission from Nature Publishing Group: [Nature Reviews Cancer] [33], Copyright (2006).)

Just like protein coding genes, miRNAs are transcribed from genome mostly by RNA polymerase II and form a loop called primer miRNA (pri-miRNA) structure. After splicing of pri-miRNA to 70-100 nucleotide long precursor miRNA (pre-miRNA) by Drosha-DGCR8 complex, they have been transported to cytoplasm *via* Exportin-5 protein. Their double strand structure then has been spliced further with DICER-TRBP complex and their mature single strand form is placed on RISC complex. With the assembly of mature form of miRNA with RISC complex, miRNA gets activated to bind 3'UTR of target mRNA. Targeted mRNA has been degraded or blocked at transcriptional level [34, 35]. 3'UTR of targeted mRNA must contain 8-10 nucleotide length of matched sequence with miRNA, called seed sequence, for RISC complex to bind [36].

There has been more than 2600 miRNAs have been identified, up to date and it is been estimated that miRNAs can regulate up to 60% of human genome (mirBase version 20) [37]. 14 years ago, the role of miRNAs in cancer progression was first reported and over the years, growing number of research on miRNAs have indicated their effects on cancer cell proliferation, invasion, metastasis and drug resistance [38, 39]. Our recent findings showed that several miRNAs functions as regulators of cell invasion and tamoxifen resistance [40-42]. It has been shown that miRNAs can target more than one gene in a network manner. In this regard, our group showed that instead of focusing just on one miRNA-one gene interactions, studying miRNA-protein networks can be more effective in the field of cancer therapy [43].

Recent clinical findings showed that treatment of chronic hepatitis C patients with a miR-122 inhibitor (Miravirsen) is now on phase-2a (NCT01200420) on clinical trials, and until now no toxicity has been reported. Furthermore, miR-34 (MRX34) was developed as a drug for liver cancer and it has been tested on patients in phase-1 in clinical trials (NCT01829971) [44]. These studies indicate that miRNAs have the potential to be non-toxic drugable targets in future.

1.5. Aims of the Study

Aims of the thesis are:

- To identify potential miRNAs acting as a dual inhibitor of PI3K and MAPK signaling pathways in breast cancer cells,
- To inhibit cell proliferation, migration and invasion *via* using miRNA(s) in breast cancer cells,
- To find mechanism of action of miRNA(s) that inhibits PI3K and MAPK pathways simultaneously.

CHAPTER 2

Materials and Methods

2.1. Materials

2.1.1. Buffers

1x Anode Buffer I	300mM Tris, 20% (v/v) methanol
1x Anode Buffer II	25mM Tris, 20% (v/v) methanol
1x Cathode Buffer	40mM 6-aminocaproic acid, 20% (v/v) methanol
1x SDS-PAGE Running Buffer	25mM Tris, 14.41g/l glycine, 1% (v/v) SDS
1x TAE	40mM Tris, 20mM acetic acid, 1mM EDTA
1x TBST	20mM Tris, 8g/l NaCl 0.2% (v/v) Tween20
RIPA lysis buffer	150mM NaCl, 1% (v/v) NP-40, 0.5% (v/v) Sodium DOC, 50mM TrisHCl (pH:8.0), 50mM NAF, 1mM NAVO4, 4% (v/v) Protease inhibitor, 4% (v/v) Phosphatase inhibitor

2.1.2. Chemicals and Reagents

4x Protein Loading Dye	250mM Tris HCl (pH:6.8), 10% (w/v) SDS, 0.1% (w/v) Bromophenol blue, 50% Glycerol (v/v), 25% (v/v) mercaptoethanol
6-aminocaproic acid	Sigma Aldrich, St Louis, MO, USA
6x DNA loading Dye	New England Biolabs, Ipswich, MA, USA
Acetic acid	Sigma Aldrich, St Louis, MO, USA
Acrylamide/bisacrylamide	Applchem, Darmstadt, Germany
Agar	Sigma Aldrich, St Louis, MO, USA
Agarose	Promega, Madison, WI, USA
Alexo Fluor 488 Phalloidin	Invitrogen, Carlsbad, CA, USA
Ammonium peroxisulfate	Carlo Erba, Cornaredo, Italy
Ampicillin	Sigma Aldrich, St Louis, MO, USA
Bovine Serum Albumin	Santa Cruz, Dallas, TX, USA
Protease inhibitor cocktail	Roche Applied Science, Mannheim, Germany
DAPI	Sigma Aldrich, St Louis, MO, USA
dNTPs	Thermo Fisher Scientific, Waltham, MA, USA
ECL	Amersham Pharmacia Biotech, Amersham, UK

Ethanol	Sigma Aldrich, St Louis, MO, USA
Ethidium Bromide	Thermo Fisher Scientific, Waltham, MA, USA
Gene Ruler 1kb DNA ladder	New England Biolabds, Ipswich, MA, USA
Isopronapol	Sigma Aldrich, St Louis, MO, USA
Puromycin	Thermo Fisher Scientific, Waltham, MA, USA
LightCycler 480 SYBR Green I Master	Roche Applied Science, Mannheim, Germany
Lipofectamine 2000	Invitrogen, Carlsbad, CA, USA
Methanol	Sigma Aldrich, St Louis, MO, USA
Milk powder	Sigma Aldrich, St Louis, MO, USA
Nuclease free water	Applied Biosystems/Ambion, Austin, TX, USA
Page Ruler Protein Ladder	Thermo Fisher Scientific, Waltham, MA, USA
Phosstop	Roche Applied Science, Mannheim, Germany
Ponceu S	Sigma Aldrich, St Louis, MO, USA
Shandon Immu-mount	Thermo Fisher Scientific, Waltham, MA, USA
Sodium Chloride	Merck, Darmstadt, Germany
Sodium Dodecyl Sulfate (SDS)	Merck, Darmstadt, Germany
TEMED	Serva, Heidelberg, Germany
TRIsure	Bioline, Luckenwalde, Germany
Triton X-100	Sigma Aldrich, St Louis, MO, USA
Trizma base	Sigma Aldrich, St Louis, MO, USA
Trypton	Sigma Aldrich, St Louis, MO, USA
Tween-20	VWR, Radnor, PA, USA
WST-1	Roche Applied Science, Mannheim, Germany
Yeast Extract	Sigma Aldrich, St Louis, MO, USA

2.1.3. Enzymes and Enzyme Buffers

10x Cut Smart Buffer	New England Biolabds, Ipswich, MA, USA
NotI Restriction enzyme	New England Biolabds, Ipswich, MA, USA
Phusion Polymerase	New England Biolabds, Ipswich, MA, USA
Taqman Abgene universal mix	Thermo Fisher Scientific, Waltham, MA, USA
XhoI Restriction enzyme	New England Biolabds, Ipswich, MA, USA

2.1.4. Media and Supplements

DMEM	Lonza, Basel, Switzerland
Fetal bovine serum (FBS)	Biowest, Nuaille, France
LB Agar	1% (v/v) agar, 10g/l tryptone, 5g/l yeast extract, 5g/l NaCl
LB Broth	10g/l tryptone, 5g/l yeast extract, 5g/l NaCl
Matrigel	BD, Franklin Lakes, NJ, USA
Non-essential aminoacids	Lonza, Basel, Switzerland
optiMEM	Invitrogen, Carlsbad, CA, USA
Penicillin/streptomycin	Lonza, Basel, Switzerland

2.1.5. Kits

BCA Protein Assay Kit	Pierce, Rockford, IL, USA
BrdU FITC Flow Kit	BD, Franklin Lakes, NJ, USA
Cell Titer-Glo cell viability assay kit	Promega, Madison, WI, USA
Dual Luciferase Reporter Kit	Promega, Madison, WI, USA
Gel and PCR clean up kit	MN, Düren, Germany
MycoAlert detection kit	Lonza, Basel, Switzerland
Plasmid isolation kit	MN, Düren, Germany
Plasmid Maxi Kit	Qiagen, Hilden, Germany
First strand cDNA synthesis kit	Fermentas, St. Leon-Roth, Germany
Taqman miRNA Assays	Applied Biosystems, Foster City, CA, USA

2.1.6. Equipments

Accuri FACS	BD, Franklin Lakes, NJ, USA
Axiovision 4.3 microscopy	Carl Zeiss, Munich, Germany
Centrifuges	Thermo Fisher Scientific, Waltham, MA, USA Beckman, Pasadena, CA, USA
Cell culture hood	Nüve, Ankara, Turkey
Cell culture incubator	Nüve, Ankara, Turkey
Counting chamber	Marienfeld, Königshofen, Germany
Freezer (-80)	Hettich, Geldermansen, Holland
Freezer (-20)	Bosch, Stuttgart, Germany
Fridge	Bosch, Stuttgart, Germany
Horizontal Shakers	FinePCR, Seoul, South Korea

	Bellco, Vineland, NJ, USA
LightCycler 96	Roche Applied Science, Mannheim, Germany
Mini-PROTEAN Gel casting module	Biorad, Hercules, CA, USA
Mini-PROTEAN Tetra Cell	Biorad, Hercules, CA, USA
Multichannel Pipette	Thermo Fisher Scientific, Waltham, MA, USA
Nanodrop 1000	Thermo Fisher Scientific, Waltham, MA, USA
Nikon TS300 Inverted microscope	Nikon, Tokyo, Japan
PCR	Thermo Fisher Scientific, Waltham, MA, USA
Power supplies for electrophoresis	Biorad, Hercules, CA, USA
qPCR Machine	Roche Applied Science, Mannheim, Germany
Semidry western blot transfer unit	Biorad, Hercules, CA, USA
Synergy HT Multireader	Biotek, Winooski, VT, USA
UV-Reader	Vilber Lourmat, Eberhardzell, Germany
Vortex	Isolab, Wertheim, Germany
Water bath	Nüve, Ankara, Turkey
X-ray cassette	Amersham Pharmacia Biotech, Amersham, UK
X-ray hyper processor	Amersham Pharmacia Biotech, Amersham, UK

2.1.7. Consumables

100mm dishes	Greiner bio-one, Frickenhausen, Germany
145mm dishes	Greiner bio-one, Frickenhausen, Germany
96-well plates	Greiner bio-one, Frickenhausen, Germany
6-well plates	Greiner bio-one, Frickenhausen, Germany
E-Plates	Acea Biosciences, San Diego, CA, USA
Filtered pipette tips (10ul, 20ul, 200ul, 1000ul)	Greiner bio-one, Frickenhausen, Germany
Cell scrapers	Greiner bio-one, Frickenhausen, Germany
CIM-Plates	Acea Biosciences, San Diego, CA, USA
Coverslips	Marienfeld, Königshofen, Germany
Cryovials	Greiner bio-one, Frickenhausen, Germany
Microscope slides	Marienfeld, Königshofen, Germany
Parafilm	VWR, Radnor, PA, USA
PCR tubes	Axygen, Corning, NY, USA
Plastic pipettes (10ml, 25ml)	Corning Incorporated, Corning, NY, USA
PVDF Membrane	Biorad, Hercules, CA, USA

Reaction tubes (500ul, 1.5ml, 2ml)	Axygen, Corning, NY, USA
Storage bottles (250ml, 500ml, 1L)	Corning Incorporated, Corning, NY, USA
Whatmann paper	GE Healthcare, Little Chalfont, UK
X-ray films	Kodak, Rochester, NY, USA
Cuvettes	VWR, Radnor, PA, USA
qPCR Plates	Roche Applied Science, Mannheim, Germany
White plates	Costar, Corning, NY, USA



2.2.Methods

2.2.1. RPPA data re-analysis on R Bioconductor

Starting from the data of Uhlmann et al. 2012 Supp. Table 5; PI3K, MAPK and cell cycle pathways genes that are most upregulated in triple negative breast cancer type are used for analysis. Our dataset composes of 16 genes and 733 miRNA expression values. Genes were assigned labels of 1s and 0s for oncogenes and tumor suppressor genes respectively, shown in Table 2.1. MiRNA prediction and classification are performed using R 3.0.2 and then miRNA expression data was correlated with these labels to find out the predicted regulatory miRNAs for these classes.

Initially normalized dataset was filtered such that those miRNAs which have less variability in between two classes (oncogenes and tumor suppressor) is removed. Coefficient of variation (CV) threshold was set to 0.25. Removing miRNAs having less then 0.25 coefficient of variation, dataset was reduced to have 581 miRNAs. Analysis was concluded using genefilter package.

From these 581 candidate miRNAs, potential ones having differential expression between two classes were chosen using Pearson correlation measure. Correlation was measured between each miRNA and class labels. Correlation threshold was set to $|0.5|$ for miRNA selection. miRNAs having correlation >0.5 are supposed to be upregulated in oncogenes and downregulated in tumor suppressor genes. While threshold of correlation <-0.5 gave us the miRNAs downregulated in oncogene and upregulated in tumor suppressor. Using gplot package in R, these 50 miRNAs are clustered together, and heatmap is plotted as shown in Figure 3.1. SVM was used to classify these miRNAs in two classes. SVM classified accuracy selected miRNA with 93.75 and good sensitivity and specificity as shown in Table 2.1 and Table 2.2.

Table 2.1. Key proteins from PI3K, ERK and Cell cycle pathways, which are upregulated in TNBC cell, line MDA-MB-231.

	PI3K pathway	ERK pathway	Cell Cycle Pathway
Oncogene	AKT1	KRAS	CycD1
	AKT2	ERK1	CycD3
	PI3KCA	ERK2	CDK4
	PI3KCB		CDK2
Tumor suppressor	PTEN	MIG6	p27
		PTPN11	
		DUSP6	

Table 2.2. SVM class prediction with 93.75 percent accuracy.

	tumor suppressor	oncogene
tumor suppressor	5	0
oncogene	0	11

2.2.2. Cell culturing

2.2.2.1. Culturing Human Breast Cancer Cell lines

MDA-MB-231, MCF-7, BT-474 and MDA-MB-436 human breast cancer cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were grown in 100mm petri dishes at 37°C 5% CO₂ condition. Cells were split two times per week in 1-to-3 ratio. For splitting cells were washed with 2ml PBS after culture media has been aspirated, trypsinized with 1.5ml trypsin in 37°C for 5 minutes and resuspended in appropriate culture medium. Cells were cultured until the passage number 50 and all cell lines were tested for mycoplasma contamination periodically.

2.2.2.2. miRNA mimics, hairpin inhibitors, siRNAs and expression construct transfections

miRNA mimic, hairpin inhibitors, siRNAs and 3'UTR expression construct transfections were carried out using Lipofectamine 2000TM and OptiMEM with the final concentrations of 40nM, 100nM, 40nM and 25ng/well, respectively (Table 2.3). Pool of 4 individual siRNA of gene of interest used for knockdown experiments.

For transfection cells were seeded 8000 cells/well for 96-well plates and 150,000-250,000 cells/well for 6-well plates with 150ul or 1,5ml penicillin/Streptomycin free (P/S-free) media respectively.

For one well in 6-well format transfection, 2ul of lipofectamine was diluted in 250ul optiMEM and incubated at room temperature for 5 minutes after 20 seconds vortexing (mix A). miRNA mimic/hairpin inhibitor/siRNA/expression constructs were also diluted in 250ul optiMEM alongside with lipofectamine (mix B). Two vials (mix A and mix B) were mixed in 1:1 (v/v) ratio and incubated at room temperature for 20 minutes after 1 minute of vortexing. After aspiration of media and addition of 1ml fresh P/S-free media on cells 500ul of transfection mixture was added on top of the cells resulting final volume of 1,5ml transfection medium. Cells were incubated at 37°C, 5% CO₂ for further processes.

Table 2.3. List of siRNAs, mimics and hairpin inhibitors.

siRNAs	Catalog number	Firm
siGENOME Set of 4: AKT2	D-003001-05, D-003001-21, D-003001-07, D-003001-08	Dharmacon
siGENOME Set of 4: GNA12	D-008435-01, D-008435-02, D-008435-03, D-008435-04	Dharmacon
siGENOME Set of 4: GYS1	D-017726-01, D-017726-02, D-017726-03, D-017726-04	Dharmacon
siGENOME Set of 4: SRF	D-009800-01, D-009800-03, D-009800-05, D-009800-06	Dharmacon

miRNA mimics	Catalog number	Sequence	Firm
miRIDIAN mimic Negative Control #1	CN-001000-01	UCACAACCUCCUAGAAAGAGUAGA	Dharmacon
hsa-miR-564 miRIDIAN mimic	C-300882-01	AGGCACGGUGUCAGCAGGC	Dharmacon
hsa-miR-490-3p miRIDIAN mimic	C-300750-05	CAACCUGGAGGACUCCAUGCUG	Dharmacon
hsa-miR-200b-3p miRIDIAN mimic	C-300582-07	UAAUACUGCCUGGUAAGAUGA	Dharmacon

miRNA inhibitors	Catalog number	Sequence	Firm
miRIDIAN Hairpin Inhibitor Negative Control #1	IN-001005-01	UCACAACCUCCUAGAAAGAGUAGA	Dharmacon
miR-564 inhibitor	IH-300882-03	AGGCACGGUGUCAGCAGGC	Dharmacon

2.2.2.3. Lentiviral stable transfections

SMARTchoice human lentiviral hsa-miR-564 shMIMIC hCMV-turboRFP and GIPZ non-silencing lentiviral shRNA control were purchased from Dharmacon (Lafayette, CO, USA) and used for stable cell line constructs. In 24- well plate format, MDA-MB-231.luc cells (a kind gift from Dr. Dihua Yu (MD Anderson, TX)) were transduced with miR-564 viral particles by direct addition of purchased viral particles in to culture media, and cells were

selected with 1 µg/ml of puromycin 96 hours after transduction. For control group non-silencing shRNA empty GIPZ vector was used. HEK293FT cells were seeded with 50% confluency on 6-well plates. 6 µg empty GIPZ vector together with 4.3µl of trans-lentiviral packaging mix (Dharmacon, Lafayette, CO, USA) were transfected with CaCl₂ reagent (Dharmacon, Lafayette, CO, USA). Transfected cells were incubated in 37°C, 5% CO₂ and culturing media were collected after 24, 48 and 72 hours post-transfection for harvesting viral particles and fresh media added on cells. Alongside with HEK293FT cells, MDA-MB-231 cells were seeded with 50% confluency. Viral particle containing harvested media filtered and supplemented to MDA-MB-231.luc cells 24 hour after cell seeding for transduction. 96 hour after last media supplementation, stably transfected cells were selected with 1 µg/ml of puromycin.

2.2.3. Cell-based assays

2.2.3.1. *In vitro* viability

For end point viability Cell Titer-Glo cell viability assay kit was used for MDA-MB-231 cell line and WST-1 reagent was used for MCF-7 and BT-474 cell lines. 3 individual technical replicas of each experiment and on every experiment 4 biological replicates in 96-well format were used for each treatment condition. 48 hour after transfection of cells, 25ul of Cell titer glo reagent was added on culturing media. After incubation of cells for 2 minutes on shaker, 100ul of media on top of cells were placed in opaque white flat bottom 96-well plates and luminescence measured on Biotek Multireader. For MCF-7 and BT-474 cells, after 48 hours incubation, 10ul of WST-1 reagent was added each well and absorbance was measured in 1h, 2h, 3h and 4h time points. Percent viability was calculated on Microsoft Excel (Albuquerque, NM, USA) and Student's two-tailed t-test was used for statistical analysis.

2.2.3.2. Real-time cell Analyzer (RTCA) Experiments

2.2.3.2.1. Real-time viability

16 well E-Plates (Acea Biosciences, San Diego, CA, USA) were used for real-time cell viability analysis. 75ul of P/S-free media were added on empty wells and plates were normalized in RTCA machine before cell seeding. 8000cells/75ul of MDA-MB-231, MCF-7 and MDA-MB-436 cells were seeded in each well of E-plates. 3-to-4 technical replicates were used for each treatment condition. Before placing E-plate on RTCA machine, E-plates were incubated in room temperature for 30 minutes for cells to settle down. Program was set to read cell index every 30 minutes for 120 hours.

For transfection, after preparation of transfection reagent (described in section 3.2.2.) E-plate was removed from RTCA machine and 50ul of transfection reagent was added on each well. After placing E-plate back on the RTCA machine, system automatically starts reading cell

index with the same protocol selected before. Cell index was normalized to transfection time. After 72 hours of normalized cell index reading, E-plate was taken out of the system and statistical analysis continued with selected time points using Student's two-tailed t-test.

2.2.3.2.2. Real-time migration and invasion

16 well CIM-Plates (Acea Biosciences, San Diego, CA, USA) were used for real-time cell migration and invasion analysis. For migration and invasion assays on RTCA systems cells were seeded and transfected in 6-well format.

For invasion assay, top chamber was coated with 800ug/ml matrigel diluted in serum-free media and incubated in 37°C for 4 hours. Bottom chamber was filled with 160ul of complete media (containing 10% FBS) and top chamber was placed tightly on it. After addition of 50ul of starvation media (containing 1% FBS) on top chamber and CIM-plate was incubated in 37°C for 1 hour. After 1 hour incubation CIM-plate was placed on RTCA machine for plate normalization. Same protocol was used for migration assay on RTCA machine except matrigel coating of top chamber.

24 hours after transfection cells were counted and seeded on CIM-plates with the concentration of 30,000cells/well. 3-to-4 technical replicates were seeded on CIM-plate for each treatment condition. After cell seeding CIM-plate was incubated in room temperature for 30 minutes for cells to settle down. Program was set to read cell index every 15 minutes for 72 hours. After 48 hours of cell index reading, CIM-plate was taken out of the system and statistical analysis continued with selected time points using Student's two-tailed t-test.

2.2.3.3. Cell Cycle Analysis

For cell cycle analysis cells were seeded in 6-well plates with 3 technical replicates and transfection was continued as previously described in section 3.2.2. After 24 hours of transfection, culture media was aspirated and 1ml fresh media was added on top of the cells. 10ul of BrdU solution (containing 1mM of BrdU diluted in 1xPBS) was carefully added in culture media and incubated for 45 minutes in 37°C 5% CO₂

After incubation cells were trypsinized, collected in FAC tubes and pulled down by centrifugation at 300g +4°C for 5 minutes. After carefully removing supernatant cells were washed with 1ml staining buffer and centrifuged at 300g +4°C for 5 minutes. After removal of supernatant, cells were permeabilized with 100ul/tube Cytoperm buffer and incubated for 30 minutes on ice. After incubation 1ml wash buffer was added on centrifuged at 300g +4°C for 5 minutes. After carefully removing of supernatant permeabilization and fixation steps were repeated. Cells were treated with 100ul/tube of DNase solution (30ul DNase and diluted in 70ul PBS) and incubated in 37°C for 1 hour. After incubation cells were washed with 1ml wash buffer and after centrifugation resuspended at 50ul/tube BrdU solution (1ul BrdU

diluted in 49ul wash buffer). After washing the cells with 1ml wash buffer, cells were resuspended in 20ul of 7-AAD solution and 250ul staining solution (1xPBS in 3% FBS) and read on Accuri FACS.

2.2.3.4. Migration (Wound Healing) Assay

For migration assay cells were seeded on 24- well plates with the concentration of 250,000 cells/well and transfected as described previously (Section 3.2.2.). 48 hour incubation after transfection, a scratch was formed at the middle of each well and cells were observed with 5x magnification using Nikon Eclipse inverted microscope (Nikon, Japan) for 48 hours. Distance between cells were measured using Adobe Photoshop CC and statistical analysis were carried out in GraphPad software (GraphPad software Inc., La Jolla, CA, USA) using Student's two-tailed t-test.

2.2.3.5. Poly-HEMA Assay

To measure anchorage-independent growth, cells were cultured in poly-HEMA coated 96-well plates. miR-564/Control stably transfected MDA-MB-231.luc were seeded with the concentration of 8000cells/well and growth of cells were examined. Images were taken at 1,3,5 and 7th day after seeding with 5X magnification using Nikon Eclipse inverted microscope (Nikon, Japan). 4 hours after 10ul WST-1 reagent addition to each well, cell growth was quantified by measuring absorbance at 450nm using SynergyHT microplate reader (Biotek, VT, USA). Statistical analysis was preceded on GraphPad software (GraphPad software Inc., La Jolla, CA, USA) using Student's two-tailed t-test.

2.2.3.6. Immunofluorescence

To analyze cell morphology by immunofluorescence, cells were seeded on square cover slips in 6-well plates in the concentration of 250,000cells/well and transfected as previously described (3.2.2.). 24 hour incubation at 37°C 5%CO₂ after transfection, cells were fixed with 2% paraformaldehyde for 15 minutes and permeabilized with 0.2% Triton X-100 solution in PBS for 5 minutes. Samples were blocked with 3%BSA/PBS for 30 minutes. For f-actin staining, cells were incubated in Alexa Fluor 488 phalloidin (1:40 diluted in 3% BSA/PBS) for 30 minutes in room temperature and for nuclear staining, cells were incubated in 1ug/ul 4',6-Diamidin-2-phenylindol (DAPI, diluted in 3%BSA/PBS) for 10 minutes. Cover slips were mounted with Shandon Immu-Mount reagent and images were taken with 20X and 40x focus on Axiovision 4.3 Florescent microscopy (Zeiss, Munich, Germany). Quantification of cell morphology was calculated with the ratio of long axis to short axis and further statistical analysis was performed using Student's two-tailed t-test.

2.2.3.7. Dual Luciferase Reporter Assay

MDA-MB-231 and MCF-7 cells were seeded in P/S-free media to 96-well plates with the concentration of 8000cells/well. 6 technical replicates were used for each treatment condition. After transfection performed as previously described (3.2.2.) cells were incubated for 48 hours in 37°C 5% CO₂. Cells were lysed with 60ul of 1x passive lysis buffer and incubated on shaker at room temperature for 15 minutes. 40ul cell lysate from each well were placed to 96-well opaque flat bottom white plates. 40ul of reagent I and 40ul of reagent II was added on top of the cells. After each substrate addition luminescence was measured with Synergy HT microplate reader (BioTek, Vermont, US). Renilla luciferase activity was normalized to firefly luminescence activity of psiCHECK™-2 vector and further statistical analysis was performed using Student's two- tailed t-test.

2.2.4. Molecular Biology

2.2.4.1. Cloning of 3'UTR for target expression constructs

psiCHECK™-2 dual luciferase reporter vector was kindly provided by Stefan Weinmann, DKFZ, Heidelberg and cloning of 3'UTR of gene of interests described below.

2.2.4.1.1. Insertion of 3'UTR

3'UTR of AKT2, GNA12, GYS1 and SRF genes were amplified using specifically designed primers for predicted miR-564 binding sites with addition of *XhoI* and *NotI* restriction sites (Table 2.4).

Table 2.4. List of cloning primers designed in Primer3.

Name	Sequence
AKT2_3'UTR_F	5'-GCGCGCCTCGAGATGACAGCCTGGGCTTACT-3'
AKT2_3'UTR_R	5'-GCGCGCGCGGCCGCTACAGATGGATAGCTAGTTTATTACAG-3'
GNA12_3'UTR_F	5'-GGGTGTCTCGAGTCTGTGTGGTCCTTGAGTGG-3'
GNA12_3'UTR_R	5'-GAAAGCGCGGCCGAGGTAGCTGGACAAGTGAGC-3'
GYS1_3'UTR_F	5'-CTCCAGCCCTCGAGAGAGTGAGGACGAGGAGGAT-3'
GYS1_3'UTR_R	5'-TCACACCCGCGGCCGCTCCCTCTCAACTGCAAAC-3'
SRF_3'UTR_F	5'-CGGGAGCTCGAGCAACCTACCGAGCTACAGG-3'
SRF_3'UTR_R	5'-CTGGGCGCGGCCGCAATCTCCCCACACACTGG-3'

Both psiCHECK™-2 vector and PCR product were double digested according to protocol in Table 2.5. PCR tubes containing restriction mixture were placed in PCR machine to incubate at 37°C for 90 minutes.

Table 2.5. Protocol of double restriction digestion.

Name	Concentration/Volume
DNA	2ug (Xul)
NotI	1.25ul
XhoI	1.25ul
Cut Smart Buffer	10ul
H2O	(37.5-X)ul

Restriction enzymes were deactivated by additional 10 minutes 65°C PCR step. At the end of each step, end products were cleaned up with Gel and PCR clean up kit and concentrations were measured with Nanodrop 1000. For ligation mixture vector and insert concentrations were calculated according to formula given below.

$$\text{Insert (ng)} = \text{Vector (ng)} \times 5 \times (\text{Insert (bp)} / \text{Vector (bp)})$$

Ligation mixture was incubated in 22°C for 60 minutes and ligation was checked on acrylamide gel electrophoresis.

2.2.4.1.2. Transformation

10 ul of ligation end product was transformed to DH5α bacteria strain 42°C 45 seconds heat shock. 1ml of LB added on top of transformed DH5α and incubated at 37°C shaker for 1 hour. Transformed DH5α containing LB was spread on 500ug/ml ampicillin containing agar plates and incubated at 37°C overnight. 5 colonies from each transformation was selected and amplified in 5ml LB on 37°C shaker overnight. Plasmids from amplified bacteria were isolated using Plasmid isolation kit.

2.2.4.1.3. Sequence Verification and Plasmid Maxi Prep

To verify the sequence of the created construct, isolated vectors from previous step was digested with *NotI* and *XhoI* restriction enzymes using the protocol previously described (3.4.1.1.). After validation by restriction digestion, vector was amplified in 100ml LB culture and isolated *via* using Maxi prep isolation kit and sent to sequencing with psiCHECK™-2 vector universal sequencing primers (Table 2.6).

Table 2.6. Universal primers of psiCHECK™-2 reporter vector

Name	Sequence
psicheck2_Rluc_F	5'-CGCTCCAGATGAAATGGGTAAG-3'
psicheck2_R	5'-CGAGGTCCGAAGACTCATTT-3'

2.2.4.2. Site-Directed Mutagenesis

Luciferase reporter constructs containing 3'UTR sequences of AKT2, GNA12, GYS1 and SRF were constructed as previously described. Each miR-564 target sequence was mutated using primers (Table 2.7) containing point mutations for every second nucleotide (4 nucleotides in total).

Table 2.7. List of primers used for site-directed mutagenesis of miR-564 target 3'UTR constructs.

Name	Sequence
AKT2_mut_F	5'-AAACCTGCCTGCCTCCCAGCCACTTTCA TTACTAGGGCTTCCTTCCAGCTGGCCTTACCT-3'
AKT2_mut_R	5'-AGGTAAGGCCAGCTGGAAGGAAGCCCTA GTAATGAAAGTGGCTGGGAGGCAGGCAGGTTT-3'
GNA12_mut_F	5'-AAAGGGAGCACGTTGCCTTTTCAAACCTCAC TTTCAGATTTCTTAAGAGCCCCTAGTCCAA-3'
GNA12_mut_R	5'-TTGGACTAGGGGCTCTTAGGAAATCTGAAAG TGAGTTTGAAAAGGCAACGTGCTCCCTTT-3'
GYS1_mut_F	5'-TTTCCAAGTTCCTGCACTCCAGAATCCACAAAG ACTTTCATTTCTCTGGCTCCAGAATATGCATAAT-3'
GYS1_mut_R	5'-ATTATGCATATTCTGGAGCCAGAGAAATGAAAG TCTTTGTGGATTCTGGAGTGCAGGAAGTTGGAAA-3'
SRF_mut_F	5'-ATGTTTGCCATGAGTATTAGCTTACCCAATGGG AACTTTCACCACCTCCCCACACAGGCCTT-3'
SRF_mut_R	5'-AAGGCCTGTGTGTGGGGAGGTGGTGAAAGTTC CCATTGGGTAAGCTAATACTCATGGCAAACAT-3'

Two PCR mixes were prepared according to Table 2.8 and two-step PCR protocol was used for each mutation primers (forward and reverse) that are targeting one seed sequence. After PCR-1 step, 25ul from each PCR mix were pooled and run for PCR-2. After PCR-2 protocol (Table 2.9), 1ul of *DpnI* enzyme was added in each PCR reaction for digestion of wild type vectors and incubated overnight in 37°C.

Table 2.8. PCR mixes for site-directed mutagenesis of miR-564 target sequences.

Reagent	PCR Mix-1 (ul)	PCR Mix-2 (ul)
Template DNA(100ng/ul)	1.5	1.5
Forward primer (20uM)	1	-
Reverse primer (20uM)	-	1
dNTPs (10mM)	1	1
5x Phusion HF buffer	10	10
DMSO	1.5	1.5
Phusion polymerase	1	1
Nuclease-free H ₂ O	34	34
Total	50	50

Table 2.9. Two-step PCR protocol for site-directed mutagenesis.

Temperature	PCR-1		PCR-2	
98°C	1 minute		1 minute	
98°C	30 seconds		30 seconds	
63-72 Δ °C	1 minute	6x	1 minute	18x
72°C	4 minutes		4 minutes	
72°C	10 minutes		10 minutes	

After enzyme inactivation at 80°C for 10 minutes, PCR was run in gel electrophoresis for product validation. 1ul of each reaction was transformed in competent DH5α strain of *E.coli*, colonies were picked after overnight incubation on amp⁺ LB-plates and inoculated in 5ml LB media. DNA was isolated from the colonies using MN Plasmid Isolation Kit. Mutations in miR-564 seed sequences were verified by sequencing.

2.2.4.3. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

2.2.4.3.1. RNA isolation

Cells were seeded in 6-well plates with the concentration of 250,000cells/well and transfected as previously described above (3.2.2.). After 48 hour incubation in 27oC 5% CO₂, cells were collected with trypsin and RNA was isolated using 0.5ml TRIsure reagent according to manufacturer's instructions.

2.2.4.3.2. cDNA synthesis

RevertAid RT Reverse Transcription kit was used for reverse-transcription of total RNA. Total of 2ug RNA from each sample was used to synthesize cDNA according to the protocol given at Table 2.10 and the reaction was incubated in thermocycler using the program given at Table 2.11.

Table 2.10. Components of reverse transcription reaction.

Reagent	Concentration/Volume
RNA	2ug (X ul)
oligoDT	1ul
5x Revert Aid reaction buffer	4ul
Ribolock Ribonuclease Inhibitor	1ul
dNTPs(10mM)	2ul
Revert Aid H Minus M-MuLV RT	1ul
H2O	(20-X) ul

Table 2.11. Thermocycler program for cDNA synthesis

Temperature	Time
37°C	5 minutes
42°C	60 minutes
70°C	10 minutes
4°C	∞

For qRT-PCR usage, all samples were diluted to 1:10 after cDNA synthesis.

2.2.4.3.3. qRT-PCR for mRNA expressions

For qRT-PCR, SYBR Green Master mix was used together with the primers listed on Table 2.12.

Table 2.12. List of qRT-PCR primers

Name	Gene ID	Forward Primer	Reverse Primer
CDH1	999	5'-CCCGGGACAACGTTTATTAC-3'	5'-GCTGGCTCAAGTCAAAGTCC-3'
ZO1	7082	5'-CAGAGCCTTCTGATCATTCCA-3'	5'-CATCTCTACTCCGGAGACTGC-3'
FN	2335	5'-CTGGCCGAAAATACATTGTAAA-3'	5'-CCACAGTCGGGTCAGGAG-3'
SNAI2	6591	5'-TGGTTGCTTCAAGGACACAT-3'	5'-GTTGCAGTGAGGGCAAGAA-3'
ZEB1	6935	5'-GGGAGGAGCAGTGAAAGAGA-3'	5'-TTTCTTGCCCTTCCTTTCTG-3'
ZEB2	9839	5'-AAGCCAGGGACAGATCAGC-3'	5'-CCACACTCTGTGCATTGAACT-3'
AKT2	208	5'-CCATGAATGAGGTGTCTGTC-3'	5'-GGCCTCCAGGTCTTGATGTA-3'
GNA12	2768	5'-TCAAGAAGCACTTCCCGGACTTCA-3'	5'-TTTCACAGCATGGAACACGAAGCG-3'
GYS1	2997	5'-CCGCTATGAGTTCTCCAACAAGG-3'	5'-AGAAGGCAACCACTGTCTGCTC-3'
SRF	6722	5'-AGCACAGACCTCACGCAGA-3'	5'-GTTGTGGGCACGGATGAC-3'
CREB5	9586	5'-CGTGCCTCCTTGAAACAAGCCATT-3'	5'-ATGAAACACCAGCACCTGCCTAGA-3'
ACTB	60	5'-CCAACCGCGAGAAGATGA-3'	5'-CCAGAGGCGTACAGGGATAG-3'
HPRT	3251	5'-TGACCTTGATTTATTTGCATACC-3'	5'-CGAGCAAGACGTTTCAGTCCT-3'

Reaction was carried out in 96-well plate and a reaction mix was prepared for each primer pair (Table 2.13). After addition of 8ul of mastermix, 2ug (final concentration of 20ng) of cDNA added separately into each well and carefully mixed by pipetting.

Table 2.13. Mastermix for qRT-PCR reaction

Reagent	Volume
SYBR Green	5ul
Forward Primer (20uM)	1ul
Reverse Primer (20uM)	1ul
H2O	1ul

Plate was tightly sealed, centrifuged at 1000rpm for 1minute at 4oC before running on LightCycler® 96 qRT-PCR thermocycler (Roche Applied Science, Mannheim, Germany) with thermocycler program shown on Table 2.14.

Table 2.14. qRT-PCR program.

Pre-incubation							
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
95	None	00:05:00	4.4	5	0	0	0
Amplification							
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
95	None	00:00:10	4.4	5	0	0	0
58	Single	00:00:20	2.2	5	0	0	0
72	None	00:00:20	4.4	5	0	0	0
Melting Curve							
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
95	None	00:00:05	4.4	5	0	0	0
55	None	00:01:00	2.2	5	0	0	0
95	Continuous	00:00:00	0.11	5	0	0	0
Cooling							
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
40	None	00:00:30	2.2	5	0	0	0

2.2.4.3.4. Taqman qRT-PCR for miRNA expressions

After RNA isolation cDNA synthesis and qPCR protocols for miRNAs were performed using AB Taqman miRNA Assays (Foster City, CA, USA). RT master mix was prepared for each sample using Table 2.15. 15ul of RT reaction consists of 7ul master mix, 3ul RT primer from Taqman miRNA Assay kit and 5ul (2ng/ul) RNA sample. Table 2.16 was used to perform miRNA reverse transcription.

Table 2.15. Mastermix for Taqman miRNA reaction

Reagent	Volume for each 15ul reaction (ul)
dNTPs (100nM)	0.15
MultiScribe™ Reverse Transcriptase (50U/ul)	1
10x RT Buffer	1.5
RNase Inhibitor (20U/ul)	0.19
Nuclease-free H ₂ O	4.16
Total	7ul

Table 2.16. PCR protocol for Taqman miRNA reverse transcription.

Temperature	Time
16°C	30 minutes
42°C	30 minutes
85°C	5 minutes
4°C	∞

For qPCR amplification of miRNA reverse transcription samples, Taqman 2x Master Mix was used as described in Table 2.17. 0.5ul of 20x Taqman miRNA ASSay mix (labeled as Real time) was added on top of 17.67 ul of PCR master mix for each well and 1.33ul of RT product for each sample was added into each well of 96-well qPCR opaque plates. After preparation of qPCR plate using 3 technical replicates for each experimental condition, PCR amplification was performed using PCR protocol on Table 2.18.

Table 2.17. Mastermix for Taqman miRNA PCR amplification.

Reagent	Volume for each 20ul reaction (ul)
Taqman 2X Universal PCR Master Mix	10
Nuclease-free H ₂ O	7.67
Total	17.67

Table 2.18. qPCR protocol for Taqman miRNA amplification.

Parameter	Value			
Run mode	9600 emulation (Default)			
Sample volume	20ul			
Thermal cycling parameters	Step	Type	PCR	
		Hold	40 cycles	
			Denature	Anneal
	Time	10min	15sec	60sec
	Temp (°C)	95	95	60
Auto Increment Settings	Accept default. (Default is 0.)			
Ramp Rate Settings	Accept default. (Default is Standard.)			
Data Collection	Accept default. (Default is 60°C.)			

2.2.4.3.5. qRT-PCR Data analysis

Each qRT-PCR experiment was containing at least two housekeeping genes and qRT-PCR data analysis was performed according to $\Delta\Delta C_t$ method with using Microsoft Excel (Albuquerque, NM, USA). Statistical analysis was performed using Student's two-tailed t-test. For each treatment condition 3 technical replicates were used and for each qRT-PCR experiment was repeated 3 times for individual biological replicates.

2.2.5. Protein Biochemistry

2.2.5.1. Protein Isolation

MDAMB-231 and MCF7 cells were seeded in 6-well plates with the concentration of 250,000cells/well and transfection was performed as described above (3.2.2.). 48 hours after incubation, culture media was aspirated and cells were washed with 1ml 1XPBS. After aspiration of 1XPBS, 50ul RIPA lysis buffer was added on top of the cells. Cells are collected to 1.5ml tubes after scraping on ice and centrifuged at 13000rpm 4°C for 20 minutes. Supernatant was collected and stored at -20°C for further experiments.

2.2.5.2. Protein Quantification

Protein quantification was performed by using the BCA Assay Kit according to manufacturer's instructions. BSA standard solutions were prepared in the range of 0 to 2ug/ul and 25ul of each standard was placed in 96-well plate with two replicates. Dilution factor between standard and protein samples were arranged as 5. By mixing reagent A and B in 50:1 ratio, working solution was prepared and 200ul of working solution was added to each well. After 30 minutes of incubation in 37°C, absorbance of samples was measured on SynergyHT microplate reader (Biotek, VT, USA) at 562nm. Using the BSA standards, a calibration curve was drawn and sample concentrations were quantified from line graph of the curve.

2.2.5.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Concentrations of protein samples were equalized using 4X protein loading dye and boiled at 95°C for 5 minutes. Polyacrylamide stacking and resolving gels were prepared according to Table 2.19.

Table 2.19. Mixture for stacking and resolving gels in different concentrations

Reagent	8% Resolving gel	10% Resolving gel	12% Resolving gel	5% Stacking gel
H ₂ O	2.3ml	1.9ml	1.6ml	1.36ml
30% acrylamide mix	1.3ml	1.7ml	2ml	340ul
1 M Tris	(pH 8.8) 1.3ml	(pH 8.8) 1.3ml	(pH 8.8) 1.3ml	(pH 6.8) 260ul
10% SDS	50ul	50ul	50ul	20ul
10% APS	50ul	50ul	50ul	20ul
TEMED	5ul	5ul	5ul	2ul
Total volume	5ml	5ml	5ml	2ml

First resolving gel was prepared and overlaid with isopropanol until it is fully polymerized. After polymerization, isopropanol was removed and stacking was cast on top of resolving gel and 10 or 15 well comb was placed. Samples were loaded as 15ug per well and empty wells were filled with diluted protein loading dye. Electrophoresis was performed at 130V for 90 minutes.

2.2.5.4. Western blotting

For semi-dry transfer 3mm whatmann papers were cut in 7cm to 9cm and 4 of them moistened in anode buffer I, 2 of them in anode buffer II and 6 of them in cathode buffer. PVDF membrane was first placed in 100% methanol for 3 minutes and then placed in anode buffer II for 1 minute. 4 anode I, 2 anode II, PVDF membrane, stacking gel, 6 cathode papers were stacked from anode to cathode respectively and placed in Semi-dry Turbo Blot machine for 30 minutes in 25V.

After transfer, PVDF membrane was stained with Ponceu S solution for 3 minutes and washed with ddH₂O. After removal of Ponceu stain by washing with TBST, membrane was cut for specific kDa for interest and placed on 5% (w/v) milk:TBST blocking or on 5% (w/v) BSA:TBST blocking for 1 hour room temperature on slow shaking. After removal of blocking solution membranes were incubated with primary antibody either for 1-hour in room temperature or overnight in 4°C (Table 2.20).

Table 2.20. List of primary and secondary antibodies used in western blot.

Gene Name	Firm	Catalog Number	Dilution
phospho-AKT (Ser473)	Cell Signaling Technology	CST4058	1:1000
phospho-AKT (Thr308)	Cell Signaling Technology	CST4056	1:1000
total-AKT	Cell Signaling Technology	CST9272	1:1000
phospho-ERK1/2 (Thr202/Tyr204)	Cell Signaling Technology	CST4376	1:1000
ERK1/2	Cell Signaling Technology	CST4695	1:1000
p27/Kip1	BD Biosciences	610241	1:1000
p21	BD Biosciences	554228	1:1000
CDK4	Epitomics	2341-1	1:1000
CDK2	Sigma Aldrich	C5223	1:1000
phospho-Rb (Ser807/811)	Cell Signaling Technology	CST8516	1:1000
AKT2	Cell Signaling Technology	CST5239	1:1000
GNA12	Santa Cruz	sc-409	1:500
GYS1	Cell Signaling Technology	CST3886	1:500
SRF	Cell Signaling Technology	CST5147	1:500
Beta-actin	MP Biomedicals	69100	1:5000
HRP-coupled anti-rabbit IgG	Cell Signaling Technology	CST7074	1:10000
HRP-coupled anti-mouse IgG	Cell Signaling Technology	CST7076	1:10000

After primary antibody incubation membranes were washed with TBST three times 10 minutes on shaker. Membranes were then incubated in secondary antibody either for 1-hour in room temperature or overnight in 4°C. After secondary antibody incubation membranes were again washed with TBST three times 10 minutes on shaker. ECL reagents were mixed 1:1 and applied to membrane, which is placed on X-ray cassette after washing steps. X-ray films were exposed to membranes for different time periods ranging from 3 seconds to 30 minutes.

2.2.6. Bioinformatic analysis

2.2.6.1. Target prediction and KEGG pathway analysis

For target prediction analysis of four different target prediction algorithms were used. In TargetScan database, only targets with conserved sites were included. In DIANA and PITA databases, targets with a miTG score greater than 0.7 and a score of less than -10 respectively were selected. In miRWalk database, no cut-off was assigned. Out of 4 databases the predictions that were shown at least in two of them were selected for further analysis.

2.2.6.2. Cell line and patient dataset analysis

For miRNA and mRNA expression analysis, NCI60, NCBI Gene Expression Omnibus (GEO) database (GSE40059, GSE40525, GSE19536, GSE22220, GSE40267, GSE37407, GSE38867, GSE31376, GSE40355, GSE28425, GSE14985, GSE29542, GSE37053 and GSE3494) was analyzed. The Cancer Genome Atlas (TCGA) mRNA and RPPA databases were analyzed. mRNA and RPPA data from TCGA were downloaded from cBioPortal for Cancer Genomics[45, 46] and TCPA databases[47], respectively and GSE datasets were downloaded from GEO database[48].

To calculate target-network expression of AKT2, GNA12, GYS1 and SRF, z-scores of individual genes were summed up and sum z-score was created for each patient in each database. Conventional z-score formula was used to calculate z-scores of each gene within patients[49, 50].

In GSE40059 breast cancer cell line dataset, normalization of log2 expression values and sum of z-scores was done by subtracting each value from its minimum and then dividing by the range of data.

Kaplan-Meier patient survival graphs were generated in Graphpad software (GraphPad software Inc., La Jolla, CA, USA) using the patient information given in GSE datasets and patients with no survival information or "dead of non-cancerous cause" were removed. Both for miRNA, mRNA expression and target sum z-scores, patients were separated from median of group. Further statistical analysis was done using Student's two-tailed t-test.

Statistical significance of the data provided in this thesis was shown as * = $p < 0.05$ and ** = $p < 0.01$.

CHAPTER 3

Results

3.1. miR-564 inhibits PI3K and MAPK signaling pathways simultaneously and suppresses cell proliferation via G1 arrest in breast cancer cell lines

To identify a potential PI3K and MAPK pathway dual inhibitor miRNA, our previously published RPPA[43] data containing 26 protein/phosphorylation level changes in the presence of 733 different miRNAs has been re-analyzed. 16 proteins (also shown in Table 2.1) functioning in either PI3K, MAPK or cell cycle pathway has been selected and separated according to their function in the pathway (either tumor suppressor as 1 or oncogene as 0). Then each miRNA has been correlated to PI3K and MAPK pathways regarding 0/1-classification of proteins (details are given in Methods section). Out of 733 ectopically expressed miRNAs, top 50 miRNAs clustered according to their degree of proximity to PI3K and MAPK pathways as well as cell cycle modulation (Figure 3.1). Among these 50 miRNAs, 23 were identified as potential tumor suppressor miRNAs, which negatively correlates with PI3K, MAPK and cell cycle pathways (Table 3.1). hsa-miR-564 and hsa-miR-490 were selected as top candidates due to their novelty in tumor formation and progression. miR-564 and miR-490 then further tested for their anti-proliferative effects on breast cancer cell lines representing different clinical subtypes (TNBC, ER+ and HER2-overexpressed). miR-200b, as a highly studied tumor suppressor miRNA, was used as a positive control [40, 41, 51]. After ectopic expression of miRNAs, miR-564 showed the most prominent and superior anti-proliferative effect on breast cancer cells and thus selected for further studies (Figure 3.2).

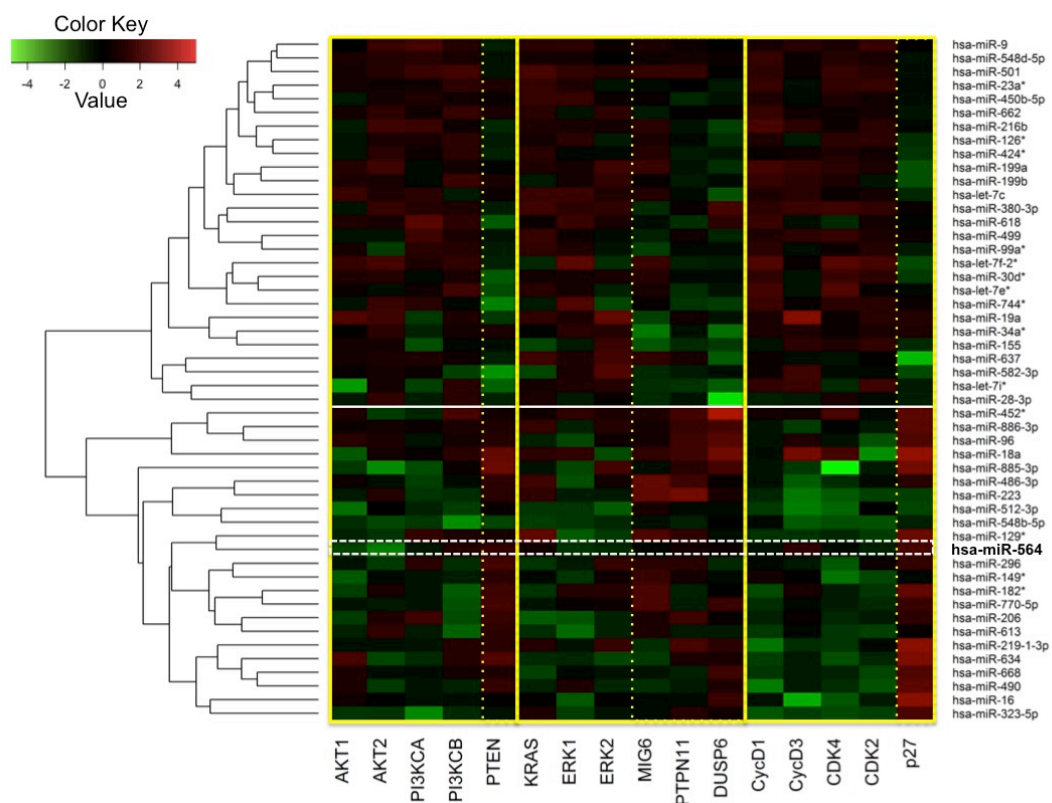


Figure 3.1. Heatmap of top 50 miRNAs from RPPA data regulating PI3K, MAPK and cell cycle pathway proteins.

Table 3.1. Correlation scores of 23 predicted tumor suppressor miRNAs from RPPA data selected by their correlation scores with proteins of PI3K and MAPK pathways as well as cell cycle.

Potential tumor suppressor miRNAs	Heatmap Correlation scores (cut off: -0.5)
hsa-miR-770-5p	-0.738
hsa-miR-323-5p	-0.688
hsa-miR-486-3p	-0.660
hsa-miR-96	-0.656
hsa-miR-206	-0.642
hsa-miR-886-3p	-0.623
hsa-miR-885-3p	-0.620
hsa-miR-223	-0.577
hsa-miR-634	-0.557
hsa-miR-182*	-0.545
hsa-miR-18a	-0.545
hsa-miR-129*	-0.541
hsa-miR-613	-0.539
hsa-miR-149*	-0.533
hsa-miR-512-3p	-0.529
hsa-miR-668	-0.522
hsa-miR-564	-0.520
hsa-miR-16	-0.517
hsa-miR-296	-0.516
hsa-miR-219-1-3p	-0.513
hsa-miR-452*	-0.513
hsa-miR-490	-0.505
hsa-miR-548b-5p	-0.502

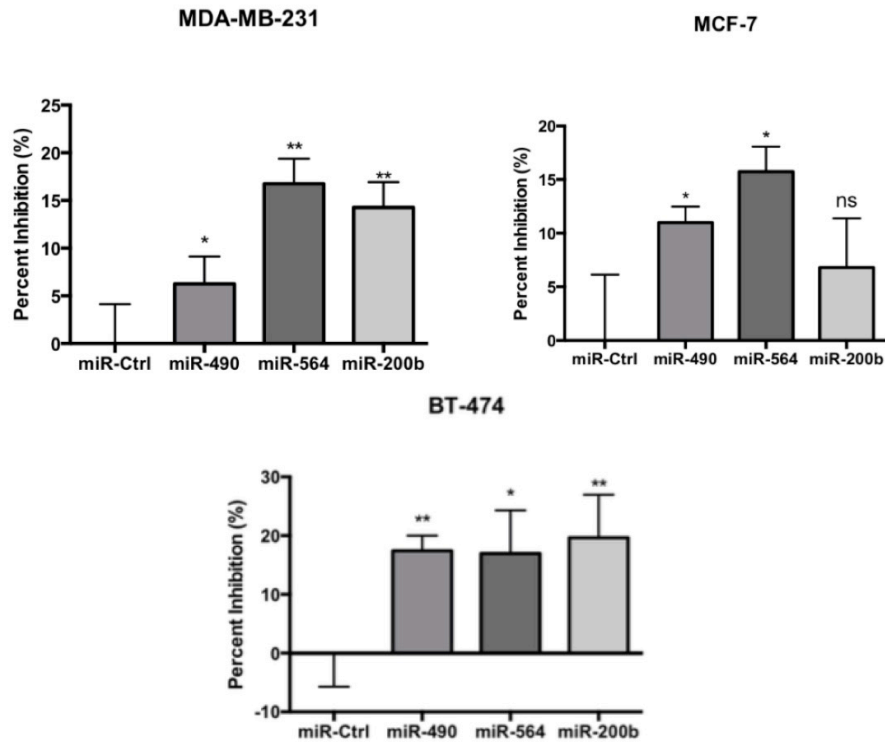


Figure 3.2. Effect of potential tumor suppressor miRNAs on cell viability of MDA-MB-23 (TNBC), MCF-7 (ER+) and BT-474 (ER+, HER2+) breast cancer cell lines representing three clinical subtypes.

To confirm transient and stable transfection efficiencies, we performed miRNA Taqman qPCR in MDA-MB-231, MCF-7 and MDA-MB-436 cell lines. Both stable and transient transfections showed significant increase in miR-564 expression (Figure 3.3).

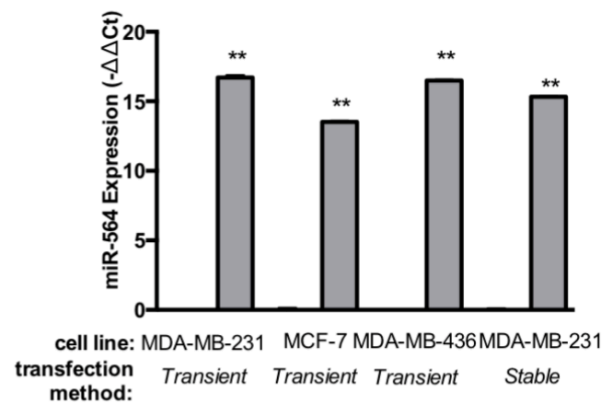


Figure 3.3. miR-564 expression levels in transiently transfected MDA-MB-231, MCF-7 and MDA-MB-436 cell lines and stably transfected MDA-MB-231 with miR-564 mimics or lentiviral vectors.

To validate predicted dual inhibitory effect of miR-564, phosphorylation levels of PI3K and MAPK pathway elements were examined by western blot. Increase in phosphorylation levels

of AKT and ERK1/2, directly correlates with activation of PI3K and MAPK pathways, respectively. MDA-MB-231 and MCF-7 breast cancer cells were transiently transfected with miR-564 and miR-Negative Control #1 (Dharmacon) as the negative control group that was shown as miR-Ctrl. A significant decrease was observed in both AKT and ERK1/2 phosphorylation levels, whereas total AKT and ERK1/2 protein levels remained same in presence of miR-564 overexpression (Figure 3.4).

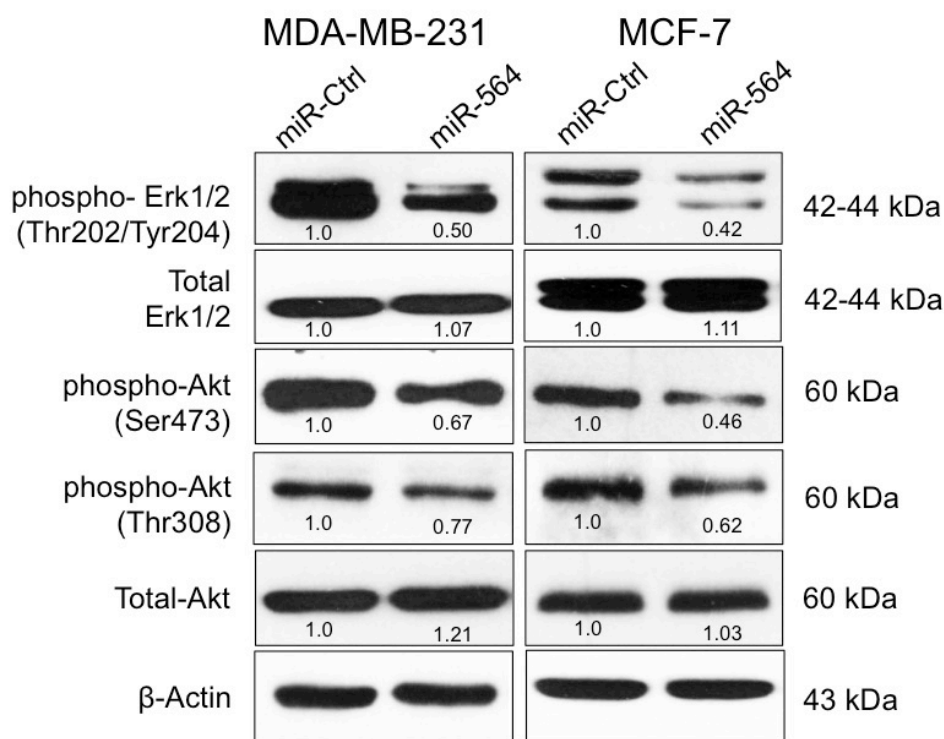


Figure 3.4. miR-564 acts as a dual inhibitor of PI3K and MAPK pathways by downregulating phospho-AKT and phospho-ERK1/2 levels. Western blots showing expression and phosphorylation of key proteins in PI3K and MAPK pathways.

Re-analysis of RPPA data has also revealed prominently decreased Cyclin D1, CDK2 and CDK4 levels as well as increased p27 levels in miR-564 overexpressing MDA-MB-231 cells (Figure 3.1). To further validate these findings, we performed a cell cycle analysis with BrdU/7-AAD double staining after mimic transfection. In miR-564 overexpressed MDA-MB-231 and MCF-7 cells, cells in S phase were decreased and G1 phase increased compared to control group, which is indicating that miR-564 induced a G1 arrest. It has been further validated by Western Blot analysis of G1 and S phase associated cyclin-dependent kinases (CDKs), CDK inhibitors as well as phosphorylation levels of Retinoblastoma (Rb) protein (Figure 3.5). G1-arrest induced by miR-564 overexpression in MDA-MB-231 cells was shown to be primarily mediated by CDK4 downregulation and p27 upregulation, whereas in MCF-7 cells it resulted in p21 and CDK2 mediated G1 arrest.

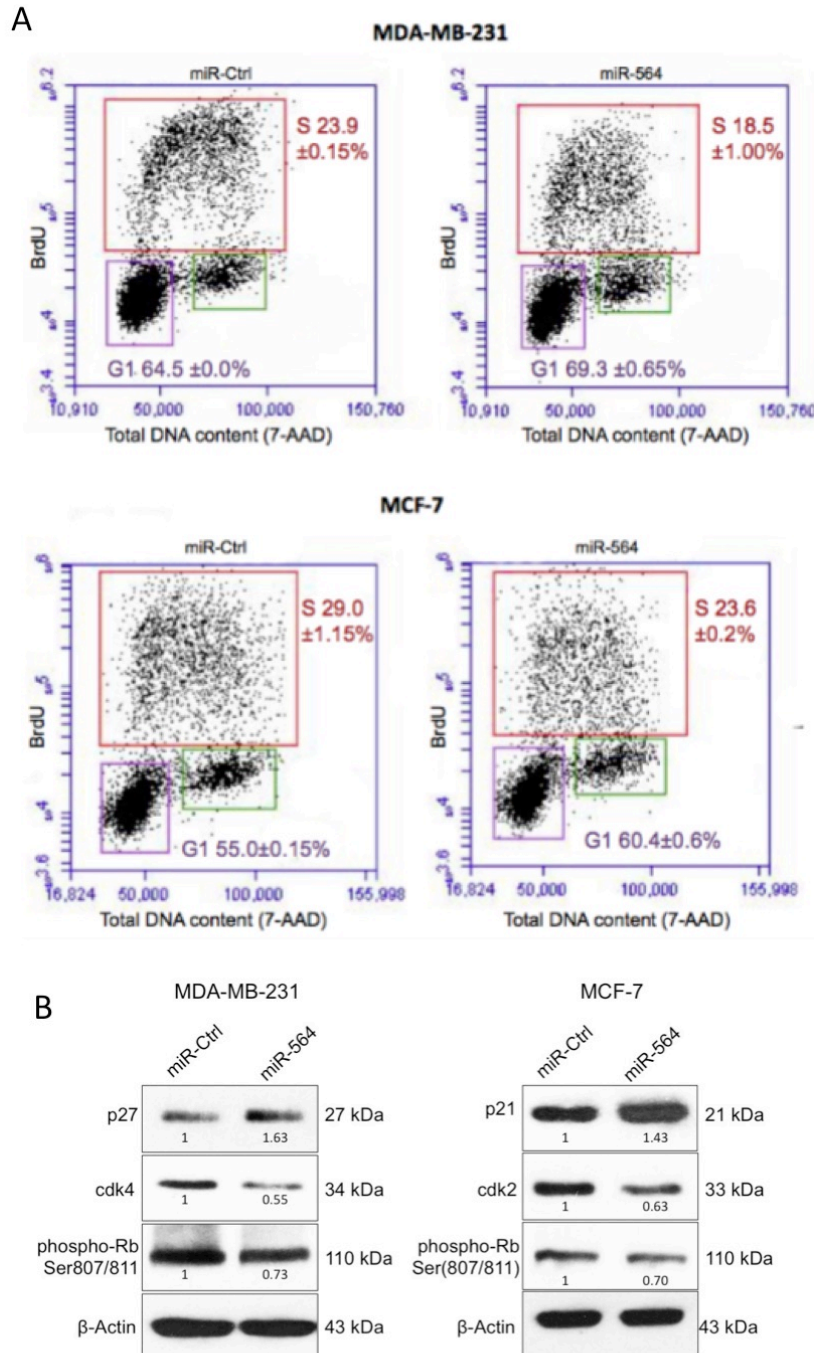


Figure 3.5. miR-564 overexpression blocks cell proliferation via G1 arrest. **A.** BrdU/7-AAD staining of miR-564 overexpressed MDA-MB-231 and MCF-7 cell lines. **B.** Western blot analyses of G1/S transition proteins in MDA-MB-231 and MCF-7 cell lines transiently transfected with miR-564 or miR-Negative control.

To validate the growth inhibitory effect of miR-564 overexpression on breast cancer cell lines, we performed both end-point and real-time cell proliferation analysis. miR-564 overexpression conferred a 25% inhibition of cell proliferation in both MCF-7 and MDA-MB-231 cell lines. In line with these results, RTCA demonstrated a significantly lower level of cell growth in MDA-MB-231 and MCF-7 cells transfected with miR-564 (Figure 3.6A,C).

Subsequently, proliferation of MCF-7 cells that are transfected with miR-564 antagonist showed a significant increase compared to negative hairpin inhibitor transfected cells (Figure 3.6B).

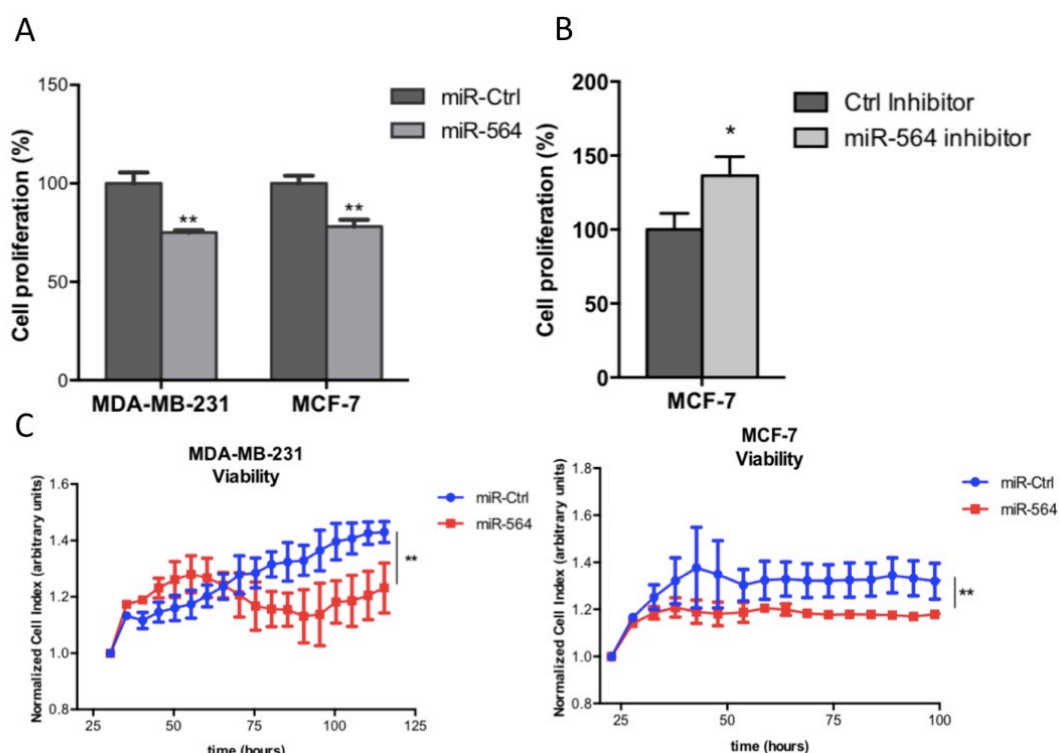


Figure 3.6. In vitro viability assays in miR-564 overexpressing MDA-MB-231 and MCF-7 cell lines. **A.** End-point cell proliferation assay performed on MDA-MB-231 and MCF-7 cell lines transiently transfected with miR-564 or miR-Negative control. **B.** End-point viability assay performed in miR-564 inhibitor transfected MCF-7 cells. **C.** Real-time cell analysis of miR-564 overexpressing MDA-MB-231 and MCF-7 cell lines.

In order to further support the potential tumor suppressor role of miR-564 in breast cancer, we have analyzed several breast cancer cell line datasets from Gene Expression Omnibus (GEO) database. In GSE40059[40] dataset, containing mRNA and miRNA expression profiles of 12 different breast cancer and non-cancerous cell lines, miR-564 expression was shown to be higher in normal breast cell lines as compared to breast cancer cell lines (Figure 3.7).

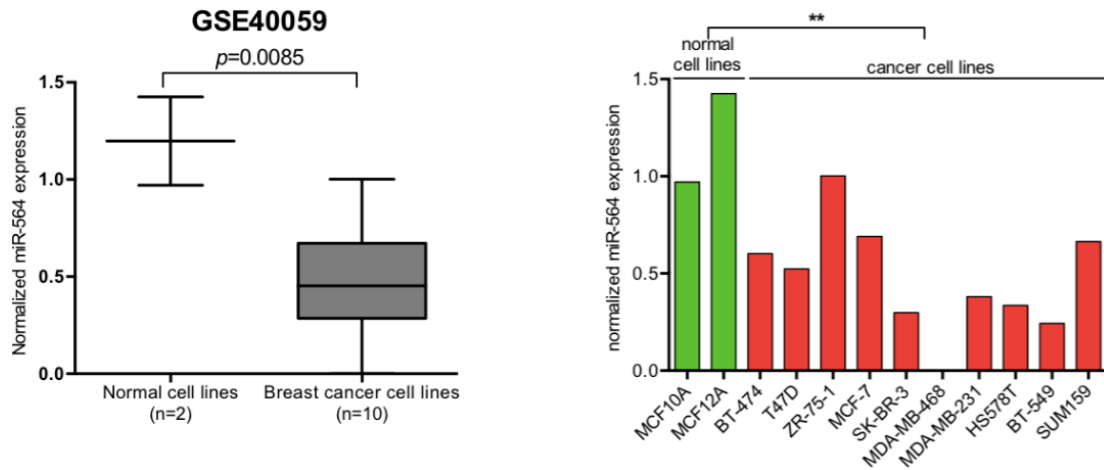


Figure 3.7. Expression of miR-564 in 12 different breast cell lines from GSE40059 dataset, classified as cancerous versus normal (left panel). Individual expressions of miR-564 in each cell line shown as relative expression with respect to MDA-MB-468 (right panel).

These results indicate that miR-564 suppresses PI3K and MAPK signaling pathways thereby inhibits proliferation of breast cancer cells *via* reducing cell population in S-phase.

3.2. miR-564 blocks EMT and consequently inhibits migration and invasion in breast cancer cell lines

PI3K and MAPK signaling pathways are associated with EMT, and they have been shown to induce migration and invasion in breast cancer[52]. In order to test the effects of miR-564 on breast cancer migration, we performed a wound-healing assay as well as a real-time cell migration assay with RTCA. Ectopic expression of miR-564 in an aggressive breast cancer cell line, MDA-MB-231, significantly reduced the migratory function. In addition, miR-564 inhibitor transfected cells showed increased migration capacity compared to control hairpin inhibitor transfection. RTCA migration analysis on MDA-MB-231 and MDA-MB-436 cell lines further supported these findings and suggested an important role of miR-564 in inhibiting migration in breast cancer (Figure 3.8A-D).

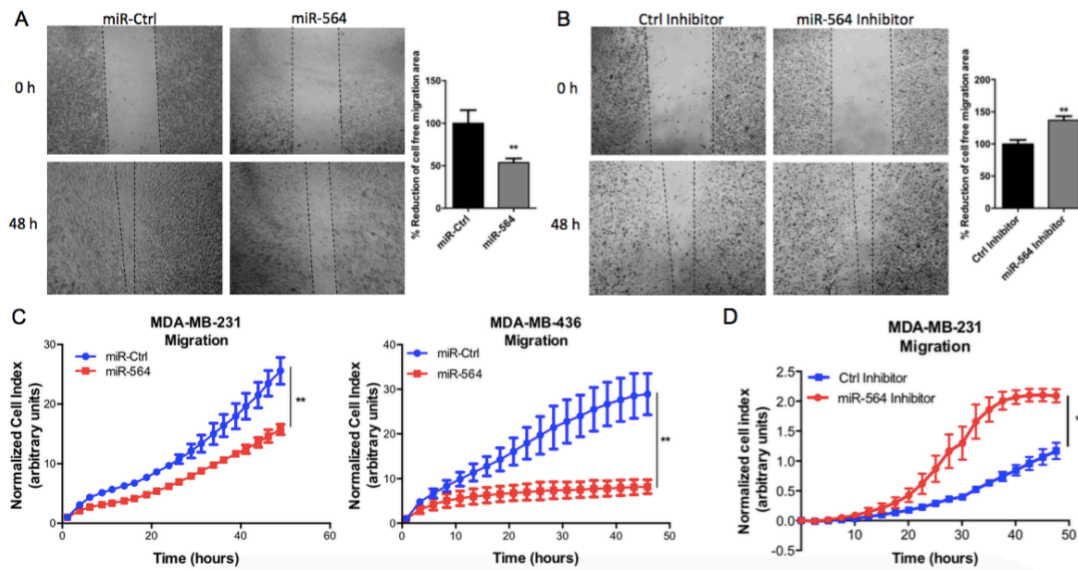


Figure 3.8. miR-564 inhibits migration in breast cancer cell lines **A.** Wound healing assay performed using miR-564 overexpressed MDA-MB-231 cell line at 0 and 48 hours post-transfection **B.** Wound healing assay performed using miR-564 antagonist transfected MDA-MB-231 cells. **C.** Real-time migration assay with miR-564 overexpressed MDA-MB-231 and MDA-MB-436 cells. **D.** Real-time migration assay with miR-564 inhibitor transfected MDA-MB-231 cells.

Anchorage-independent growth is the ability of cancer cells to proliferation regardless of any surface interference, and it is the key aspect of tumor phenotype as well as metastatic potential [53]. To test the effects of miR-564 on anchorage-independent growth, we used MDA-MB-231 cells stably overexpressing miR-564 determined as suitable for poly-HEMA assay. 7 days after cell seeding on poly-HEMA coated 96-well plates; miR-564 overexpressing MDA-MB-231 cells showed significant reduction in anchorage-independent growth as compared to non-silencing vector overexpressed MDA-MB-231 cells (Figure 3.9).

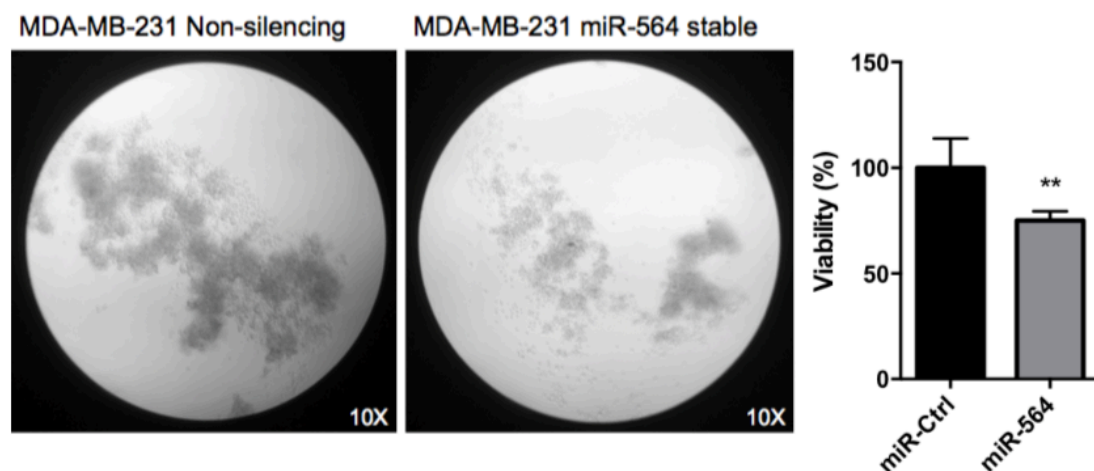
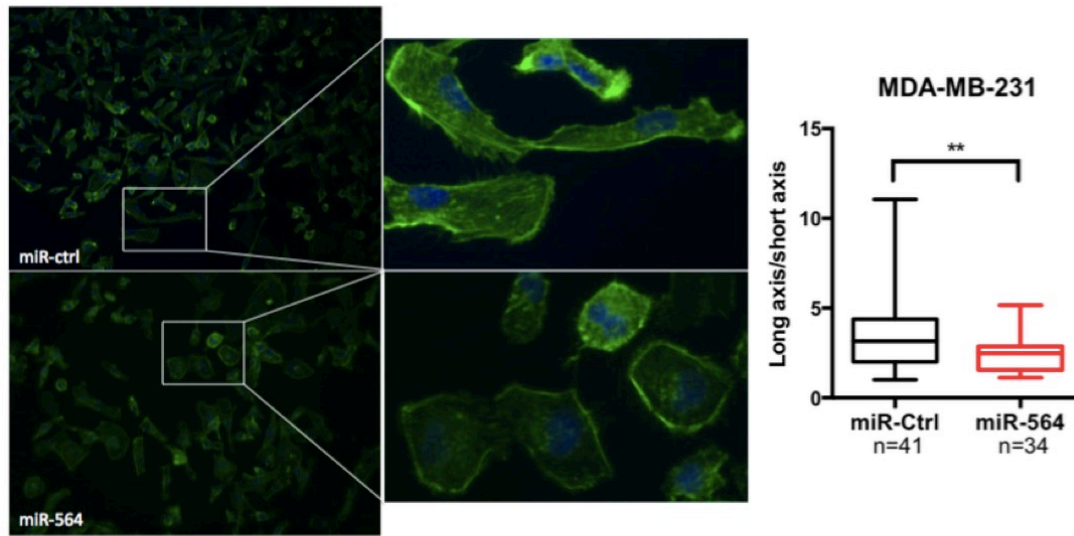


Figure 3.9. Anchorage-independent growth analysis on miR-564 stably transfected MDA-MB-231-luc cells by Poly-HEMA assay. Cells were incubated for 48 hours and viability was measured with WST-1 cell proliferation reagent. Control group was stably transfected with empty vector.

While performing cell viability assay on MDA-MB-231 cells, we saw that the cells showed morphological changes and more epithelial-like morphology after miR-564 overexpression. To test the effect of miR-564 on EMT, we stained MDA-MB-231 cells with Phalloidin, an f-actin marker, together with nuclear dye DAPI. 24 hours after transient transfection with miR-564, rather than regular MDA-MB-231 mesenchymal-like structure, we observed a more epithelial-like morphology as characterized by loss of stress fibers in MDA-MB-231 cells, which normally possesses a mesenchymal-like morphology. We observed a significant decrease in the ratio of long axis to short axis with miR-564 overexpression, which again confirmed the blockage of EMT induced by miR-564 in aggressive breast cancer cells (Figure 3.10A). We further validated these results by checking the RNA expressions of known epithelial and mesenchymal markers by qRT-PCR. As expected, CDH1 and ZO1 showed a significant increase, whereas FN, SNAI2, ZEB1 and ZEB2 showed a significant decrease in the presence of miR-564 overexpression (Figure 3.10B).

A



B

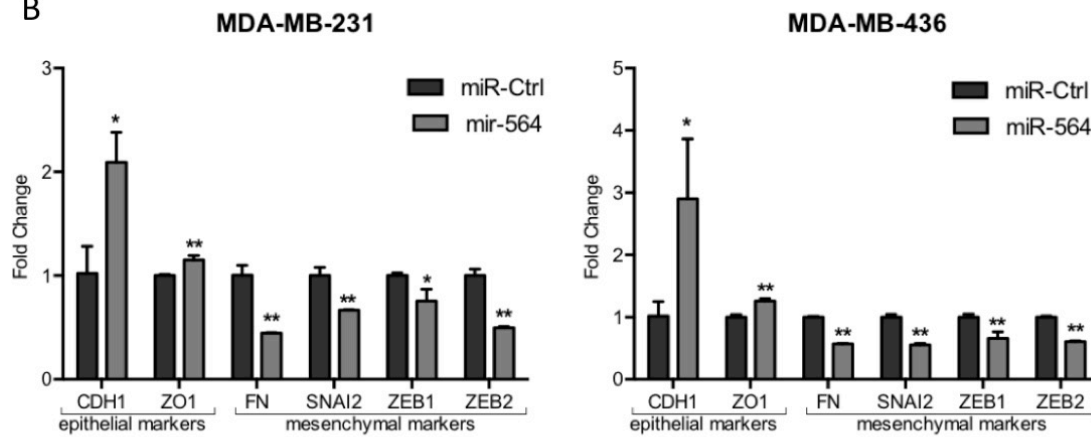


Figure 3.10. miR-564 blocks EMT in aggressive breast cancer cell lines. A. DAPI/Phalloidin staining of miR-564 overexpressing MDA-MB-231 cells. Images were taken 24 hours after transfection. Morphological changes were quantified as ratio of long axis to short axis for each cell. B. Changes in expression of epithelial and mesenchymal markers in MDA-MB-231 and MDA-MB-436 cell upon miR-564 transfection.

So far, we were able to show an inhibitory effect of miR-564 on migration and invasion *via* blockage of EMT *in vitro*. To further corroborate these results with a patient relevance, GSE40059[40] dataset has been re-analyzed by grouping the cell lines according to their invasiveness. miR-564 was found to be higher in less invasive cell lines as compared to invasive breast cancer cell lines (Figure 3.11).

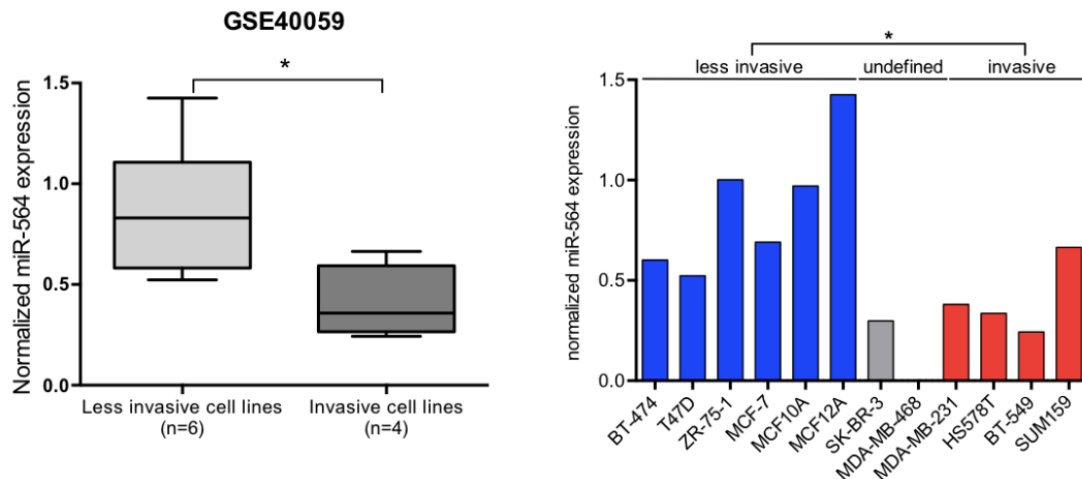


Figure 3.11. miR-564 expression is inversely correlated with cell invasiveness in breast cancer cell lines. Expression of miR-564 in 12 different breast cell lines from GSE40059 dataset, classified as invasive and less-invasive (left panel). Individual expression of miR-564 in each cell line shown as relative expression with respect to MDA-MB-468 (right panel).

Overall, these results show that miR-564, as a dual PI3K-MAPK inhibitor, blocks EMT and inhibit anchorage-independent growth, migration and invasion in breast cancer cells.

3.3. miR-564 suppresses PI3K and MAPK pathways via directly targeting a network of genes

After identification of miR-564 as a dual PI3K and MAPK inhibitor affecting cell proliferation, migration and invasion, it is aimed to identify the detailed tumor suppressive mechanisms of miR-564. To do this, we have started by identifying the mRNA targets of miR-564 by using four different target prediction algorithms (Targetscan (Release 7.0)[54], miRwalk (2.0)[55], DIANA[56] and PITA[57]) in combination. With the cut-offs described in methodology, list of predicted mRNA targets were obtained from each algorithm, and 37 genes that were predicted by at least two different algorithms were taken into account. By performing KEGG pathway analysis with 37 selected targets, we have shortlisted the genes that function in PI3K and MAPK pathways for further validation (Figure 3.12A). Five genes, namely; V-akt murine thymoma viral oncogene homolog 2 (AKT2), guanine nucleotide binding protein subunit alpha 12 (GNA12), glycogen synthase 1 (GYS1), serum response factor (SRF) and cAMP responsive element binding protein 5 (CREB5) were among the selected predicted targets of miR-564 that play roles in either PI3K or MAPK pathways (Figure 3.12B).

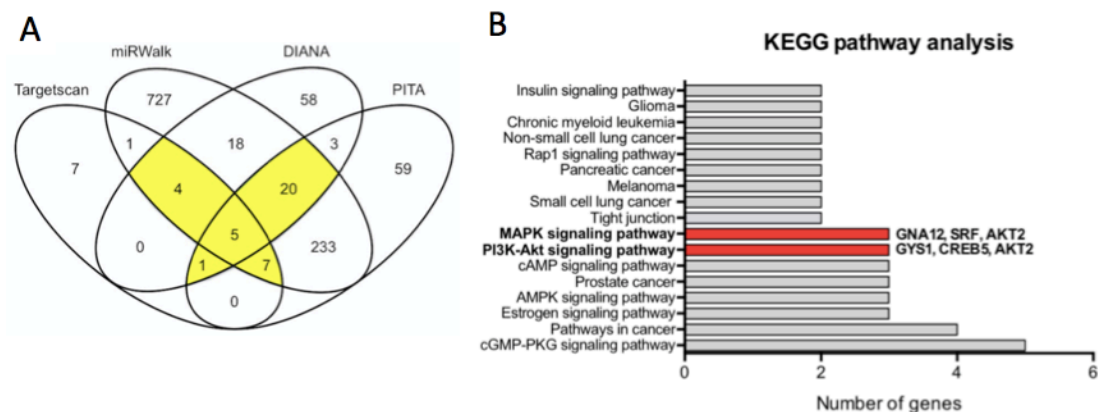


Figure 3.12. AKT2, GNA12, GYS1, SRF and CREB5 are all in PI3K and MAPK pathways and are predicted targets of miR-564. **A.** Venn schema showing potential targets of miR-564 from 4 different target prediction databases. **B.** KEGG pathway analysis of predicted miR-564 targets selected from four different target prediction algorithms.

To validate target predictions, mRNA levels of each potential target were measured in the presence of miR-564 overexpression in MDA-MB-231 and MCF-7 cell lines (Figure 3.13A). Out of 5 genes, AKT2, GNA12, GYS1 and SRF as predicted targets showed the expected decrease in expression; therefore were selected for further analysis. Decrease in expression was further validated in protein level with Western Blot analysis of the predicted targets in miR-564 overexpressing cells (Figure 3.13B).

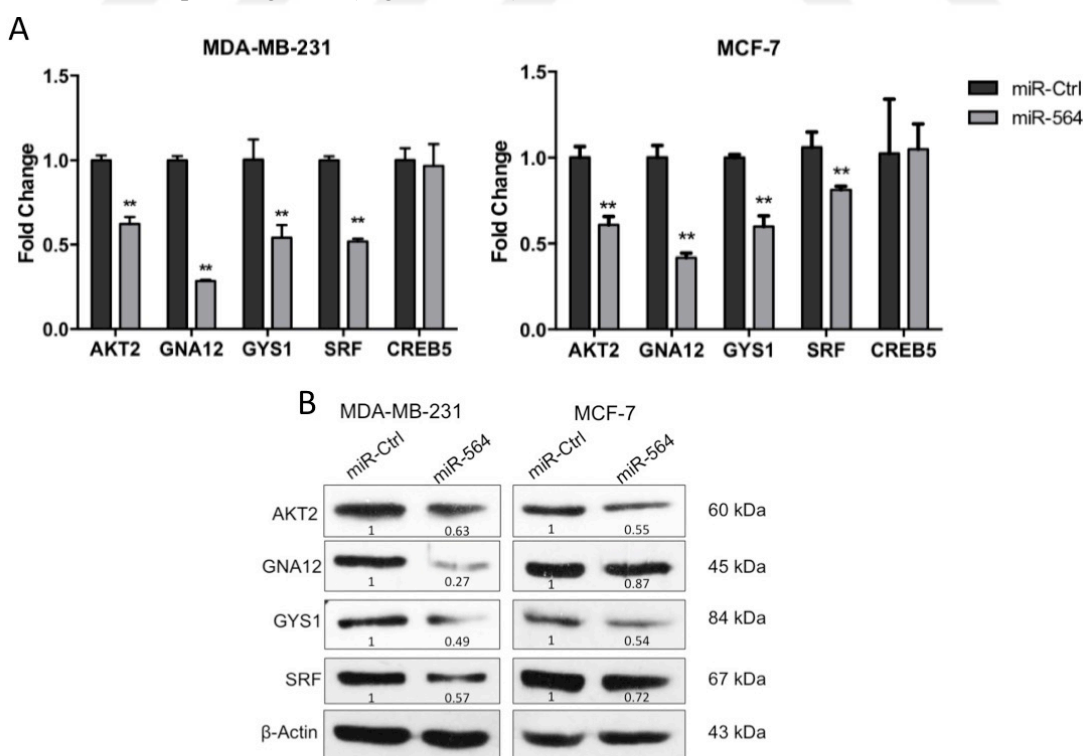


Figure 3.13. AKT2, GNA12, GYS1 and SRF expressions were downregulated at mRNA and protein levels in the presence of miR-564 overexpression. **A.** mRNA levels of 5 predicted target

genes in miR-564 overexpressing MDA-MB-231 and MCF-7 cells. **B.** Protein levels of four predicted targets of miR-564 in miR-564 overexpressing MDA-MB-231 and MCF-7 cells.

It has been known that miRNAs can simultaneously regulate the expression of more than one mRNA in a network manner[43]. Therefore, we have hypothesized that miR-564 may be functioning as a tumor suppressor by targeting the four genes that we have identified. In order to test this hypothesis, we silenced the expression of the four targets simultaneously by utilizing a cocktail of siRNAs (referred as siCocktail) against the four genes and tried to recapitulate the effects of miR-564 overexpression. 4 individual siRNA for each miR-564 target were ordered from Dharmacon and used as a pool for further experimental procedures. Contribution of each pool siRNA was 10nM with the final concentration of 40nM. Silencing of AKT2, GNA12, GYS1 and SRF by the siCocktail was confirmed in both mRNA and protein levels (Figure 3.14A and B).

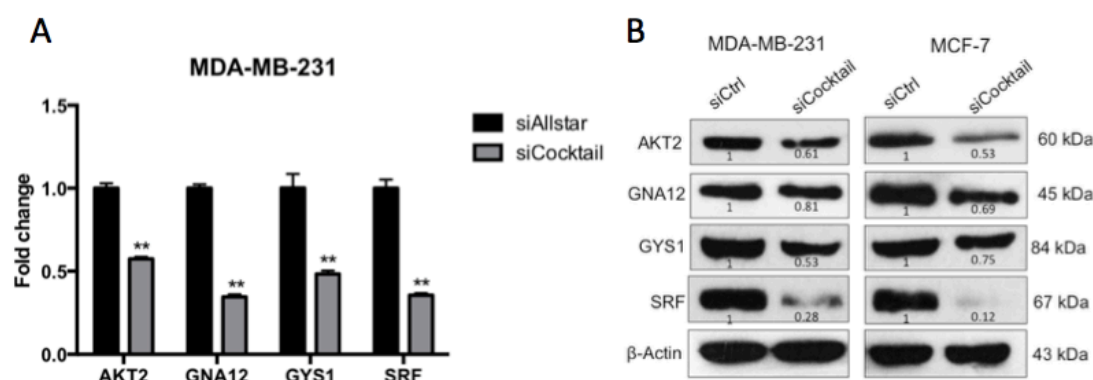


Figure 3.14. siCocktail downregulates AKT2, GNA12, GYS1 and SRF both at RNA and protein levels. **A.** Expression levels of miR-564 targets in siCocktail transfected MDA-MB-231 cell line. **B.** Protein levels of miR-564 targets in siCocktail transfected MDA-MB-231 cell line.

After confirmation of knockdowns of miR-564 targets, dual inhibitory function of miR-564 on PI3K and MAPK pathways was tested using siCocktail. Similar to the overexpression of miR-564, phosphorylation of both AKT and ERK1/2 decreased in the presence of siCocktail treatment in MDA-MB-231 and MCF-7 cells, whereas total AKT and ERK1/2 protein levels remained unchanged (Figure 3.15).

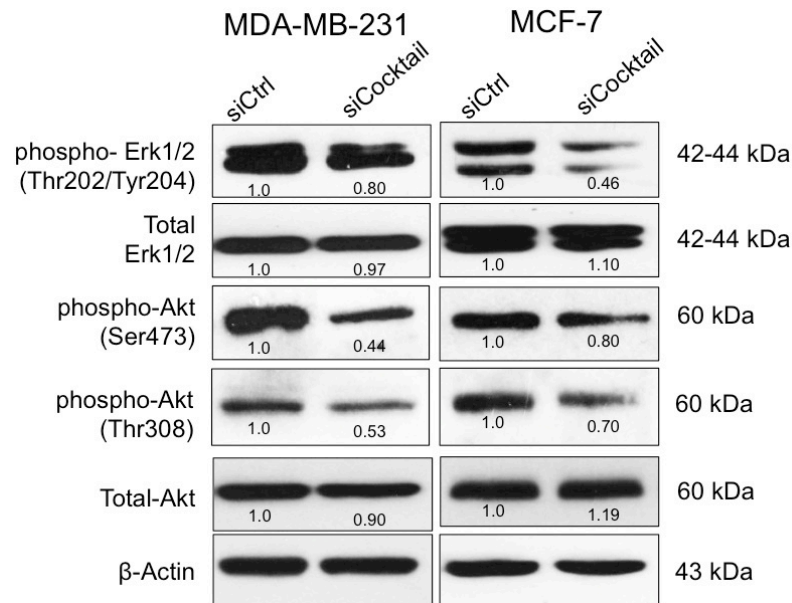


Figure 3.15. Combinatorial knockdown of miR-564 targets mimics the dual inhibitory effect miR-564 on PI3K and MAPK pathways. Western blots showing expression and phosphorylation of key proteins of PI3K and MAPK pathways in the presence of siCocktail in MDA-MB-231 and MCF-7 cells.

Validation of direct targeting of AKT2, GNA12, GYS1 and SRF was shown by dual luciferase reporter assay and validated by miRNA inhibitor overexpression as a complementary approach. For dual luciferase reporter assay, expected miR-564 seed sequence-containing and mutated miR-564 seed sequence containing 3'UTRs of each target mRNA were constructed in psiCHECK-2 vector as described in Methodology. After co-transfection of 3'UTR vectors together with miR-564 mimic, *Renilla* and *firefly* luciferase activity was measured. In the presence of miR-564 overexpression coexpressed with wild type seed sequence containing 3'UTR constructs, normalized *Renilla* luciferase activity was significantly reduced in both MDA-MB-231 and MCF-7 cells (Figure 3.16A) whereas cells transfected with miR-564 together with mutated seed sequence containing 3'UTR constructs showed no difference in luciferase activity. On the other hand with miR-564 inhibitor co-transfection, *Renilla* luciferase activity was significantly increased compared to hairpin inhibitor control in MCF-7 cells (Figure 3.16B).

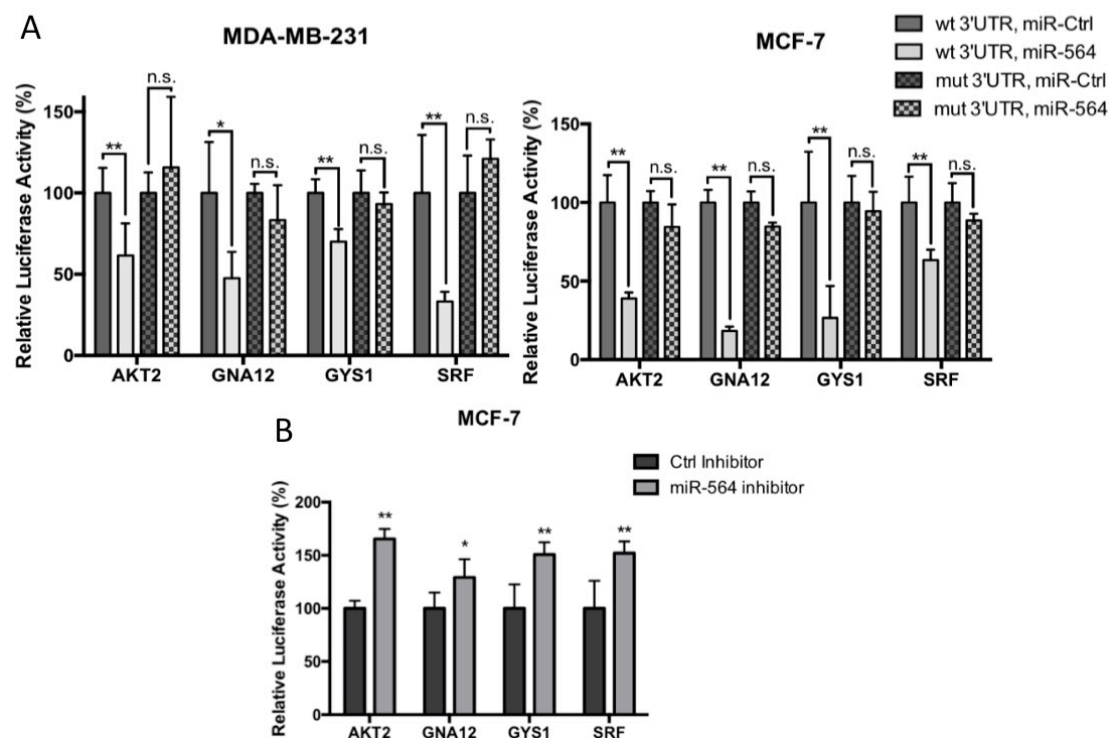


Figure 3.16. AKT2, GNA12, GYS1 and SRF are direct targets of miR-564. **A.** 3'-UTR luciferase reporter assay with dual transfection of miR-564 and psiCHECK2 vector harboring either wild type or mutated 3'-UTR sequence of each target gene in MDA-MB-231 and MCF-7 cells. **B.** 3'-UTR luciferase reporter assay with dual transfection of miR-564 inhibitor and psiCHECK2 vector harboring 3'-UTR sequence of each target gene in MDA-MB-231 and MCF-7 cells. The ratio of *renilla*/ *firefly* luciferase in each condition was taken as relative luciferase activity.

In conclusion, these data indicate that AKT2, GNA12, GYS1 and SRF are the direct targets of miR-564, and dual inhibitory function of miR-564 on PI3K and MAPK pathways is *via*, at least in part, targeting these genes as a network.

3.4. Combinatorial silencing of AKT2, GNA12, GYS1 and SRF mimics the effects of miR-564 overexpression on cell proliferation, migration and invasion in breast cancer cells

Having identified AKT2, GNA12, GYS1 and SRF as the direct targets of miR-564, we aimed to see if these four genes are the major targets of miR-564 mediating the dual inhibition of PI3K and MAPK pathways. To validate the regulation of different cellular processes such as viability, migration and invasion (mentioned in sections 5.1 and 5.2), all functional experiments were repeated using combinatorial targeting siCocktail approach. Similar to miR-564 overexpression, 20 to 25% proliferation inhibition was observed with end-point viability assays after siCocktail treatment in MDA-MB-231 and MCF-7 cells (Figure 3.17A). RTCA results showed an inhibition of proliferation in on siCocktail treated MDA-MB-231

and MCF-7 cells (Figure 3.17B). Furthermore, upon siCocktail transfection, combinatorial inhibition of miR-564 target genes led to increase in G1 population and a decrease in S phase population in MDA-MB-231 cells similar to the effect seen with miR-564 overexpression. G1 arrest of siCocktail treated MDA-MB-231 cells was mediated by CDK4 downregulation and p27 upregulation together with downregulation in Rb phosphorylation (Figure 3.18).

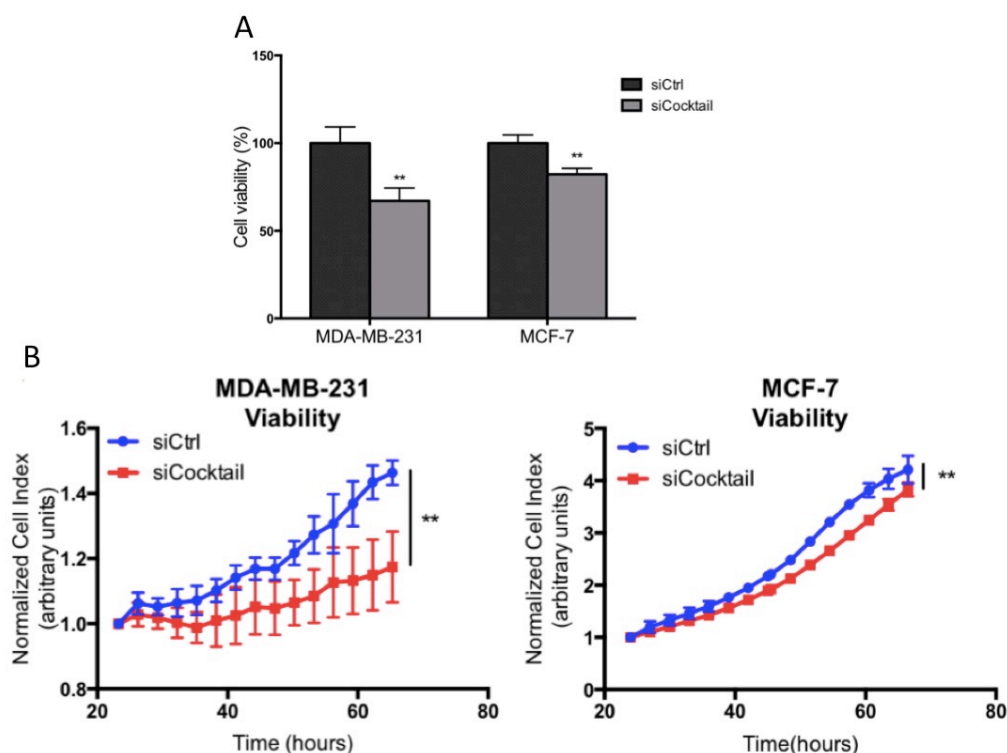


Figure 3.17. Combinatorial knockdown of miR-564 targets mimics the anti-cell proliferative effects of miR-564. **A.** End point cell proliferation assay performed on MDA-MB-231 and MCF-7 cells transiently transfected with siCocktail or siAllstar. **B.** Real-time cell proliferation analysis of MDA-MB-231 and MCF-7 cells transiently transfected with siCocktail or siAllstar.

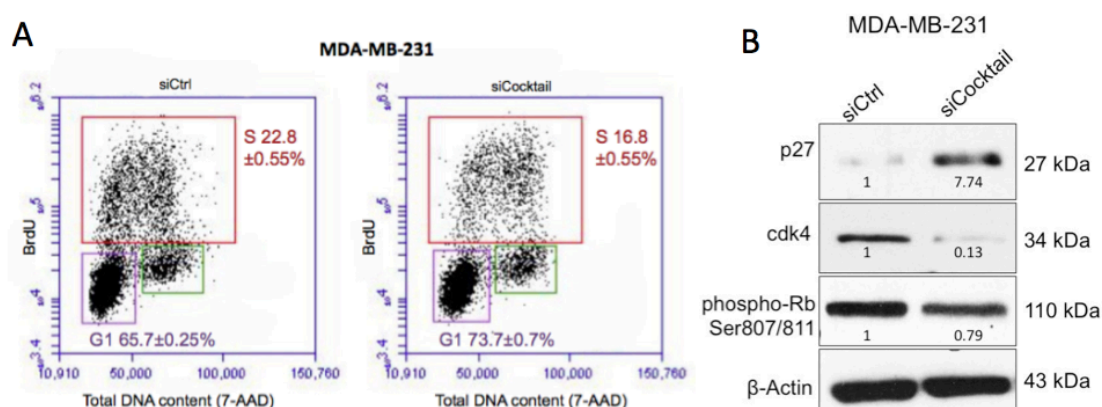


Figure 3.18. Combinatorial knockdown of miR-564 targets results in G1 arrest in MDA-MB-231 cells. **A.** BrdU/7-AAD staining of MDA-MB-231 cells transiently transfected with siAllstar or

siCocktail. **B.** Western blot analyses of G1/S transition proteins in MDA-MB-231 cells transiently transfected with siAllstar or siCocktail.

To validate that the inhibitory effect of miR-564 on migration and invasion *via* blocking EMT is mediated through targeting AKT2, GNA12, GYS1 and SRF; siCocktail transfected MDA-MB-231 cells were stained with DAPI/Phalloidin. Similar to miR-564 overexpression, MDA-MB-231 cells lost their mesenchymal morphology upon combinatorial target knockdown (Figure 3.19A). Interestingly, combinatorial knockdown of miR-564 targets by siCocktail transfection reduced the migratory and invasive characteristics of MDA-MB-231 cells in RTCA (Figure 3.19B and C).

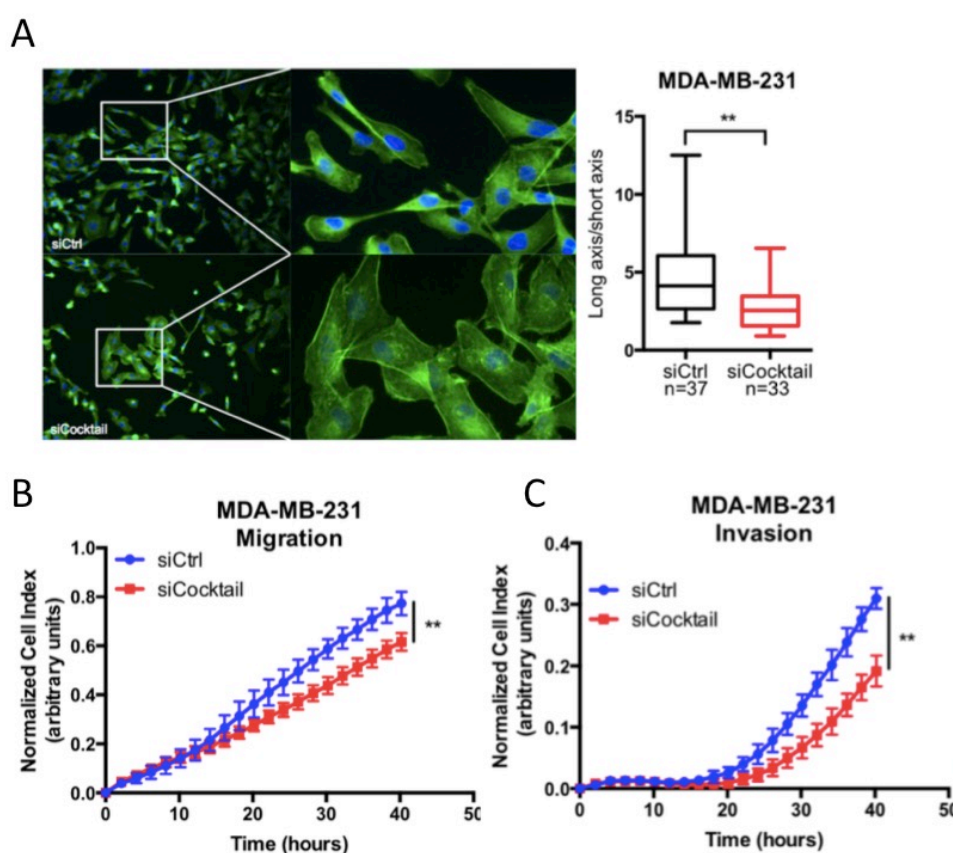


Figure 3.19. Combinatorial knockdown of miR-564 targets inhibit migration and invasion *via* blocking EMT. **A.** DAPI/Phalloidin staining of siCocktail transfected MDA-MB-231 cells. Images were taken 24 hour after transfection. Morphological changes were quantified as ratio of long axis to short axis for each cell. **B.** Real-time cell migration analysis of MDA-MB-231 cells transiently transfected with siAllstar or siCocktail. **C.** Real-time cell invasion analysis of MDA-MB-231 cells transiently transfected with siAllstar or siCocktail.

Overall, these findings indicated that simultaneous targeting of AKT2, GNA12, GYS1 and SRF mimics, at least in part, the inhibitory effect of miR-564 on cell proliferation as well as on migration and invasion.

Till this point, it has been shown that miR-564 inhibits cell proliferation, migration and invasion mainly through targeting AKT2, GNA12, GYS1 and SRF genes, and combinatorial knockdown of these genes mimicked, at least in part, miR-564 overexpression in suppressing cell proliferation, migration and invasion of breast cancer cells. To further validate these findings, publicly available cell line as well as breast cancer patient gene expression datasets have been analyzed. 58 different cancer cell lines from NCI60 panel were analyzed to further validate the combinatorial effect of miR-564 target genes. Analysis of each gene in cell lines classified in NCI60 panel as "less invasive", "unidentified" and "invasive" showed that not all target genes expressions were significantly upregulated in invasive cell lines (Figure 3.20). Later, cell lines were assigned a z-score depending upon expression of individual miR-564 target, and combinatorial expression of miR-564 targets was calculated by summing up assigned z-scores for each cell line (details are in Methodology section). This analysis revealed that invasive cell lines have higher combinatorial expression of miR-564 targets as compared to less-invasive ones (Figure 3.20). Furthermore, previously analyzed GSE40059 dataset was re-analyzed, and combinatorial expression of miR-564 targets was calculated as above mentioned for NCI60 panel (details are in Methodology section). This analysis again showed that, not alone, but combinatorial expression of miR-564 targets is significantly higher in invasive cell line as compared to less invasive ones and is exactly inverse of miR-564 expression as expected (Figure 3.21).

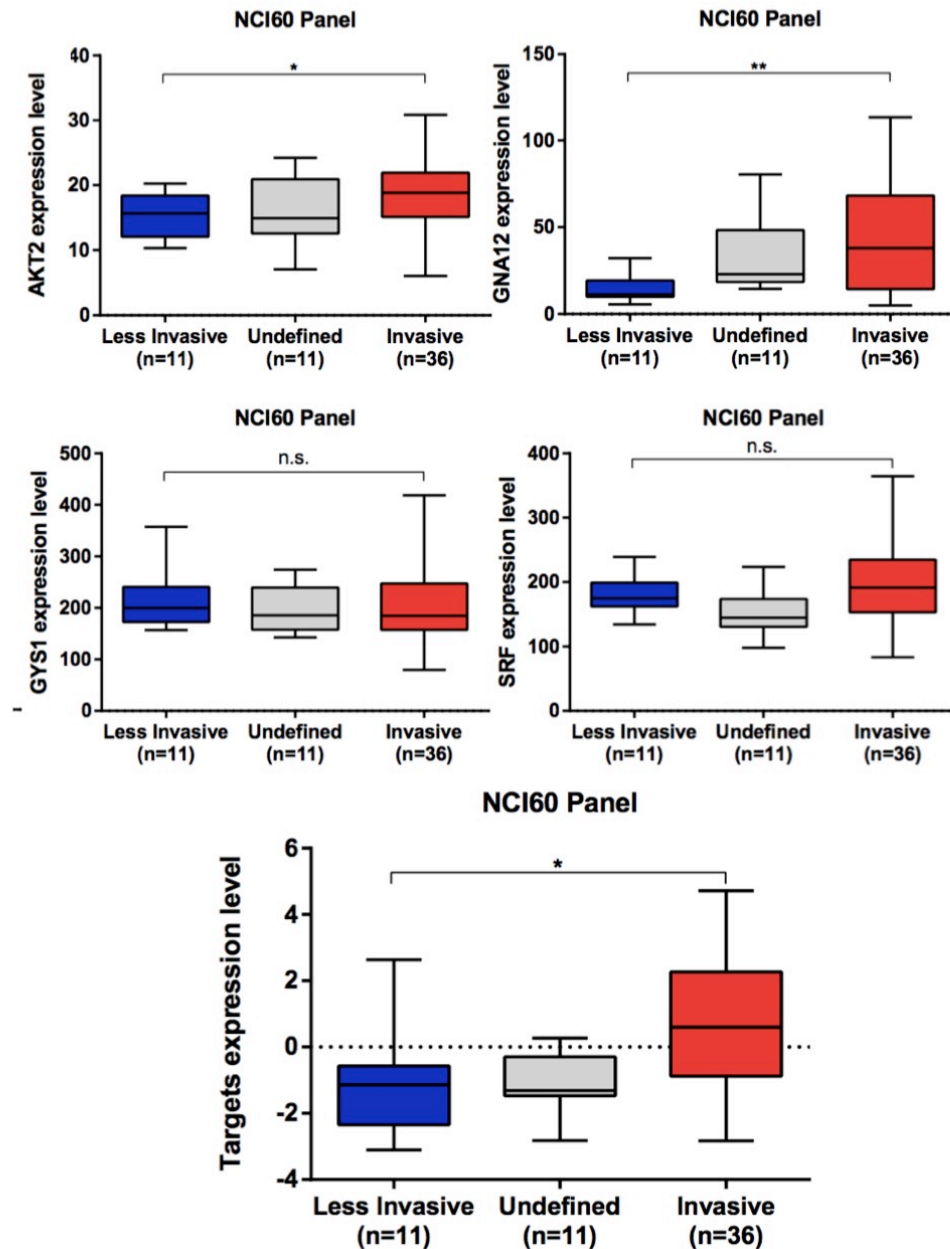


Figure 3.20. Expression of miR-564 targets in combination is inversely correlated with cell line invasiveness (NCI60 panel). Expression of each target gene was shown regarding invasiveness of cell lines. Targets expression levels were calculated as the sum of target z-scores.

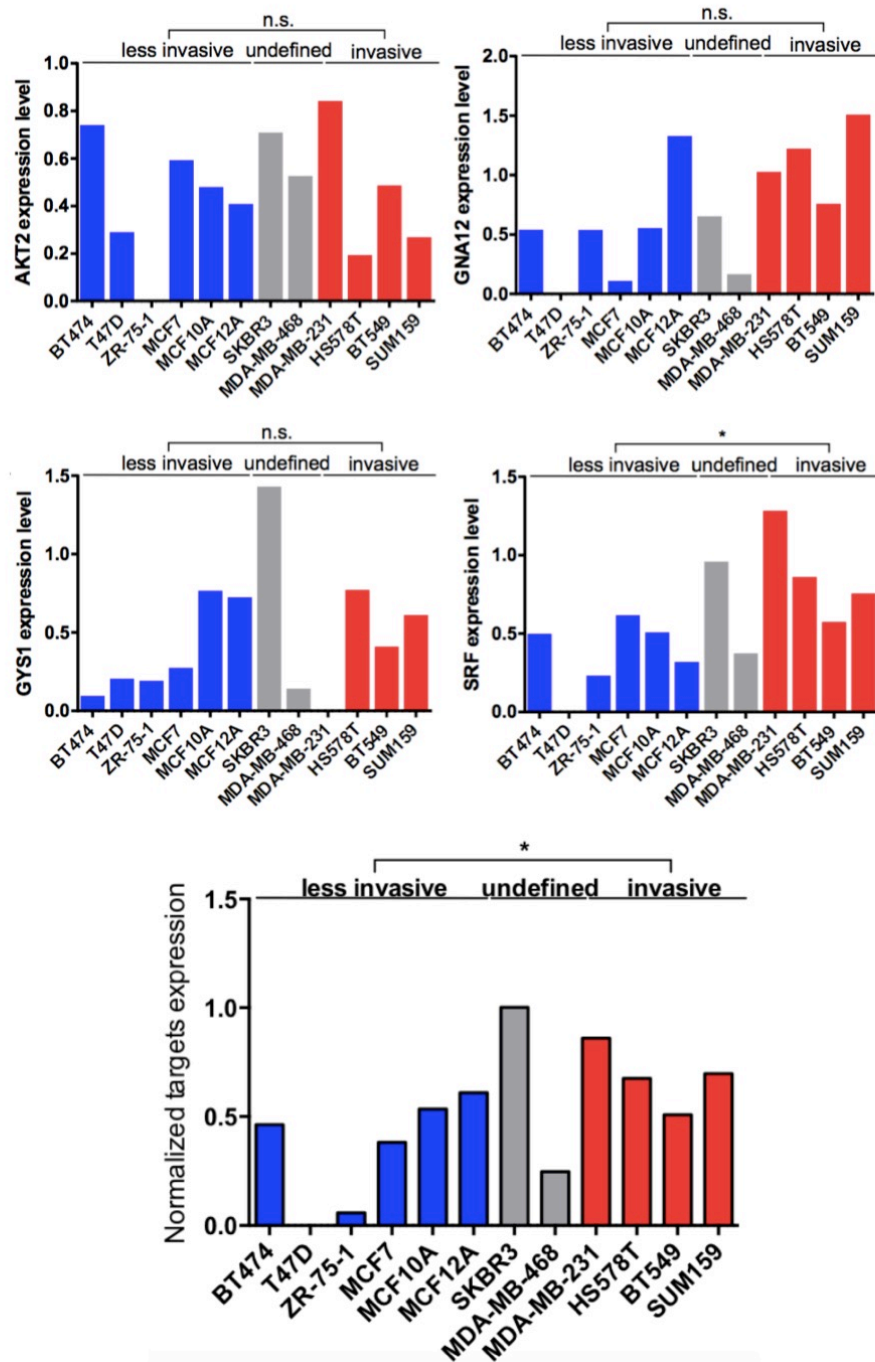


Figure 3.21. Expression of miR-564 targets in combination is inversely correlated with invasiveness of breast cancer cell lines (GSE40059 dataset). Expression of each target gene was shown regarding invasiveness of cell lines. Targets expression levels were calculated as the sum of target z-scores.

3.5. miR-564 expression is associated with tumor progression and breast cancer patient survival

Until now, it has been shown that miR-564 inhibits PI3K and MAPK pathways and results in blockage of cell proliferation *via* G1 arrest. miR-564 also inhibits migration and invasion *via* blocking EMT in breast cancer cells. AKT2, GNA12, GYS1 and SRF were validated as the direct targets of miR-564 and as main players in suppressing cell proliferation, migration and invasion at downstream of miR-564. In order to show the effect of miR-564 and its targets on tumor progression and survival, breast cancer patient datasets from GEO database have been analyzed. In GSE40525 dataset containing miRNA expression profile of 56 breast cancer tissue samples and their matched adjacent peritumor tissue, miR-564 expression was shown to be higher in peritumor breast tissue compared to breast primary tumors (Figure 3.22A). In addition to breast cancer, several other expression datasets containing patients from different cancer types were also analyzed. Cancerous tissues express lower levels of miR-564 compared to non-cancerous tissues in various cancer types including acute lymphoblastic leukemia (ALL) (GSE31376), osteosarcoma (GSE28425), bladder (GSE40355, GSE14985), ovary, prostate, colon and lung cancer (GSE14985) (Figure 3.23A-D).

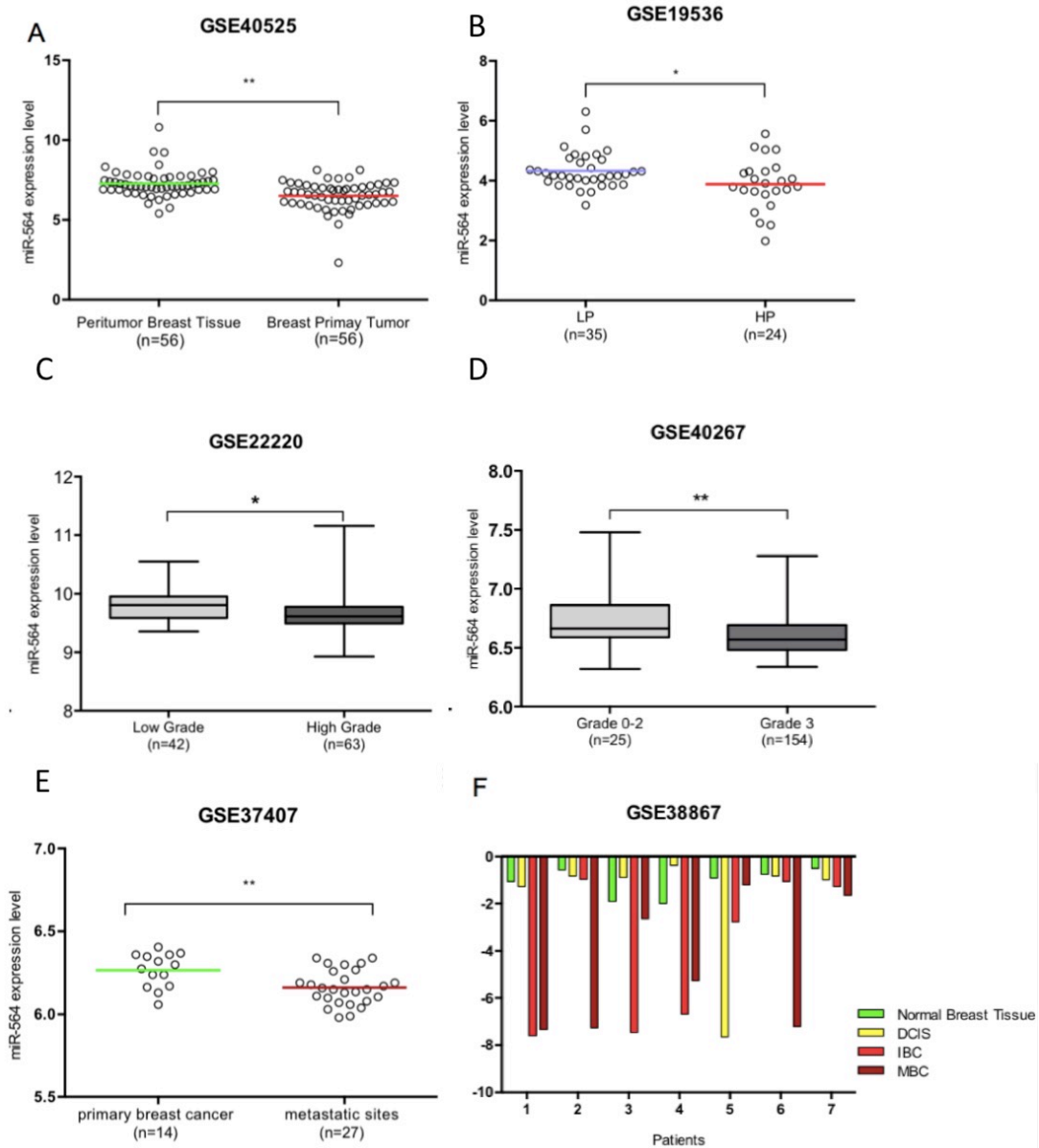


Figure 3. 22. miR-564 suppresses tumor formation, progression and metastasis in breast cancer patients. **A.** Expression analysis of miR-564 in 56 matched breast cancer patient samples from GSE40525 dataset. **B.** Expression of miR-564 in breast cancer patients with low proliferative (LP) and high proliferative (HP) tumors in GSE19536 dataset. **C.** Expression of miR-564 grouped according to tumor grades in 105 breast cancer patients from GSE19536 dataset. **D.** Expression of miR-564 grouped according to tumor grades in 179 breast cancer patients from GSE40267 dataset. **E.** miR-564 expression levels in patients from GSE37407 dataset containing 14 primary and 27 metastatic tumors expression profiles. **F.** Expression levels of miR-564 in 7 patients from GSE38867 dataset, showing different stages of breast cancer tumor progression.

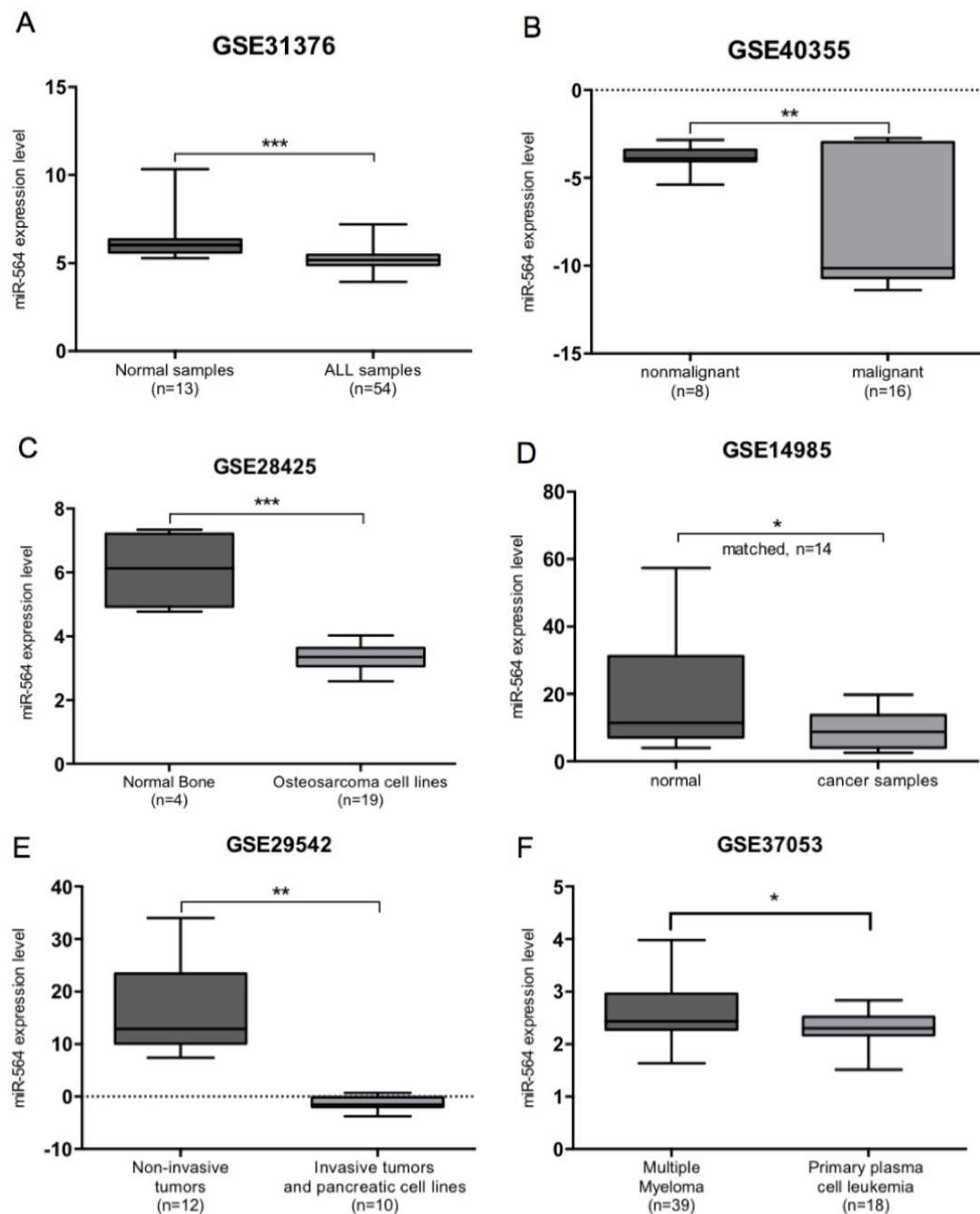


Figure 3.23. miR-564 suppresses tumor formation, progression and metastasis in several cancer types. **A.** Expression levels of miR-564 in 13 normal and 54 ALL tumor samples from GSE31376 dataset. **B.** Expression levels of miR-564 in GSE40355 dataset containing non-malignant and malignant bladder cancer patient samples. **C.** Expression analysis of miR-564 osteosarcoma cell lines compared to normal bone cell lines from GSE28425 dataset. **D.** miR-564 expression analysis of 14 normal-tumor matched patient samples of lung, breast, prostate, colon, ovary cancer and lymphoma from GSE14985 dataset. **E.** miR-564 expression levels of bladder cancer samples as well as bladder cancer cell lines, classified as non-invasive and invasive cancer (GSE29542). **F.** Expression levels of

miR-564 in GSE37053 dataset containing multiple myeloma and primary plasma cell leukemia patient samples.

In GSE19536 dataset, containing miRNA expression profiles of 59 primary breast cancer tissues, miR-564 expressions were significantly higher in low proliferative tumors (LP) compared to high proliferative tumors (HP) (Figure 3.22B) confirming a negative correlation between miR-564 expression and tumor growth. To further support these findings with other patient datasets, analysis of GSE22220 (n=105) and GSE40267[58-60] (n=179) breast cancer patient datasets have been performed, and miR-564 expression levels were found significantly higher in low-grade tumors (Grade 0-2) compared to high-grade tumors (Grade 3) (Figure 3.22C).

In GSE37407 dataset containing miRNA expression profiles of primary breast cancer tumor samples together with samples from metastatic sites, higher expression levels of miR-564 were observed in primary breast cancer tissues compared to metastatic sites (Figure 3.22D). To support these findings another dataset, GSE38867, containing expression profiles from four different stages of breast cancer from 7 patients has been analyzed. In each patient expression levels of miR-564 gradually decreased as breast cancer progresses to advanced stage (Figure 3.22E).

Furthermore, not only breast cancer but also in pancreatic cancer and multiple myeloma, aggressive forms showed lower miR-564 expression levels compared to less aggressive types (Figure 3.23E and F).

To search the effect of miR-564 expression on prognosis and patient survival, GSE22220 dataset has been re-analyzed. Patients were separated into 2 groups from median of miR-564 expression and named as "low miR-564" and "high miR-564". Patients with high miR-564 expression levels have significantly better distant relapse-free survival (DRFS) than patients with low miR-564 expressions (Figure 3.24).

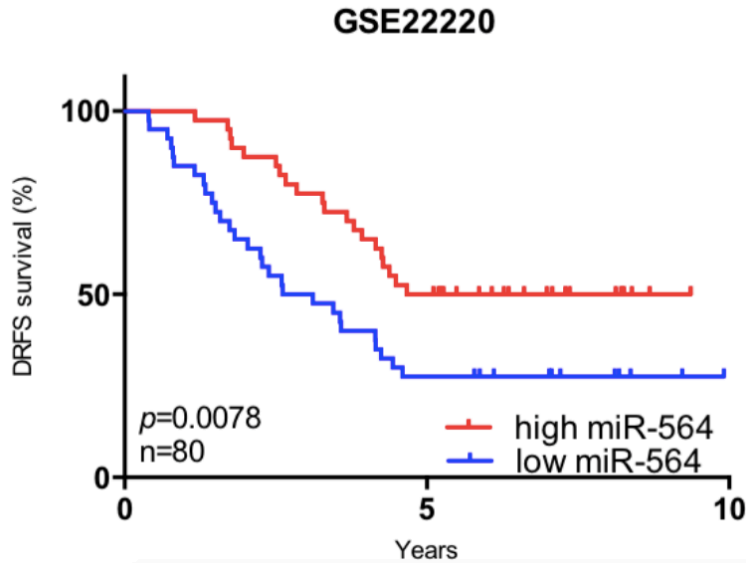


Figure 3.24. High miR-564 levels predict better distant-relapse free survival in breast cancer patients. Patients were separated from median of miR-564 expression with 5-year cut-off on survival.

Overall, these data indicate that miR-564 act as a potential biomarker on tumor progression and invasiveness in breast cancer tissues.

3.6. Combinational analysis of AKT2, GNA12, GYS1 and SRF expression mimics the effect of miR-564 on tumor progression and patient survival

Functional roles of both miR-564 and combinatorial target gene expression on PI3K and MAPK pathways as well as G1/S transition have been validated *in vitro*. To further support their association, RPPA data from The Cancer Genome Atlas (TCGA)[61] has been analyzed regarding expression/phosphorylation of PI3K pathway, MAPK pathway and cell cycle proteins. In correlation with our own RPPA data showing a negative regulation of Akt1 and Akt2 in the presence of miR-564 overexpression, positive correlation between combination of miR-564 targets expressions and both Thr308 and Ser473 phosphorylation residues of Akt was observed in TCGA data (Figure 3.25A). Furthermore, a positive correlation has been found between the combinatorial expression of miR-564 targets and the Thr246 phosphorylation of PRAS40, an mTORC1 complex element (Figure 3.25B). Phosphorylation of PRAS40 by Akt from Thr246 residue activates mTORC1 complex by dissociation of PRAS40 from the complex[62].

From our RPPA data analysis, it has been shown that miR-564 overexpression in MDA-MB-231 cells showed decrease p27 protein levels. Supporting these findings analysis performed on RPPA data of TCGA database showed that combinatorial expression of miR-564 targets

expression was negatively correlated with p27 protein and also positively correlated with T157 and T198 phosphorylations of p27 protein (Figure 3.25C & D). Both T157 and T198 phosphorylations of p27 are mediated by Akt, and it has been linked to cell cycle progression by preventing p27 nuclear localization[63]. Overall, these data indicates that miR-564 targets could be significant regulators of PI3K pathway and G1/S transition in breast cancer patients.

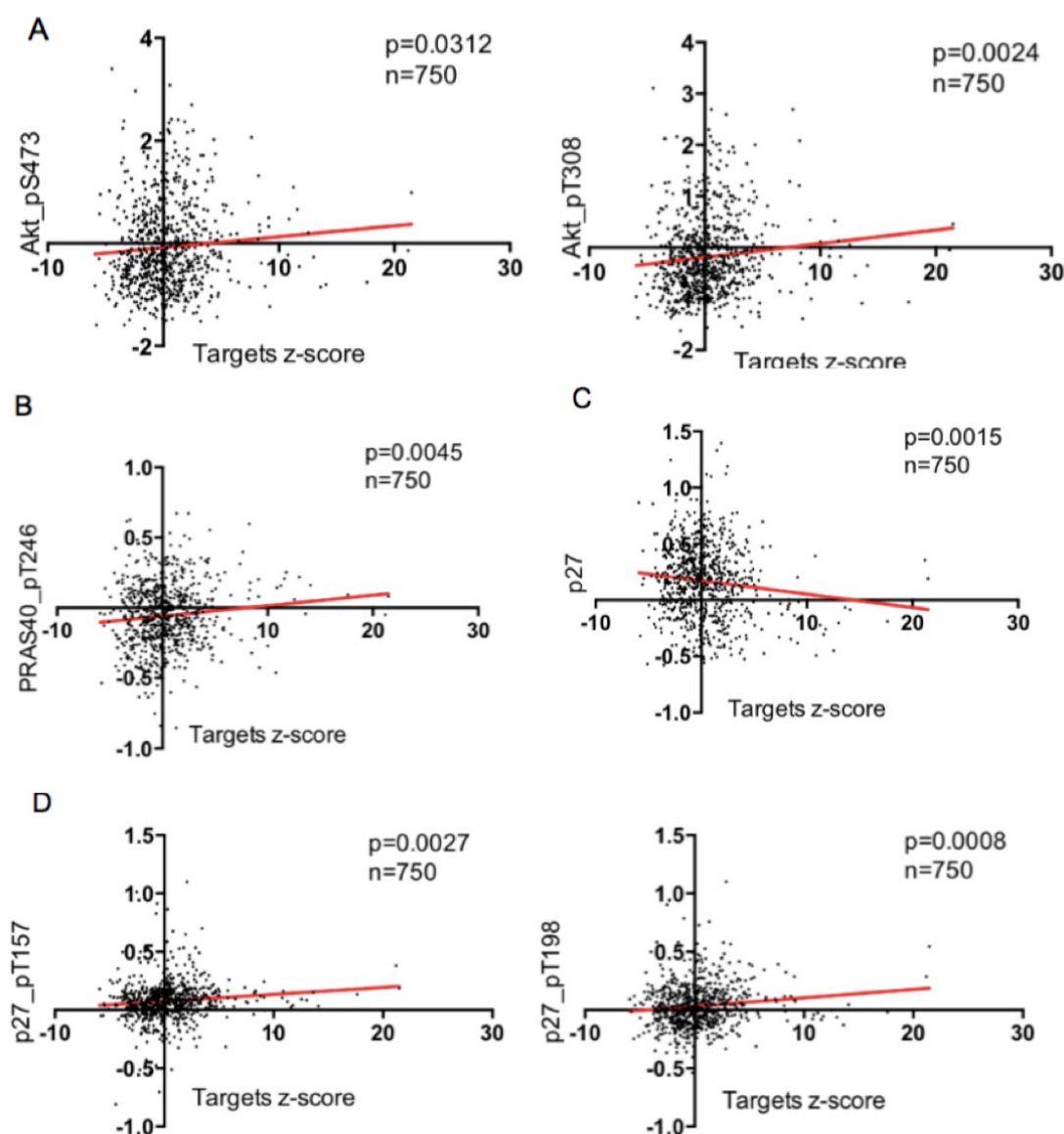


Figure 3.25. miR-564 target expression score is significantly correlated with PI3K and cell cycle pathway. **A.** Correlation of miR-564 targets z-score with Akt phosphorylations taken from TCGA RPPA data. **B.** Correlation of miR-564 targets z-score with PRAS40 T246 site phosphorylation. **C.** Correlation of miR-564 targets z-score with p27 protein levels in patient from TCGA database. **D.** Correlation of miR-564 targets z-score with p27 phosphorylations.

We have previously shown that high expression of miR-564 predicts better DRFS survival in breast cancer patients. To test if also the targets of miR-564 can predict survival,

combinatorial target expression was analyzed in two independent breast cancer patient microarray datasets: GSE3494 (n=234) and GSE22220 (n=216). Survival analysis for individual miR-564 targets as well as combinatorial expression was done. As a result it has been shown that combined expression, rather than individual target expressions, predicts the survival of breast cancer patients with higher significance. Patients with low expression of miR-564 target genes showed better survival as compared to the patients with high miR-564 target genes expression (Figure 3.26 and Figure 3.27). In conclusion, combinatorial expression analysis of AKT2, GNA12, GYS1 and SRF, rather than individual genes mimics the effects of miR-564 on cancer progression and survival of breast cancer patients.

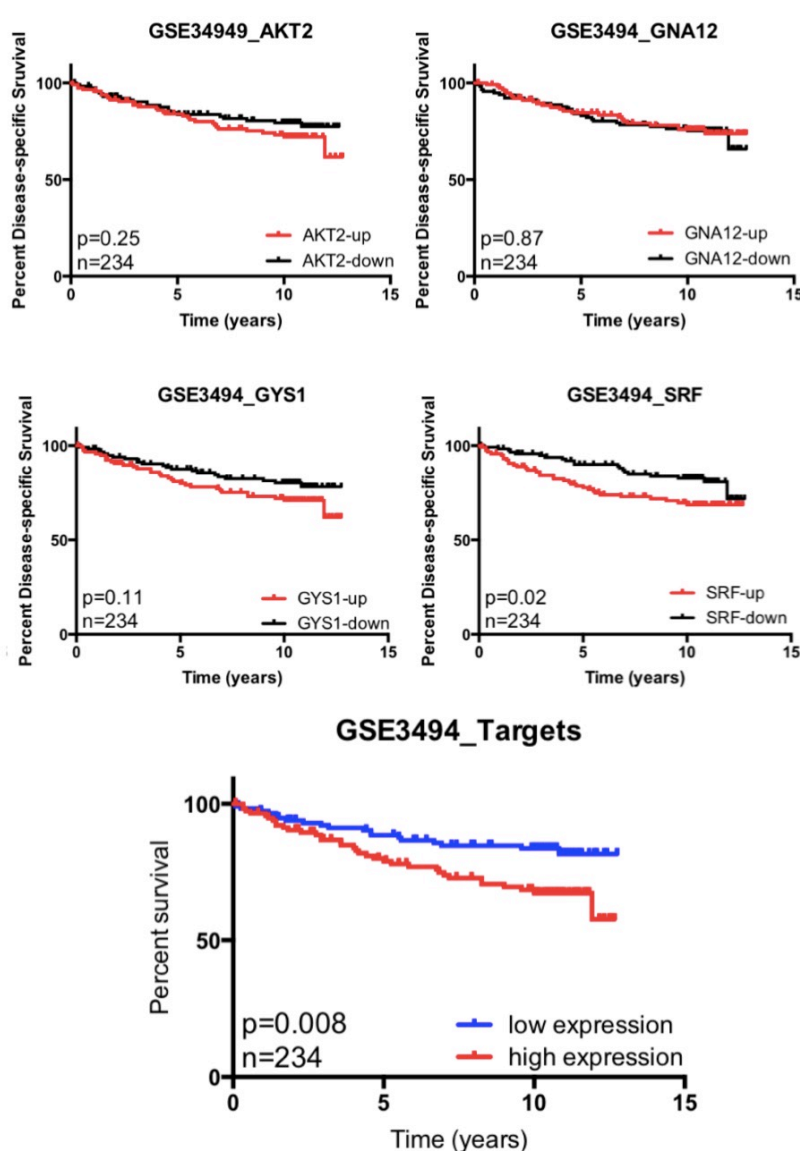


Figure 3.26. Combinational target expression analysis predicts better survival in breast cancer patients (GSE3494). Expression of target genes was both individually and in combination and patients were separated from median of expression.

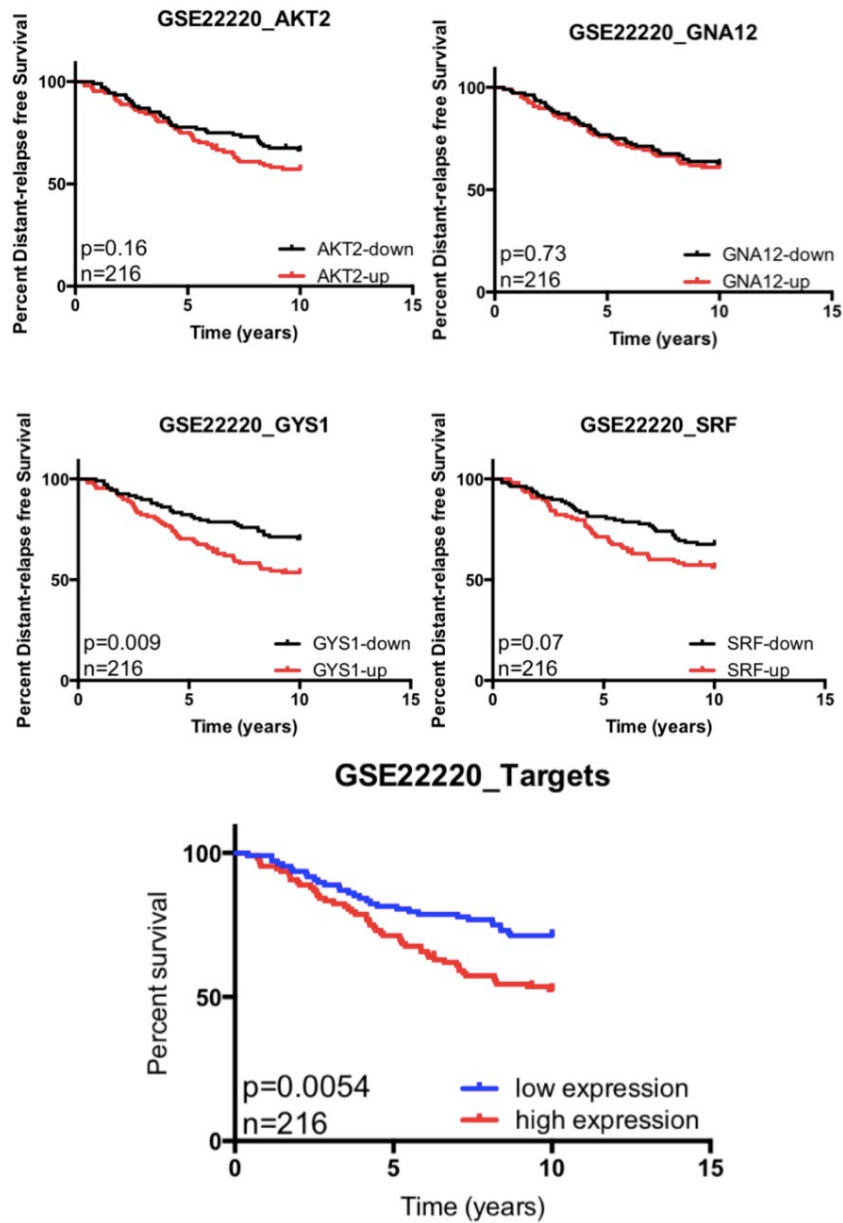


Figure 3.27. Combinational target expression analysis predicts better survival in breast cancer patients (GSE22220). Expression of target genes was both individually and in combination and patients were separated from median of expression.

Chapter 4

Discussion

In this thesis, inhibitory effect of miR-564 on PI3K and MAPK pathways has been shown for the first time in breast cancer. It has been shown that by direct targeting AKT2, GNA12, GYS1 and SRF in combination, miR-564 works as a dual inhibitor and subsequently inhibits cell proliferation, migration and invasion. Analysis of several GEO datasets showed that low miR-564 and consequently high expression of its targets is associated with tumor progression, invasiveness as well as distant relapse-free survival of breast cancer patients. Data represented in this thesis validates a distinct novel dual inhibitor function of miR-564 and presents it as a biomarker for breast cancer.

4.1 Background information on miR-564

Together with six other miRNAs, miR-564 was first shown to be a potential biomarker in schizophrenia patients. It has been shown that miR-564 levels in blood were significantly higher in 30 schizophrenia patients compared to control group[64]. One year later, another publication showed that miR-564 expression levels, together with miR-31 and miR-155, were significantly low in chronic myeloid leukemia (CML) patients compared to non-CML patients. This report using miRNA profiling from both CML cell line K562 together with CML patients samples, was the first data showing the role of miR-564 in cancer biology. In the same publication, it has been also indicated that miR-564 may have a role on MAPK, ErbB, mTOR and VEGF signaling pathways[65].

In 2015, Yang et al. performed the first molecular functioning of miR-564, and identified it as a tumor suppressor miRNA. They showed that miR-564 directly targets and inhibits Zic family member 3 (ZIC3) protein in lung cancer cells and inhibits cell proliferation and motility[66]. Nonetheless, the role of miR-564 in breast cancer tumorigenesis and the mechanisms underneath has not been reported until now. Here, we show that miR-564 works as a dual inhibitor for PI3K and MAPK pathways in breast cancer *via* targeting network of genes.

4.2 Importance of identifying a novel regulator of PI3K and MAPK pathways

PI3K and MAPK pathways were shown to be the main regulators of cell proliferation, invasion and survival in several cancer types including breast. Over time, different kinase inhibitors targeting key regulators of PI3K and MAPK pathways were identified and used for efficient blocking of these pathways. Although the initial results were promising, most of these inhibitors led to high toxicity or resistance due to recovery either by the same pathway

or an alternative pro-survival pathway. Moreover, the intermingled structure containing positive and negative feedback loops between PI3K and MAPK pathways has been validated and their function of compensation one another results limited success with kinase inhibitors [23, 67, 68]. Several miRNAs have been shown to target PI3K or MAPK pathways through targeting different regulators of these pathways [69-75], however many few studies identified dual regulators. Recently, PI3K and MAPK pathway inhibition by miR-148a/152 was reported by direct targeting of IGF-IR and IRS1, which are at the upstream of these cascades [76]. Therefore, our study is one of the first studies that identified a dual inhibitor miRNA of PI3K and MAPK pathways.

4.3 AKT2, GNA12, GYS1 and SRF as miR-564 target genes

In this thesis; AKT2, GNA12, GYS1 and SRF as novel targets of miR-564 has been presented and proposed to act in combination to inhibit PI3K and MAPK pathways. AKT2, a serine-threonine kinase that contains SH2-like domain, is a crucial oncogene intensively studied in several cancers including breast cancer [77, 78]. Guanine nucleotide-binding protein subunit, GNA12, was first identified in cardiac physiology [79] and maintenance of blood pressure in smooth muscle [80]. It has been shown that GNA12 is controlling both upstream and downstream of MAPK pathway. GNA12 is inhibiting Ras protein *via* Rasa2, and MEK *via* RhoGEF (Rho guanine nucleotide exchange factor)/Rho/Rac protein chain (reviewed in [81]). On the other hand, GNA12 induces the expression of serum response factor (SRF) and inhibiting actomyosin assembly at the same time *via* inducing the expression of Rho associated kinase (ROCK), which is inducing actin polymerization. Cytoplasmic concentration of unpolymerized actin subunits, which are called G-actins, regulates both myocardin and myocardin related transcription factors (MRTFs). MRTFs control SRF activity as a transcription factor [82]. By upregulating IL-6 and IL-8, GNA12 has also been shown as the mediator of invasion in MDA-MB-231 breast cancer cell line [83]. Glycogen synthase 1 (GYS1) functions in glycogen metabolism and it is shown to be overexpressed in muscle tissues. As glycogen metabolism located downstream of PI3K pathway, GYS1 was shown to be phosphorylated and activated by AKT2. As a negative feedback loop AKT2 was shown to inhibit glycogen synthase kinase 3B (GSK-3B) which in turn phosphorylates and inactivates GYS1 [84, 85]. Serum response factor (SRF) is a well-known transcription factor in cancerous tissues at the downstream of MAPK pathway, regulating c-fos and cell cycle progression [86, 87]. All four target-genes of miR-564 were shown to be linked to each other and functioning in cell proliferation, migration and invasion in cancer cells. In this regard, tumor suppressive effect of miR-564 was further supported by the validation of direct targeting and inhibiting these four key genes together.

PI3K pathway induces EMT and invasion in several cancer types, including breast cancer, *via* different effectors. Suppression of PTEN through Bmi1 and EZH2 induces EMT and further activates PI3K pathway [88-90]. Activated PI3K cascade can further induce EMT *via* Snail and Slug activation[91] as well as Rho/Rac signaling through mTOR[92]. On the other hand, MAPK pathway activation *via* BRAF mutation has been shown to induce migration and invasion. In BRAF mutant colon cancers, activation of RhoA as a RAS effector protein was shown to promote EMT phenotype [93]. In this thesis, it is been shown that miR-564 overexpression in breast cancer cells reduces migration and invasion *via* blockage of EMT. Not only *in vitro* data but also *in silico* analysis supported that miR-564 can be a metastatic suppressor. Importantly, patients with high miR-564 expressions showed better distant relapse-free patient survival compared to patients with low miR-564. In line with miR-564 expression levels, combinatorial analysis of miR-564 targets expressions predicted better survival in breast cancer patients further supporting, at least in part, the functional roles of both miR-564 and its targets in clinico-pathological conditions. Importantly, combinatorial analysis of miR-564 target genes predicted patient outcome better than individual analysis, further supporting the effect miRNA-gene network on tumor prognosis and patient survival. Not only in breast cancer cell lines, but also in patient tumor-normal tissue matched dataset it has been validated that, low levels of miR-564 and high levels of its target genes were associated in tumor samples compared to non-tumor tissue. Overall, these findings indicate miR-564 as a potential tumor suppressor miRNA.

Chapter 5

Future Perspectives

In this study, miR-564 has been reported as a potential tumor suppressor miRNA, regulating cellular growth and EMT *via* inhibiting PI3K and MAPK pathways in breast cancer. As a dual inhibitor of PI3K and MAPK pathways, miR-564/AKT2-GNA12-GYS1-SRF network has been established and its role in cell proliferation, migration and invasion has been validated.

Over the years, several kinase inhibitors have been developed to target either PI3K or MAPK pathway. However, due to the cross-talk and extensive feedback loop mechanisms between these two pathways, kinase inhibitors had limited success in clinics as a targeted therapy. This study brings a new insight to the field of targeted therapy in breast cancer. miR-564 works as a dual inhibitor of PI3K and MAPK pathways by targeting a downstream gene-network making it a critical target for cancer therapy. Further understanding of underlying mechanisms of miRNA-gene-network will help in improving and re-shaping future strategies for targeted therapy.

MiRNAs has been extensively studied as blood and tissue biomarkers and as drugable targets in several cancer types due to their small size and good targeting efficiency. miR-34, one of the most deregulated and highly studied miRNA in gastric, colorectal, prostate cancer as well as in hepatocellular carcinoma (HCC), is a well-established biomarker and has been entered phase-I clinical trial (NCT01829971) for the treatment of HCC [94, 95].

In this study, it has been shown that miR-564 expression levels significantly correlated with lower grade tumors and better distant relapse-free survival of breast cancer patients, supporting its potential to be a prognostic biomarker.

With this study, AKT2, GNA12, GYS1 and SRF were identified as direct-targets of miR-564 and dual inhibitor function of miR-564 on PI3K and MAPK pathways, in part, regulated *via* combinatorial targeting of these genes. Therefore, miR-564 has high potential to become the next miRNA in clinical trials as PI3K-MAPK dual inhibitor.

Bibliography

- [1] Sorlie T.,et al., Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A*. 2003; 100(14):8418-8423.
- [2] Sorlie T.,et al., Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A*. 2001; 98(19):10869-10874.
- [3] de Ronde J, Wessels L and Wesseling J. Molecular subtyping of breast cancer: ready to use? *Lancet Oncol*. 2010; 11(4):306-307.
- [4] Hanahan D and Weinberg RA. The hallmarks of cancer. *Cell*. 2000; 100(1):57-70.
- [5] Hennessy BT.,et al., Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat Rev Drug Discov*. 2005; 4(12):988-1004.
- [6] Majumder PK.,et al., mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. *Nat Med*. 2004; 10(6):594-601.
- [7] Jimenez C.,et al., Identification and characterization of a new oncogene derived from the regulatory subunit of phosphoinositide 3-kinase. *EMBO J*. 1998; 17(3):743-753.
- [8] Chen R.,et al., Regulation of Akt/PKB activation by tyrosine phosphorylation. *J Biol Chem*. 2001; 276(34):31858-31862.
- [9] Krasilnikov MA. Phosphatidylinositol-3 kinase dependent pathways: the role in control of cell growth, survival, and malignant transformation. *Biochemistry (Mosc)*. 2000; 65(1):59-67.
- [10] Vazquez F and Sellers WR. The PTEN tumor suppressor protein: an antagonist of phosphoinositide 3-kinase signaling. *Biochim Biophys Acta*. 2000; 1470(1):M21-35.
- [11] Vlahos CJ.,et al., A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem*. 1994; 269(7):5241-5248.
- [12] Crul M.,et al., Phase I and pharmacological study of daily oral administration of perifosine (D-21266) in patients with advanced solid tumours. *Eur J Cancer*. 2002; 38(12):1615-1621.
- [13] Gills JJ.,et al., Phosphatidylinositol ether lipid analogues that inhibit AKT also independently activate the stress kinase, p38alpha, through MKK3/6-independent and -dependent mechanisms. *J Biol Chem*. 2007; 282(37):27020-27029.
- [14] Berndt N.,et al., The Akt activation inhibitor TCN-P inhibits Akt phosphorylation by binding to the PH domain of Akt and blocking its recruitment to the plasma membrane. *Cell Death Differ*. 2010; 17(11):1795-1804.
- [15] LoPiccolo J.,et al., Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations. *Drug Resist Updat*. 2008; 11(1-2):32-50.

- [16] McCubrey JA.,et al., Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. *Adv Enzyme Regul.* 2006; 46:249-279.
- [17] Chuang CF and Ng SY. Functional divergence of the MAP kinase pathway. ERK1 and ERK2 activate specific transcription factors. *FEBS Lett.* 1994; 346(2-3):229-234.
- [18] Dudley DT.,et al., A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc Natl Acad Sci U S A.* 1995; 92(17):7686-7689.
- [19] Britten CD. PI3K and MEK inhibitor combinations: examining the evidence in selected tumor types. *Cancer Chemother Pharmacol.* 2013; 71(6):1395-1409.
- [20] Saini KS.,et al., Targeting the PI3K/AKT/mTOR and Raf/MEK/ERK pathways in the treatment of breast cancer. *Cancer Treat Rev.* 2013; 39(8):935-946.
- [21] Yu CF.,et al., ERK negatively regulates the epidermal growth factor-mediated interaction of Gab1 and the phosphatidylinositol 3-kinase. *J Biol Chem.* 2002; 277(22):19382-19388.
- [22] Hoeflich KP.,et al., In vivo antitumor activity of MEK and phosphatidylinositol 3-kinase inhibitors in basal-like breast cancer models. *Clin Cancer Res.* 2009; 15(14):4649-4664.
- [23] Carracedo A.,et al., Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. *J Clin Invest.* 2008; 118(9):3065-3074.
- [24] Andreu-Perez P.,et al., Protein arginine methyltransferase 5 regulates ERK1/2 signal transduction amplitude and cell fate through CRAF. *Sci Signal.* 2011; 4(190):ra58.
- [25] Wei TY.,et al., Protein arginine methyltransferase 5 is a potential oncoprotein that upregulates G1 cyclins/cyclin-dependent kinases and the phosphoinositide 3-kinase/AKT signaling cascade. *Cancer Sci.* 2012; 103(9):1640-1650.
- [26] Frau M, Feo F and Pascale RM. Pleiotropic effects of methionine adenosyltransferases deregulation as determinants of liver cancer progression and prognosis. *J Hepatol.* 2013; 59(4):830-841.
- [27] Du Y, Peyser ND and Grandis JR. Integration of molecular targeted therapy with radiation in head and neck cancer. *Pharmacol Ther.* 2014; 142(1):88-98.
- [28] Shimizu T.,et al., The clinical effect of the dual-targeting strategy involving PI3K/AKT/mTOR and RAS/MEK/ERK pathways in patients with advanced cancer. *Clin Cancer Res.* 2012; 18(8):2316-2325.
- [29] Sahin O.,et al., Biomarker-guided sequential targeted therapies to overcome therapy resistance in rapidly evolving highly aggressive mammary tumors. *Cell Res.* 2014; 24(5):542-559.
- [30] Wang Q.,et al., Concomitant targeting of tumor cells and induction of T-cell response synergizes to effectively inhibit trastuzumab-resistant breast cancer. *Cancer Res.* 2012; 72(17):4417-4428.

- [31] Weigelt B and Downward J. Genomic Determinants of PI3K Pathway Inhibitor Response in Cancer. *Front Oncol.* 2012; 2:109.
- [32] Burris HA, 3rd. Overcoming acquired resistance to anticancer therapy: focus on the PI3K/AKT/mTOR pathway. *Cancer Chemother Pharmacol.* 2013; 71(4):829-842.
- [33] Esquela-Kerscher A and Slack FJ. Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer.* 2006; 6(4):259-269.
- [34] Lujambio A and Lowe SW. The microcosmos of cancer. *Nature.* 2012; 482(7385):347-355.
- [35] Wienholds E and Plasterk RH. MicroRNA function in animal development. *FEBS Lett.* 2005; 579(26):5911-5922.
- [36] Kurisetty VV, Lakshmanaswamy R and Damodaran C. Pathogenic and therapeutic role of miRNAs in breast cancer. *Front Biosci (Landmark Ed).* 2014; 19:1-11.
- [37] Kozomara A and Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res.* 2011; 39(Database issue):D152-157.
- [38] Calin GA.,et al., Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A.* 2002; 99(24):15524-15529.
- [39] Raza U, Zhang JD and Sahin O. MicroRNAs: master regulators of drug resistance, stemness, and metastasis. *J Mol Med (Berl).* 2014; 92(4):321-336.
- [40] Jurmeister S.,et al., MicroRNA-200c represses migration and invasion of breast cancer cells by targeting actin-regulatory proteins FHOD1 and PPM1F. *Mol Cell Biol.* 2012; 32(3):633-651.
- [41] Uhlmann S.,et al., miR-200bc/429 cluster targets PLCgamma1 and differentially regulates proliferation and EGF-driven invasion than miR-200a/141 in breast cancer. *Oncogene.* 2010; 29(30):4297-4306.
- [42] Ward A.,et al., Re-expression of microRNA-375 reverses both tamoxifen resistance and accompanying EMT-like properties in breast cancer. *Oncogene.* 2013; 32(9):1173-1182.
- [43] Uhlmann S.,et al., Global microRNA level regulation of EGFR-driven cell-cycle protein network in breast cancer. *Mol Syst Biol.* 2012; 8:570.
- [44] Agostini M and Knight RA. miR-34: from bench to bedside. *Oncotarget.* 2014; 5(4):872-881.
- [45] Gao J.,et al., Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal.* 2013; 6(269):p11.
- [46] Cerami E.,et al., The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer discovery.* 2012; 2(5):401-404.
- [47] Luo D.,et al., A systematic evaluation of miRNA:mRNA interactions involved in the migration and invasion of breast cancer cells. *J Transl Med.* 2013; 11:57.

- [48] Barrett T.,et al., NCBI GEO: archive for functional genomics data sets--update. *Nucleic Acids Res.* 2013; 41(Database issue):D991-995.
- [49] Geng C.,et al., Androgen receptor is the key transcriptional mediator of the tumor suppressor SPOP in prostate cancer. *Cancer Res.* 2014; 74(19):5631-5643.
- [50] Dancik GM.,et al., A cell of origin gene signature indicates human bladder cancer has distinct cellular progenitors. *Stem Cells.* 2014; 32(4):974-982.
- [51] Mutlu M.,et al., miR-200c: a versatile watchdog in cancer progression, EMT, and drug resistance. *J Mol Med (Berl).* 2016; 94(6):629-644.
- [52] Larue L and Bellacosa A. Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. *Oncogene.* 2005; 24(50):7443-7454.
- [53] Mori S.,et al., Anchorage-independent cell growth signature identifies tumors with metastatic potential. *Oncogene.* 2009; 28(31):2796-2805.
- [54] Agarwal V.,et al., Predicting effective microRNA target sites in mammalian mRNAs. *Elife.* 2015; 4.
- [55] Dweep H.,et al., miRWalk--database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. *J Biomed Inform.* 2011; 44(5):839-847.
- [56] Paraskevopoulou MD.,et al., DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows. *Nucleic Acids Res.* 2013; 41(Web Server issue):W169-173.
- [57] Kertesz M.,et al., The role of site accessibility in microRNA target recognition. *Nat Genet.* 2007; 39(10):1278-1284.
- [58] de Rinaldis E.,et al., Integrated genomic analysis of triple-negative breast cancers reveals novel microRNAs associated with clinical and molecular phenotypes and sheds light on the pathways they control. *BMC Genomics.* 2013; 14:643.
- [59] Enerly E.,et al., miRNA-mRNA integrated analysis reveals roles for miRNAs in primary breast tumors. *PLoS One.* 2011; 6(2):e16915.
- [60] Buffa FM.,et al., microRNA-associated progression pathways and potential therapeutic targets identified by integrated mRNA and microRNA expression profiling in breast cancer. *Cancer Res.* 2011; 71(17):5635-5645.
- [61] Ciriello G.,et al., Comprehensive Molecular Portraits of Invasive Lobular Breast Cancer. *Cell.* 2015; 163(2):506-519.
- [62] Wiza C, Nascimento EB and Ouwens DM. Role of PRAS40 in Akt and mTOR signaling in health and disease. *American journal of physiology Endocrinology and metabolism.* 2012; 302(12):E1453-1460.
- [63] Morishita D.,et al., Pim kinases promote cell cycle progression by phosphorylating and down-regulating p27Kip1 at the transcriptional and posttranscriptional levels. *Cancer Res.* 2008; 68(13):5076-5085.

- [64] Lai CY.,et al., MicroRNA expression aberration as potential peripheral blood biomarkers for schizophrenia. *PLoS One*. 2011; 6(6):e21635.
- [65] Rokah OH.,et al., Downregulation of miR-31, miR-155, and miR-564 in chronic myeloid leukemia cells. *PLoS One*. 2012; 7(4):e35501.
- [66] Yang B.,et al., MiR-564 functions as a tumor suppressor in human lung cancer by targeting ZIC3. *Biochem Biophys Res Commun*. 2015; 467(4):690-696.
- [67] Serra V.,et al., PI3K inhibition results in enhanced HER signaling and acquired ERK dependency in HER2-overexpressing breast cancer. *Oncogene*. 2011; 30(22):2547-2557.
- [68] Carracedo A and Pandolfi PP. The PTEN-PI3K pathway: of feedbacks and cross-talks. *Oncogene*. 2008; 27(41):5527-5541.
- [69] Ward A.,et al., MicroRNA-519a is a novel oncomir conferring tamoxifen resistance by targeting a network of tumour-suppressor genes in ER+ breast cancer. *J Pathol*. 2014; 233(4):368-379.
- [70] Wang F, Li L, Chen Z, Zhu M and Gu Y. MicroRNA-214 acts as a potential oncogene in breast cancer by targeting the PTEN-PI3K/Akt signaling pathway. *Int J Mol Med*. 2016.
- [71] Fang Y.,et al., MicroRNA-7 inhibits tumor growth and metastasis by targeting the phosphoinositide 3-kinase/Akt pathway in hepatocellular carcinoma. *Hepatology*. 2012; 55(6):1852-1862.
- [72] Schmitt DC.,et al., ErbB2-intronic microRNA-4728: a novel tumor suppressor and antagonist of oncogenic MAPK signaling. *Cell Death Dis*. 2015; 6:e1742.
- [73] Nagaraja AK.,et al., A link between mir-100 and FRAP1/mTOR in clear cell ovarian cancer. *Mol Endocrinol*. 2010; 24(2):447-463.
- [74] Wang W.,et al., MicroRNA-497 suppresses angiogenesis by targeting vascular endothelial growth factor A through the PI3K/AKT and MAPK/ERK pathways in ovarian cancer. *Oncol Rep*. 2014; 32(5):2127-2133.
- [75] Bao L.,et al., MicroRNA-21 suppresses PTEN and hSulf-1 expression and promotes hepatocellular carcinoma progression through AKT/ERK pathways. *Cancer Lett*. 2013; 337(2):226-236.
- [76] Xu Q.,et al., A regulatory circuit of miR-148a/152 and DNMT1 in modulating cell transformation and tumor angiogenesis through IGF-IR and IRS1. *J Mol Cell Biol*. 2013; 5(1):3-13.
- [77] Chau NM and Ashcroft M. Akt2: a role in breast cancer metastasis. *Breast Cancer Res*. 2004; 6(1):55-57.
- [78] Crowell JA, Steele VE and Fay JR. Targeting the AKT protein kinase for cancer chemoprevention. *Mol Cancer Ther*. 2007; 6(8):2139-2148.
- [79] Kilts JD.,et al., Selective activation of human atrial Galpha12 and Galpha13 by Galphaq-coupled angiotensin and endothelin receptors. *J Cardiovasc Pharmacol*. 2007; 50(3):299-303.

- [80] Wirth A.,et al., G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. *Nat Med.* 2008; 14(1):64-68.
- [81] Olson EN and Nordheim A. Linking actin dynamics and gene transcription to drive cellular motile functions. *Nat Rev Mol Cell Biol.* 2010; 11(5):353-365.
- [82] Moulleron S.,et al., Molecular basis for G-actin binding to RPEL motifs from the serum response factor coactivator MAL. *EMBO J.* 2008; 27(23):3198-3208.
- [83] Chia CY, Kumari U and Casey PJ. Breast cancer cell invasion mediated by Galpha12 signaling involves expression of interleukins-6 and -8, and matrix metalloproteinase-2. *J Mol Signal.* 2014; 9:6.
- [84] Mitsuuchi Y.,et al., Translocation and activation of AKT2 in response to stimulation by insulin. *J Cell Biochem.* 1998; 70(4):433-441.
- [85] Iida Y.,et al., Hypoxia promotes glycogen synthesis and accumulation in human ovarian clear cell carcinoma. *Int J Oncol.* 2012; 40(6):2122-2130.
- [86] Dalton S.,et al., Isolation and characterization of SRF accessory proteins. *Philos Trans R Soc Lond B Biol Sci.* 1993; 340(1293):325-332.
- [87] Thuerauf DJ.,et al., p38 Mitogen-activated protein kinase mediates the transcriptional induction of the atrial natriuretic factor gene through a serum response element. A potential role for the transcription factor ATF6. *J Biol Chem.* 1998; 273(32):20636-20643.
- [88] Song LB.,et al., The polycomb group protein Bmi-1 represses the tumor suppressor PTEN and induces epithelial-mesenchymal transition in human nasopharyngeal epithelial cells. *J Clin Invest.* 2009; 119(12):3626-3636.
- [89] Ferraro A.,et al., EZH2 is regulated by ERK/AKT and targets integrin alpha2 gene to control Epithelial-Mesenchymal Transition and anoikis in colon cancer cells. *Int J Biochem Cell Biol.* 2013; 45(2):243-254.
- [90] Benoit YD.,et al., Pharmacological inhibition of polycomb repressive complex-2 activity induces apoptosis in human colon cancer stem cells. *Exp Cell Res.* 2013; 319(10):1463-1470.
- [91] Wang H.,et al., Acquisition of epithelial-mesenchymal transition phenotype and cancer stem cell-like properties in cisplatin-resistant lung cancer cells through AKT/beta-catenin/Snail signaling pathway. *Eur J Pharmacol.* 2014; 723:156-166.
- [92] Gulhati P.,et al., mTORC1 and mTORC2 regulate EMT, motility, and metastasis of colorectal cancer via RhoA and Rac1 signaling pathways. *Cancer Res.* 2011; 71(9):3246-3256.
- [93] Makrodouli E.,et al., BRAF and RAS oncogenes regulate Rho GTPase pathways to mediate migration and invasion properties in human colon cancer cells: a comparative study. *Mol Cancer.* 2011; 10:118.
- [94] Li XJ, Ren ZJ and Tang JH. MicroRNA-34a: a potential therapeutic target in human cancer. *Cell Death Dis.* 2014; 5:e1327.

- [95] Wang L.,et al., The analysis of microRNA-34 family expression in human cancer studies comparing cancer tissues with corresponding pericarcinous tissues. *Gene*. 2015; 554(1):1-8.



**Curriculum Vitae
and
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Curriculum Vitae

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RESEARCH INTEREST

My research interests are single or combinatorial cancer targeting with the systems biology point of view and I've been working on in both bioinformatics and in vitro, in vivo experimenting. As a targeted therapy approach, I'm seeking in my master's education, for a drugable target on breast cancers that will be targeting essential pathways deregulated in tumorigenesis and metastasis. I believe to achieve a goal one should use all the information is present, that's why usage of bioinformatics together with wetlab is essential.

EDUCATION

2013-current	M.Sc degree TURKEY Sahin lab (Supervisor: Özgür Şahin) Department of Molecular Biology and Genetics Bilkent University	Ankara,
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09/2005- 06/2009	High school TURKEY Tarsus American College	Mersin,

PERSONAL SKILLS AND COMPETENCE

Wetlab

- Cell culturing, fluorescent staining, RTCA, transient transfection, lentiviral stable

transfection, qPCR, western blot, cloning, luciferase reporter assays, in vivo bioluminescent and fluorescent live imaging

Computer

- R Bioconductor, MacOS, Cytoscape, Image J, GSEA,
- Online bioinformatic tools such as cBIO, StringDB, GEO2R, KEGG, GO, KMPlotter

Language

- Turkish, Mother tongue
- English, professional user (TOEFL score: 101)

ACADEMIC HONORS AND AWARDS

- *2nd place in 2nd Ankara Science Slam Competition*, Bilkent University
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- *Best Oral Presentation Award*, MOKADs 3rd Anticancer Agent Development Congress
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- *2nd place in 1st Ankara Science Slam Competition*, German Embassy
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- *Student of the Year in Science*, Tarsus American College
Mersin, 2009

RESEARCH EXPERIENCE

09/2013-	<i>Masters student</i> "Combinatorial targeting of PI3K and MAPK signaling pathways by microRNAs to inhibit tumor growth and metastasis in breast cancer" (supported by TUBITAK 1001 Grant) Şahin Lab, Department of Molecular Biology and Genetics Bilkent University Ankara, TURKEY
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CONFERENCES ATTENDED

- 4th International Congress of Molecular Biology Association, *Middle East Technical University*, Ankara, TURKEY (November 27-29,2015)
- MOKADs 3rd Anticancer Agent Development Congress, *9 Eylul University*, Izmir, TURKEY (May 17-19 2015)
- Winter retreat. *Bilkent University* (January 16-17, 2015)
- U.S. National Cancer Institute Seminar Prof. Dr. Douglas Lowy, Prof. Dr. Philip Taylor. *Bilkent University*, Ankara, TURKEY (April 1, 2014)
- İhsan Doğramacı Memorial Lecture 2014, Prof. Dr. Evan E. Eichler. *Bilkent University*, Ankara, TURKEY (April 2-3, 2014)
- 2nd International Congress of Molecular Biology Association of Turkey. *Istanbul Technical University*, Istanbul, TURKEY (November 22-23, 2013)
- İhsan Doğramacı Memorial Lecture 2013, Prof. Dr. Jeffrey Friedman. *Bilkent University*, Ankara, TURKEY (September 20, 2013)
- İhsan Doğramacı Memorial Lecture 2012, Prof. Dr. Peter Walter and Prof. Dr. Gökhan Hotamışlıgil. *Bilkent University*, Ankara, TURKEY (November 12, 2012)
- İhsan Doğramacı Memorial Lecture 2010, Prof. Dr. Sir Timothy Hunt. *Bilkent University*, Ankara, TURKEY (December 4, 2010)

PUBLICATIONS

- **Mutlu M**, Saatci O, Ansari SA, Yurdusev E, Raza U, Şahin Ö. miR-564 acts as a dual inhibitor of PI3K and MAPK pathways by targeting a network of genes and inhibits proliferation and invasion in breast cancer. *Sci Rep.* (under revision) (Impact factor: 5.578)
- Raza U, Saatci Ö, Uhlmann S, Ansari S, Eyüpoğlu E, Yurdusev E, **Mutlu M**, Ersan P, Altundağ M, Zhang J, Doğan H, Güler G, Şahin Ö. The miR-644a/CTBP1/p53 axis mediates drug resistance by simultaneous modulation of cell survival and epithelial-mesenchymal transition in breast cancer. *Oncotarget.* 2016 Jul 8. doi: 10.18632/oncotarget.10489. [Epub ahead of print] (Impact factor: 6.359)

- **Mutlu M**, Raza U, Saatci O, Eyupoglu E, Yurdusev E, Sahin O. miR-200c: A versatile watchdog in cancer progression, EMT and drug resistance. *J. Mol. Med* 2016 Jun;94(6):629-44 (Impact factor: 5.107)
- Bax NM, Sangermano R, Roosing S, Thiadens AA, Hoefsloot LH, van den Born LI, Phan M, Klevering BJ, Westeneng-van Haaften C, Braun TA, Zonneveld-Vrieling MN, de Wijs I, **Mutlu M**, Stone EM, den Hollander AI, Klaver CC, Hoyng CB, Cremers FP. Heterozygous deep-intronic variants and deletions in ABCA4 in persons with retinal dystrophies and one exonic ABCA4 variant. *Hum Mutat.* 2015 Jan;36(1):43-7. (Impact factor: 5.05)
- **Mutlu M**, Saatçi Ö, Raza U, Eyüpoglu E, Yurdusev E, Sahin Ö* (2014). MIR200C (microRNA 200c). *Atlas Genet Cytogenet Oncol Haematol.* July 2014 (online)

COURSES TAKEN

Molecular Genetics, Genetics, Molecular Biology of the Cell, Molecular Biology of Gene, Biochemistry, Bioinformatics, Neuropsychology, Physiology, Microbiology, Introduction to Stem cell, Immunology, Genomics, Special Techniques in Molecular Biology, Cancer Biology, Advanced Cellular Biology, Advanced Molecular Biology, Advanced Molecular Genetics

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The miR-644a/CTBP1/p53 axis suppresses drug resistance by simultaneous inhibition of cell survival and epithelial-mesenchymal transition in breast cancer

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Keywords: miRNAs, CTBP1, p53, EMT, therapy resistance

Received: January 04, 2016

Accepted: June 26, 2016

Published: July 08, 2016

ABSTRACT

Tumor cells develop drug resistance which leads to recurrence and distant metastasis. MicroRNAs are key regulators of tumor pathogenesis; however, little is known whether they can sensitize cells and block metastasis simultaneously. Here, we report miR-644a as a novel inhibitor of both cell survival and EMT whereby acting as pleiotropic therapy-sensitizer in breast cancer. We showed that both miR-644a expression and its gene signature are associated with tumor progression and distant metastasis-free survival. Mechanistically, miR-644a directly targets the transcriptional co-repressor C-Terminal Binding Protein 1 (CTBP1) whose knock-outs by the CRISPR-Cas9 system inhibit tumor growth, metastasis, and drug resistance, mimicking the phenotypes induced by miR-644a. Furthermore, downregulation of CTBP1 by miR-644a upregulates wild type- or mutant-p53 which acts as a 'molecular switch' between G1-arrest and apoptosis by inducing cyclin-dependent kinase inhibitor 1 (p21, CDKN1A, CIP1) or pro-apoptotic phorbol-12-myristate-13-acetate-induced protein 1 (Noxa, PMAIP1), respectively. Interestingly, an increase in mutant-p53 by either overexpression of miR-644a or downregulation of CTBP1 was enough to shift this balance in favor of apoptosis through upregulation of Noxa. Notably, p53-mutant patients, but not p53-wild type ones, with high CTBP1 have a shorter survival suggesting that CTBP1 could be a potential prognostic factor for breast cancer patients with p53 mutations. Overall, re-activation of the miR-644a/CTBP1/p53 axis may represent a new strategy for overcoming both therapy resistance and metastasis.

INTRODUCTION

Breast cancer is the most common malignancy and the second leading cause of cancer deaths among women [1]. Depending on breast cancer subtype [2], patients are treated with chemotherapy and/or targeted agents; however, intrinsic or acquired resistance is inevitable in almost all cases. As tumor cells develop *de novo* or acquired drug resistance, residing cancer

cells undergo epithelial mesenchymal transition (EMT), evade from primary tumor site and metastasize to distant organs leading to death of the patients [3]. Therefore, it is necessary to identify novel targets which do not only inhibit tumor growth, but also sensitize refractory cells to therapy and prevent metastasis.

MicroRNAs (miRNA) are 20–22 nucleotide small non-coding RNAs which regulate gene expression post-transcriptionally by preferentially binding to the seed-

matching sequence in the 3'-UTR of target mRNAs leading to either mRNA destabilization or degradation [4]. miRNAs have been classified as tumor suppressors or oncogenic ones depending on the phenotype they induce, the targets they modulate, and the tissue where they function [5, 6]. In this context, large number of oncogenic and tumor suppressor miRNAs have been shown to be associated with cancer progression, drug resistance or metastasis (reviewed in [7, 8]). However, little is known about miRNAs that can simultaneously regulate tumor proliferation and EMT whereby acting as therapy-sensitizer and metastasis blocker in breast cancer.

In this study, we identify miR-644a as a novel inhibitor of tumor cell proliferation and metastatic potential which acts as a pleotropic therapy sensitizer in breast cancer both *in vitro* and *in vivo*. These findings are further supported by the analysis of several breast cancer datasets. Mechanistically, we show that miR-644a directly targets transcriptional co-repressor CTBP1 and thereby upregulates p53 levels. We then show that this increased wild type or mutant p53 acts as a switch deciding on G1-arrest or apoptosis by inducing p21 or Noxa, respectively. Our *in silico* analyses propose CTBP1 as an important predictor for survival of breast cancer patients with p53 mutation. These results suggest that the re-activation of miR-644a/CTBP1/p53 axis may represent a new target to overcome breast cancer progression, therapy resistance, and metastasis.

RESULTS

miR-644a inhibits proliferation, promotes apoptosis, and its expression or gene signature correlates with tumor progression in breast cancer

To identify novel miRNAs regulating proliferation in breast cancer, we performed a small scale miRNA mimic cell viability screen entailing 35 miRNAs in MDA-MB-231 human breast cancer cell line (Figure 1A). As a positive control we used miR-200c, which was previously reported as a tumor suppressor miRNA by us [9] and others [10, 11]. Out of three most promising potential tumor suppressor miRNAs besides miR-200c, miR-299-3p and miR-127-5p have been reported as tumor suppressors in different cancer types [12, 13]. The other one, miR-644a, has not been characterized in the context of breast cancer. Real time cell analyzer (RTCA) assay further confirmed inhibitory role of miR-644a in viability of MDA-MB-231 cells (Figure 1B). Furthermore, miR-644a reduced viability of other cell lines representing different breast cancer subtypes and two normal breast cell lines, MCF-10A and MCF-12A, (Figure 1C).

Upon miR-644a overexpression, breast cancer cell lines with p53 mutation (p53-*mut*) only underwent apoptosis evidenced by increased cleaved caspase-3

(Figure 1D and 1E) by inducing G2/M arrest characterized by increased phosphorylation of G2/M-arrest markers Cdc2 and Cdc25C (Figure 1F and 1H). In contrast, miR-644a overexpression in p53-*wt* MCF-7 cells resulted in G1 arrest with decreased expression of G1/S transition proteins and increased expression of CDK inhibitor p21, which leads to reduced phosphorylation of Rb protein (Figure 1G and 1H).

To validate our findings *in vivo*, we engineered MDA-MB-231 cell line (referred to herein as 231.Ctrl) with lentiviral-transduced miR-644a (referred to herein as 231.miR-644a) (Supplementary Figure S1A), and observed a delayed and significantly decreased tumor growth (Figure 1I). Correspondingly, tumors collected from the 231.miR-644a group showed high levels of miR-644a expression (Figure 1L), were substantially smaller and weighed less (Figure 1J and 1K) further confirming the tumor suppressive role of miR-644a in breast cancer.

To elucidate the pathological relevance of miR-644a, we examined the expression of miR-644a in publicly available expression datasets GSE45666 and GSE58606 [14, 15], and observed significantly lower miR-644a levels in tumors as compared to normal tissues (Figure 1M and 1N). Besides breast cancer, melanoma and osteosarcoma cell lines also had lower miR-644a levels compared to their normal counterparts (Supplementary Figure S1B and S1C). In addition to gene-level analysis, we also performed gene expression profiling to derive a miR-644a signature, and used this signature to elucidate miR-644a-induced changes in a more global manner. To this end, we forced expression of miR-644a using mimics in three breast cancer cell lines representing different subtypes of breast cancer (Supplementary Figure S2A). We then collected commonly up- and down-regulated genes among these three cell lines to create a 'miR-644a signature' (Supplementary Table S1). Gene Ontology (GO) term analysis revealed association of miR-644a signature with biological processes contributing to tumor progression, such as cell cycle, apoptosis, actin cytoskeletal organization and cell adhesion (Supplementary Figure S2B). Furthermore, Gene Set Enrichment Analysis (GSEA) of GSE58644 dataset [16] showed a significant correlation between miR-644a gene signature and tumor progression in breast cancer patients. Notably a gene set containing genes downregulated in lobular carcinoma compared to normal lobular breast cells were enriched in patients with high miR-644a signature scores (Supplementary Figure S1D). Moreover, genes associated with histologic grade 1 and grade 3 in breast cancer patients were significantly enriched in high and low miR-644a signature scorers, respectively (Figure 1O and Supplementary Figure S1E). Consistent with this observation, in the same dataset, miR-644a signature score was lower in tumors with a more aggressive disease state characterized by higher tumor grade and stage (Supplementary Figure S1F and S1G). Finally, miR-644a

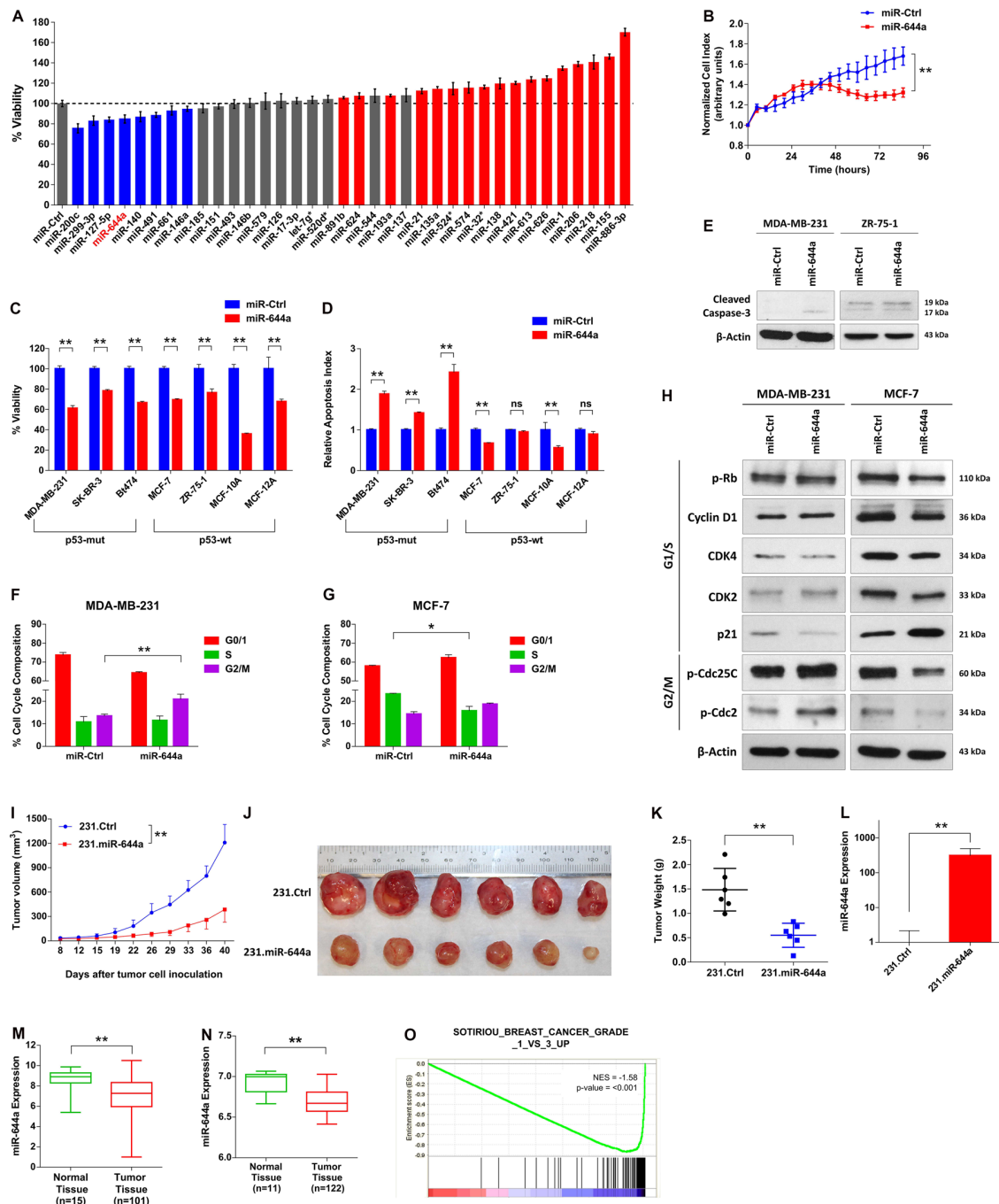


Figure 1: miR-644a reduces the viability of breast cancer cells *in vitro* and *in vivo* and miR-644a expression or its gene signature is associated with tumor progression in breast cancer. (A) miRNA mimic cell viability screen on MDA-MB-231 human breast cancer cell line comprising of 35 different miRNAs, with miR-200c as a positive control. The cells were transfected with 20 nM of mimics for 48 hours, and viability was measured using Cell titer Glo. Color coding of the bars depicts the effect of each miRNA on cell viability (blue: decreasing viability, red: increasing viability, gray: no effect on viability). (B) Real time growth of MDA-MB-231 cells transiently transfected with either a control miRNA (miR-Ctrl) or miR-644a, monitored using an RTCA (real-time cell analyzer) assay. (C) Effect of miR-644a overexpression on proliferation of 5 breast cancer cell lines and 2 normal breast cell lines transfected with either miR-Ctrl or miR-644a. $n = 4$. (D) Changes in the apoptotic index based on Caspase-3 cleavage in cells from (C). $n = 4$. (E) Western Blot Analysis showing the levels of cleaved Caspase-3 in p53-mut MDA-MB-231 (left) and p53-wt ZR-75-1 cells (right) after 72 hours transfection with either miR-Ctrl or miR-644a. (F and G) Flow cytometric analysis of cell cycle in cells transfected with miR-Ctrl or miR-644a showing G2/M arrest in miR-644a transfected MDA-MB-231 cells (F) and G1 arrest in miR-644a transfected MCF-7 cells (G). (H) Western Blot Analysis showing the levels of cell cycle proteins related to G1/S (pRb, Cyclin D1, CDK4, CDK2 and p21) and G2/M transition (p-Cdc25C and p-Cdc2) in p53-mut MDA-MB-231 (left) and p53-wt MCF7 cells (right) after 48 hours transfection with either miR-Ctrl or

miR-644a. (I) Tumor progression in xenografts generated by orthotopic subcutaneous injection of MDA-MB-231 cells stably expressing either a non-silencing control (231.Ctrl) or miR-644a (231.miR-644a) into nude mice. $n = 6$. (J) Representative images of tumors collected from xenografts of (I) on day 40. (K) Tumor weights in xenografts from (I) at day 40. (L) qRT-PCR analysis showing average miR-644a expression in 231.Ctrl and 231.miR-644a tumors collected from xenografts of (I) on day 40. $n = 3$. (M) miR-644a expression in 101 breast tumor tissues and 15 normal tissues from GSE45666 depicted as box-plot showing the median expression in all patients. (N) miR-644a expression in 122 breast tumor tissue and 11 normal tissue samples from GSE58606. (O) Enrichment plots of patients from GSE58644 ($n = 320$) with high or low miR-644a signature score. Genes up-regulated as breast tumors progress through histologic grade 3 were enriched in patients with low miR-644a signature score. Statistical significance was indicated ($*p < 0.05$; $**p < 0.01$; ns, not significant). Column data represent mean $\pm$ SD. Box-plots depict median number and the 25th to 75th quartiles. Upper and lower whiskers represent the minimum and maximum values in the corresponding group. This applies to all figures shown.

status was correlated with the formation and progression of not only breast cancer, but also of a variety of cancers, including melanoma, liver, lung and ovarian cancers (Supplementary Table S2), all supporting the tumor suppressive roles of miR-644a.

miR-644a inhibits metastasis and correlates with distant metastasis-free survival in patients

Since we observed enrichment of biological processes relevant to metastasis (Supplementary Table S3), we wanted to examine the effects of miR-644a on the metastatic potential of breast tumors. Transient or stable miR-644a overexpression significantly inhibited migration and invasion of MDA-MB-231 cells as assessed by wound healing, real-time migration and trans-well matrigel invasion assays (Figure 2A–2D) and reduced anchorage independent growth (Figure 2E). Furthermore, cells overexpressing miR-644a showed rearrangements of actin cytoskeletal structures from a mesenchymal to an epithelial-like state (Figure 2F) which is further confirmed by upregulated epithelial markers and downregulated mesenchymal markers both at mRNA and protein level upon miR-644a overexpression (Figure 2G–2I). Further, we tested the metastatic potential of 231.miR-644a cells in nude mice with tail-vein metastasis assay. Bouin's fixation of lungs and Hematoxylin and Eosin (H&E) staining indicated less colonization of 231.miR-644a cells to lungs as compared to 231.Ctrl cells (Figure 2J and 2K). As these cells are stably labelled with luciferase, we measured luciferase activity of lung lysates, and showed that substantially less number of cells reached metastasized to the lungs when miR-644a is expressed (Figure 2L). These data suggest that miR-644a also inhibits metastasis *in vivo*.

Then, to validate these *in vitro* and *in vivo* findings in human patient datasets, we first examined GSE38167 dataset [17], and found that expression of miR-644a is lower in primary tumors compared to normal adjacent tissues, and even further decreased in lymph node metastases (Figure 2M). This observation was further validated by GSEA which revealed an enrichment of genes whose expression in primary tumors of estrogen receptor positive (ER+) breast cancer positively correlates with developing distant metastases are enriched in patients with low miR-644a signature scores from GSE58644

(Supplementary Table S3). Moreover, miR-644a expression was found to be negatively associated with metastasis in cancers other than breast as well (Supplementary Figure S1H and S1I; Supplementary Table S3). Finally, we showed an enrichment of genes associated with poor outcome in patients having low miR-644a signature scores (Figure 2N). Consistent with this, breast cancer patients with high miR-644a signature scores have significantly longer distant-metastasis-free survival (Figure 2O). Overall, our data suggest that miR-644a is a novel tumor suppressor that is likely to be involved in progression and metastasis of multiple cancer types including breast cancer.

miR-644a is a pleiotropic therapy sensitizer in breast cancer

Since miR-644a inhibits both breast cancer cell survival and EMT, we hypothesized that it might also work as a therapy sensitizer. To test this hypothesis, we did GSEA with gene sets related to drug sensitivity and resistance. We observed that genes associated with doxorubicin (topoisomerase II inhibitor) and cisplatin (DNA cross linking agent; promising therapy for BRCA1/2 mutated/deficient tumors) resistance in gastric cancer cell lines and patients, respectively were significantly enriched in patients with low miR-644a signature scores (Supplementary Table S3). Indeed, miR-644a significantly sensitized p53-*mut* MDA-MB-231 and p53-*wt* MCF-7 cells to doxorubicin and *BRCA1*-mutated MDA-MB-436 breast cancer cells to cisplatin *in vitro* (Figure 3A, 3B and 3D). Furthermore, significantly higher miR-644a levels were observed in doxorubicin sensitive tumors developed *in vivo* as compared to resistant ones (Figure 3C), further supporting that miR-644a may play a role in chemotherapy resistance also *in vivo*.

In addition to chemotherapy agents, we found that patients with higher miR-644a signature scores have enhanced sensitivity to tamoxifen (Supplementary Table S3), which is the mainstay targeted therapy for ER+ breast cancer patients for over 40 years [18]. We demonstrated that forced miR-644a expression sensitizes cells to tamoxifen in ER+ MCF-7 cells *in vitro* (Figure 3E). Similarly, we saw that genes downregulated in gefitinib resistant non-small cell lung cancer cells undergoing prominent growth arrest and apoptosis upon

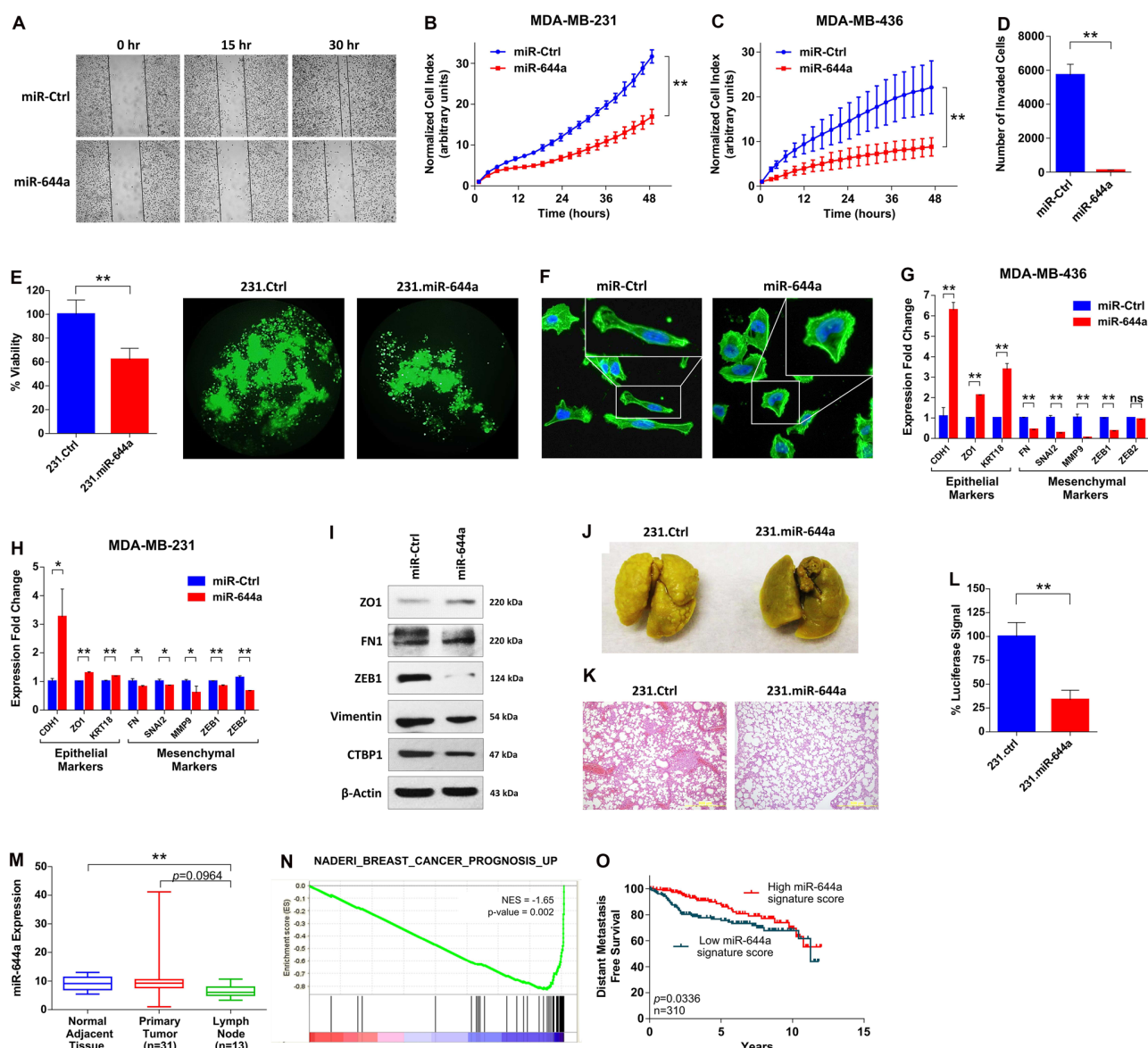


Figure 2: miR-644a inhibits metastasis, and its expression or gene signature is associated with metastasis of breast cancer patients. (A) Wound healing assay of MDA-MB-231 cells transfected with miR-Ctrl or miR-644a. Cells were scratched after 48 hours of transfection, and images were taken with 4X Magnification at 0, 15 and 30 hours after transfection. (B and C) Real-time migration of MDA-MB-231 (B) and MDA-MB-436 (C) cells transfected with either miR-Ctrl or miR-644a, monitored using an RTCA assay. (D) Number of invaded cells transfected with miR-Ctrl or miR-644a using Matrigel invasion assay. $n = 3$. (E) Viability of 231.Ctrl and 231.miR-644a cells grown in anchorage-independent conditions for 7 days, quantified by WST-1 assay (left) together with their fluorescence microscopy images with 10X magnification (right). (F) Fluorescence microscopy images of MD-MB-231 cells transfected with miR-Ctrl or miR-644a. Cell nuclei and filamentous actin were stained with 4,6-diamidino-2-phenylindole (DAPI) and Alexa Fluor 488 phalloidin, respectively. Images were taken after 72 hours of transfection with 20X magnification. Boxes at upper right corners of the images show cell morphology with higher resolution. (G and H) qRT-PCR analysis of epithelial and mesenchymal marker gene expression in MDA-MB-436 (G) and MDA-MB-231. (H) Cells transfected with miR-Ctrl or miR-644a. $n = 3$. (I) Western blot analysis of epithelial and mesenchymal marker expression in MDA-MB-231 cells transfected with miR-Ctrl or miR-644a. (J) Representative images of lungs collected from nude mice injected intravenously with 231.Ctrl or 231.miR-644a cells. Mice were sacrificed at week 7 and lungs were fixed in Bouin's Solution. (K) Hematoxylin and eosin staining of metastatic nodules in lungs from (J). (L) Luciferase signal coming from metastatic nodules in lungs of (J) as quantified by a luciferase assay. (M) miR-644a expression in 23 normal tissue, 31 primary tumor (IDC) and 13 lymph node metastasis tissues in GSE38167 depicted as box-plot. (N) Enrichment plot of patients from GSE58644 ($n = 320$) with high or low miR-644a signature score. Genes expressed higher in breast cancer patients with poor outcome as compared to those with good outcome were enriched in patients expressing low levels of miR-644a signature score. (O) Kaplan Meier survival curve representing the percentage distant metastasis-free survival in breast cancer patients based on miR-644a signature score median expression levels in GSE58644 ($n = 310$).

treatment with an irreversible EGFR inhibitor, CL-387,785 [19], were enriched in low miR-644a signature scorers (Supplementary Table S3). We validated this in EGFR overexpressing SK-BR-3 breast cancer cells, where miR-644a overexpression significantly sensitized cells to gefitinib (Figure 3F). Overall, multiple lines of evidence support the notion that miR-644a may be a pleiotropic sensitizer for both chemo- and targeted-therapy.

CTBP1 is a direct target of miR-644a

To identify the targets of miR-644a mediating these observed effects, we combined the list of genes downregulated upon miR-644a overexpression in our microarray analysis with targets of miR-644a predicted by three target prediction algorithms. This stringent analysis resulted in 3 genes: *CTBP1*, *CHMP7*, and *NDST1* (Figure 4A). We found CTBP1 as the most promising candidate since it is an established transcriptional co-repressor which preferentially represses the transcription of tumor suppressor genes and promotes tumor growth *via* playing pivotal roles in tumor pathogenesis [20–22].

Sequence analysis revealed that human *CTBP1* 3'-UTR has one binding site of miR-644a between nucleotides 453–460 (Figure 4B). Transient as well as stable overexpression of miR-644a significantly downregulated CTBP1 mRNA and protein levels in both MDA-MB-231 and MCF-7 cells (Figure 4C–4F).

Inversely, miR-644a inhibition in MDA-MB-231 and MCF-12A cells by using hairpin inhibitors upregulated CTBP1 levels (Figure 4G and 4H). We confirmed CTBP1 as a direct target of miR-644a by measuring luciferase expression from *CTBP1* 3'-UTR constructs with or without mutation (Supplementary Figure S3A) in MDA-MB-231 and MCF-7 cells upon miR-644a transfection. In both cell lines, miR-644a overexpression significantly repressed luciferase expression when co-transfected with Wild type 3'-UTR expressing vector, but not in the case of mutated 3'-UTR expressing vector (Figure 4I and 4J). Additionally, through analyzing mRNA and miRNA expression profile dataset GSE40059 [23], we observed an inverse correlation between miR-644a and CTBP1 expression in 11 different breast cancer cell lines (Figure 4K). Overall, these data confirm that CTBP1 is a direct target of miR-644a.

Loss of CTBP1 mimics tumor- and metastasis-suppressive roles of miR-644a *in vitro* and *in vivo*

To validate that CTBP1 is a major functional target of miR-644a, we first knocked down CTBP1 with two different siRNA sequences (Supplementary Figure S4A and S4B), and examined the effect on viability, apoptosis and cell cycle. We observed a significant reduction in the viability of all tested cell lines upon CTBP1 knockdown (Figure 5A).

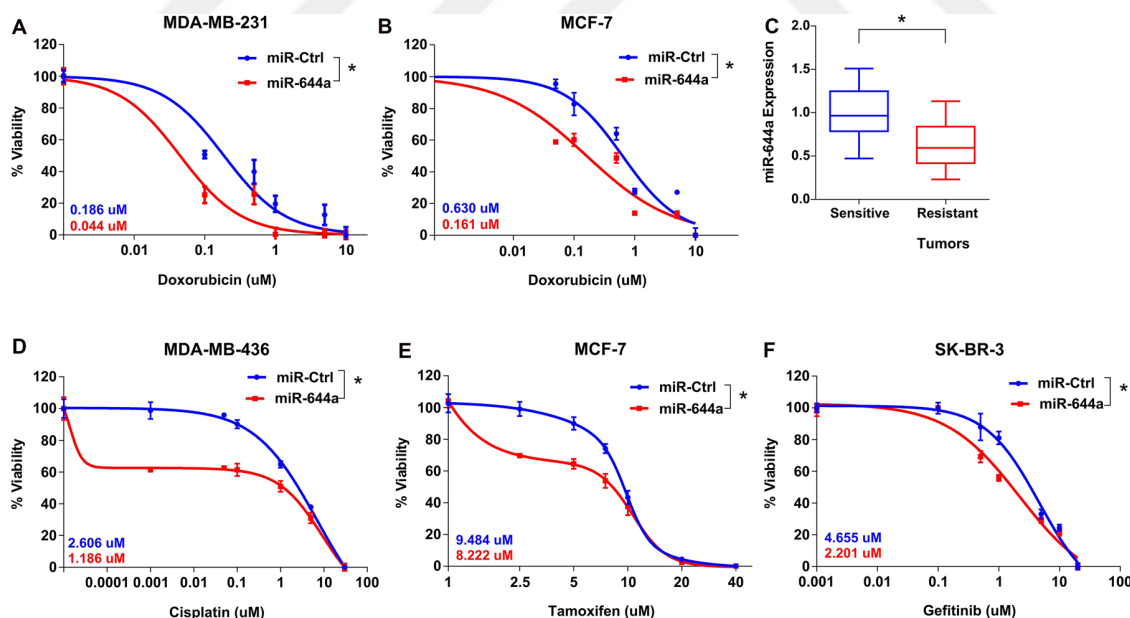


Figure 3: miR-644a overexpression acts as a therapy sensitizer in breast cancer cells and its expression correlates with doxorubicin resistance *in vivo* xenografts. (A and B) Effect of miR-644a overexpression on the response of MDA-MB-231 (A) and MCF-7 (B) cells to doxorubicin. (C) qRT-PCR analysis of miR-644a expression in xenografts sensitive or resistant to doxorubicin. Nude mice were subcutaneously injected with MDA-MB-231 cells and treated with doxorubicin. Among the treated mice, sensitive and resistant tumors were selected based on changes in tumor volumes upon successive drug treatments. $n = 4$. (D–F) Effect of miR-644a overexpression on the response of MDA-MB-436 cells to cisplatin (D), response of MCF-7 cells to tamoxifen (E), and response of SKBR-3 cells to gefitinib (F). IC50 values for each condition are given on the left bottom corners of each curve with a color code.

Interestingly, CTBP1 downregulation also mimicked miR-644a overexpression in increasing apoptotic cell death and inducing cleaved caspase-3 exclusively in p53-*mut* breast cancer cell lines, but not in p53-*wt* cell lines (Figure 5B and 5C). Moreover, cell cycle and western blot analysis confirmed a G2/M arrest in p53-*mut* MDA-MB-231 cells while a G1 arrest was observed in p53-*wt* MCF-7 cells upon CTBP1 knockdown similar to the effect of miR-644a (Figure 5D and 5E). For *in vivo* validation, we generated two independent CRISPR-Cas9 mediated CTBP1 knock-outs in MDA-MB-231 cells (referred to herein as 231.sgCTBP1_1 and 231.sgCTBP1_2) (Supplementary Figure S3B and S3C). Both

cell lines showed efficient downregulation of CTBP1 (Figure 5F), and exhibited delayed and significantly decreased tumor growth and tumor size in nude mice (Figure 5G–5I). We could also replicate these findings using an shRNA construct against CTBP1 (Supplementary Figure S5A–S5D).

Next, we tested if the loss of CTBP1 is able to mimic the effects of miR-644a on tumor metastasis. CTBP1 knockdown significantly inhibited migration and invasion of both MDA-MB-231 and MDA-MB-436 cells (Figure 5J and 5K), and induced a more epithelial-like state (Figure 5L–5N). Inversely, CTBP1 upregulation upon miR-644a inhibitor transfection promoted mesenchymal-

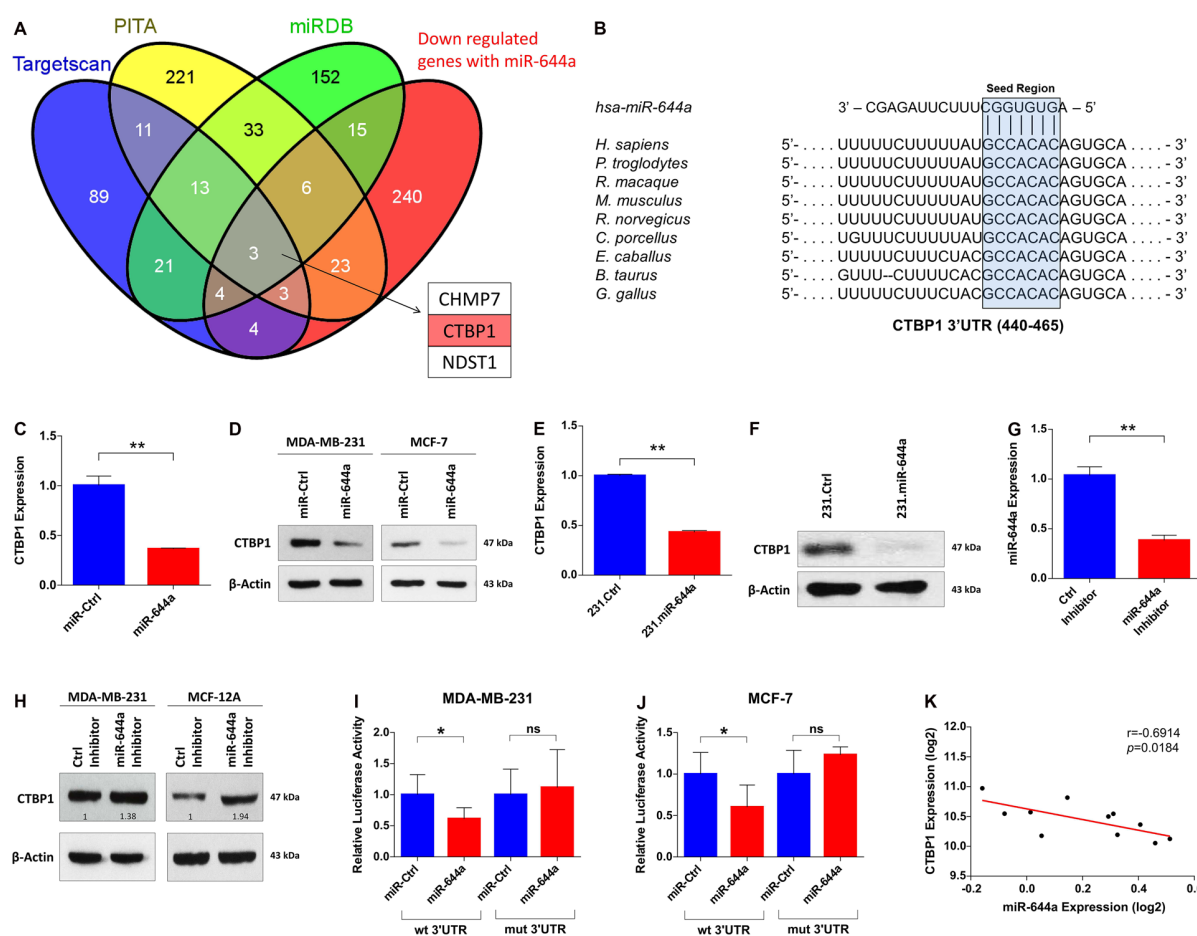


Figure 4: CTBP1 is a direct target of miR-644a. (A) Venn diagram for the combinatorial target prediction analysis. List of genes downregulated by miR-644a in the microarray analysis was combined with genes predicted to be miR-644a targets by three different target prediction algorithms namely TargetScan (blue), PITA (yellow) and miRDB (green). Three genes that are common in all four groups were depicted, with CTBP1 highlighted in red. (B) Schematic diagram showing miR-644a binding site in CTBP1 3'-UTR (453–460) in different species including human. (C and D) Confirmation of CTBP1 downregulation by miR-644a overexpression at transcript and protein levels with qRT-PCR analysis in MDA-MB-231 (C) and with Western Blot analysis in MDA-MB-231 (left) and MCF-7 (right) (D) cells transfected with either miR-Ctrl or miR-644a. $n = 3$ for (C). (E and F) qRT-PCR (E) and Western Blot (F) analysis of CTBP1 expression in 231.Ctrl and 231.miR-644a cells confirming downregulation of CTBP1 in transcript and protein levels in cells stably expressing miR-644a. $n = 3$ for (E). (G) qRT-PCR analysis of miR-644a expression in MDA-MB-231 cells transfected with either a control Inhibitor (Ctrl Inhibitor) or miR-644a Inhibitor. $n = 3$. (H) Western Blot Analysis showing the levels of CTBP1 in MDA-MB-231 and MCF-12A cells transfected with either Ctrl Inhibitor or miR-644a Inhibitor. (I and J) Luciferase activity of a reporter construct fused with either a *wt* or *mut* CTBP1 3'-UTR in MDA-MB-231 (I) and MCF-7 (J) cells transfected with miR-Ctrl or miR-644a. $n = 5$. (K) Expression of miR-644a negatively correlates with CTBP1 expression in 9 breast cancer cell lines and 2 normal breast cell lines from GSE40059.

like state (Figure 5O). Furthermore, CTBP1 knock-outs by sgRNAs or knockdown by shRNA resulted in a significant reduction in anchorage-independent growth (Figure 5P and 5Q; Supplementary Figure S5E). Re-analysis of the GSE2603 dataset [24] revealed that *in vivo* sub-lines of MDA-MB-231 with enhanced lung metastatic ability express substantially higher CTBP1 levels compared to parental cells with limited lung metastatic potential (Figure 5R). We then set to test the effect of CTBP1 in lung metastasis, and demonstrated markedly less colonization of 231.sgCTBP1_1 and 231.sgCTBP1_2 cells to lungs compared to 231.sgCtrl cells as demonstrated by Bouin's fixation and H&E staining of lungs and luciferase assay with lung lysates (Figure 5S–5U).

To elucidate the role of CTBP1 in tumor progression and metastasis in breast cancer patients, we analyzed several patient datasets. In GSE4922 [25] and GSE58644 datasets, CTBP1 levels were found to be correlated with higher tumor size and incidence of developing distant metastasis (Supplementary Figure S5F and S5G). Finally, we retrieved data from the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) project [26], and performed targeted analysis. We found that Stage 2 and 3 breast cancers where cancer starts to spread to nearby lymph nodes feature higher CTBP1 levels compared to Stage 1 breast cancer which has no or only microscopic invasion smaller than 1 mm [27]. There was also a significant increase in CTBP1 levels in Stage 4 tumors, characterized by the presence of metastases to organs other than breast (Supplementary Figure S5H). In accordance with the tumor stage, patients with 20 or more positive lymph nodes had higher CTBP1 in their primary tumors suggesting a role of CTBP1 in promoting lymph node metastasis (Supplementary Figure S5I). All of these findings suggest that CTBP1 inhibition mimics miR-644a overexpression in inhibition of breast tumor growth and metastasis.

CTBP1 is a major functional target of miR-644a mediating drug resistance and EMT

We tested if CTBP1 mediates the effect of miR-644a on drug resistance. *In silico* analysis of GSE16446 dataset [28–30] showed significantly lower levels of CTBP1 in patients with pathologic complete response (pCR) compared to patients without complete response against anthracycline treatment (Figure 6A). In the same dataset, low CTBP1 level was associated with better outcome in terms of distant metastasis-free survival (DMFS) (Figure 6B). In GSE58644, among patients treated with chemotherapy, distant metastasis incidence rate was significantly higher in patients with high CTBP1 (Figure 6C). All these suggest an important role of CTBP1 in tumor recurrence in chemotherapy treated patients.

We then tested if we can mimic the effect of miR-644a on chemotherapy response by knocking

down CTBP1. We observed that knockdown of CTBP1 sensitized MDA-MB-231 cells to doxorubicin (Figure 6D). Furthermore, rescuing CTBP1 levels along with miR-644a overexpression (Figure 6E) not only increased the viability of MDA-MB-231 cells (Figure 6F), but also rendered these cells less sensitive to doxorubicin treatment compared to those cells in which only miR-644a was overexpressed (Figure 6G). Furthermore, we observed a significantly lower levels of CTBP1 in doxorubicin sensitive tumors compared to resistant tumors of the mouse models we developed (shown in Figure 3C), which is exactly the opposite of miR-644a levels in these tumors (Figure 6H). Finally, CTBP1 rescue *via* miR-644a inhibitor transfection promoted mesenchymal-like state by upregulating mesenchymal markers and downregulating epithelial markers; and this phenotype was reversed upon CTBP1 knockdown (Figure 6I). Overall, all these data confirm that CTBP1 is a major functional target of miR-644a mediating drug resistance and EMT in breast cancer.

miR-644a/CTBP1-mediated wild type or mutant p53 upregulation acts as a switch deciding on G1 arrest or apoptosis

The *in vitro* findings that overexpression of miR-644a or loss of its target CTBP1 induces apoptosis in p53-*mut*, but not in p53-*wt* cells triggered us to investigate the relationship between miR-644a, CTBP1, and p53 mutation status. First, we performed an enrichment analysis for apoptosis-related gene sets on p53-*mut* vs p53-*wt* patients that are expressing high levels of miR-644a from GSE22220 dataset [31]. We have separated the patients as p53-*wt* and p53-*mut* according to the expression levels of a gene signature associated with p53 status [32] (details are given in Material and Methods section and Supplementary Figures S8 and S9). We observed that apoptosis genes were significantly enriched in p53-*mut* patients compared to p53-*wt* patients (Figure 7A and 7B). While investigating the underlying mechanism, we found that miR-644a overexpression or CTBP1 knockdown increased the expression of p53 level in both p53-*mut* MDA-MB-231 and p53-*wt* MCF-7 cells (Figure 7C). Furthermore, in 231.miR-644a cells, p53 level was reduced upon CTBP1 ORF expression, which shows that miR-644a mediated upregulation of p53 is *via* CTBP1 downregulation (Supplementary Figure S6A). Notably, overexpression of miR-644a or knockdown of CTBP1 in MCF-7 cells did not change p53 mRNA level, which indicates a possible post-transcriptional regulation of p53 expression (Supplementary Figure S6B and S6C). Overall, these results show that miR-644a induces p53 expression/activity in a p53 status-independent manner.

It has been shown that stabilized p53 activates several genes that induce cell cycle arrest (e.g. p21) and apoptosis (e.g. Noxa, Bax and Puma) [33]. Furthermore, CTBP1 knock-out cells were shown to express high levels

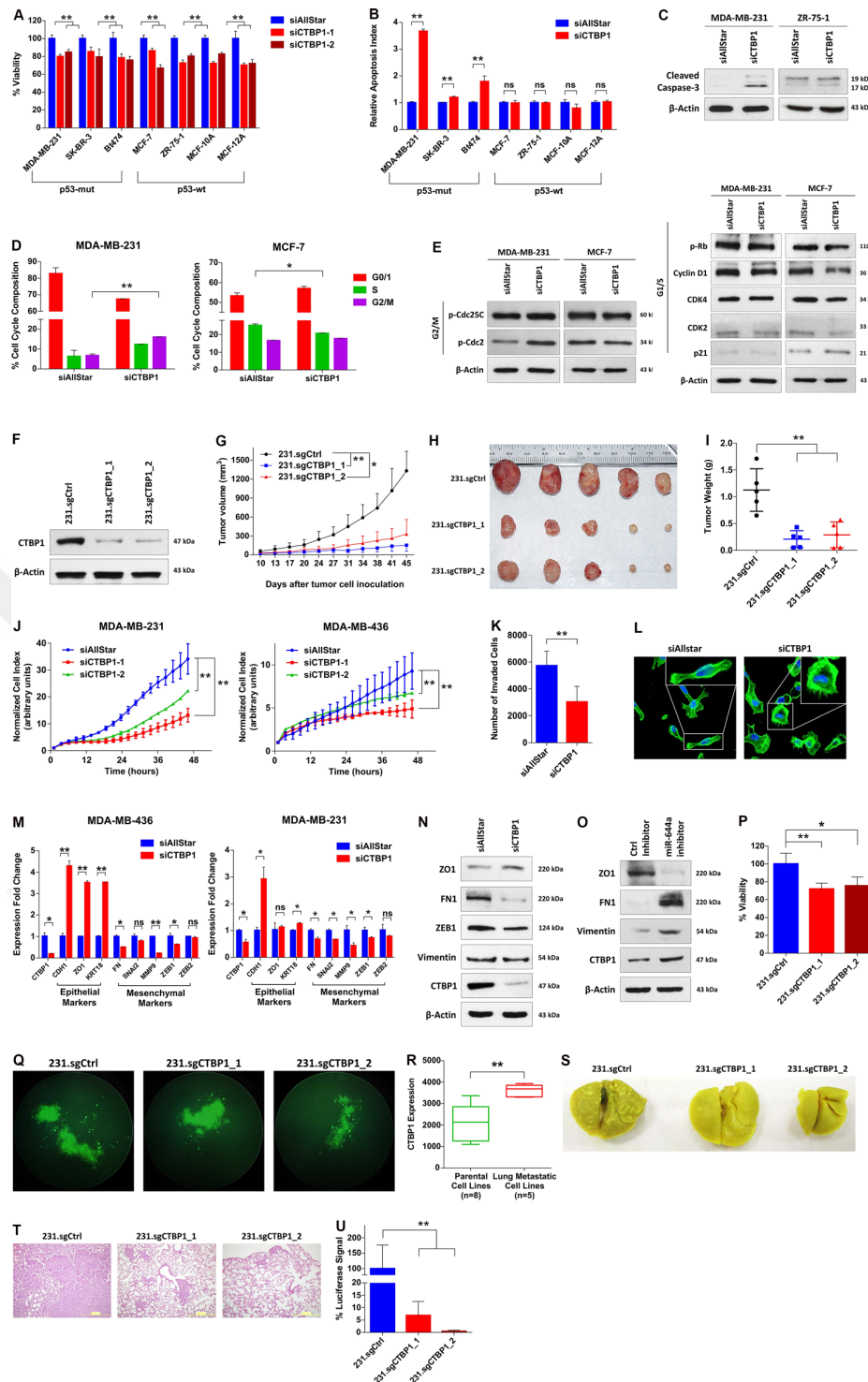


Figure 5: Loss of CTBP1 inhibits cell viability, tumor growth, migration and invasion *in vitro*, and inhibits tumor progression and metastasis *in vivo*. (A) Effect of CTBP1 knockdown on proliferation of cell lines previously used to test the effects of miR-644a overexpression on proliferation as in Figure 1C. Cells were transfected with either a non-targeting siRNA control (siAllStar) or different CTBP1 targeting siRNAs (siCTBP1-1, siCTBP1-2). $n = 4$. (B) Changes in the apoptotic index based on Caspase-3 cleavage in cells from (A) transfected with siAllStar or siCTBP1-Pool. $n = 4$. (C) Western Blot Analysis showing the levels of cleaved Caspase-3 in p53-*mut* MDA-MB-231 (left) and p53-*wt* MCF7 cells (right) after 72 hours transfection with siAllStar or siCTBP1-Pool. (D) Flow cytometric analysis of cell cycle in cells transfected with siAllStar or siCTBP1-Pool showing G2/M arrest in siCTBP1-Pool transfected MDA-MB-231 cells (left) and G1 arrest in siCTBP1-Pool transfected MCF-7 cells (right). (E) Western Blot analysis showing the levels of cell cycle proteins related to G2/M arrest (p-Cdc25C and p-Cdc2) (left) and G1/S (pRb, Cyclin D1, CDK4, CDK2 and p21) (right)

transition in p53-*mut* MDA-MB-231 and p53-*wt* MCF7 cells after 48 hours transfection with siAllStar or siCTBP1-Pool. (F) Western Blot Analysis of CTBP1 levels in MDA-MB-231.luc cells stably expressing either a non-targeting sgRNA (231.sgCtrl) or different CTBP1 targeting sgRNAs (231.sgCTBP1_1, 231.sgCTBP1_2) confirming stable knock-out of CTBP1. (G) Tumor progression in xenografts generated by orthotopic subcutaneous injection of 231.sgCtrl, 231.sgCTBP1_1 or 231.sgCTBP1_2. *n* = 5. (H) Representative images of tumors collected from xenografts of (G) on day 45. (I) Tumor weights in xenografts from (G) at day 45. (J) Real time migration of MDA-MB-231 (left) and MDA-MB-436 (right) cells transfected with siAllStar, siCTBP1-1 or siCTBP1-2, monitored using an RTCA assay. (K) Number of invaded cells transfected with siAllStar or siCTBP1-Pool using Matrigel invasion assay. *n* = 3. (L) Fluorescence microscopy images of MDA-MB-231 cells transfected with either siAllStar or siCTBP1-Pool. Cell nuclei and filamentous actin were stained with DAPI and Alexa Fluor 488 phalloidin, respectively. Images were taken after 72 hours of transfection with 20× magnification. Boxes at the upper right corners of the images show cell morphology with higher resolution. (M) qRT-PCR analysis of epithelial and mesenchymal marker gene expression in MDA-MB-436 (left) and MDA-MB-231 (right) cells transfected with either siAllStar or siCTBP1-Pool. *n* = 3. (N) Western blot analysis of epithelial and mesenchymal marker expression in MDA-MB-231 cells transfected with either siAllStar or siCTBP1-Pool. (O) Western blot analysis of epithelial and mesenchymal marker expression in MCF-12A cells transfected with either Ctrl inhibitor or miR-644a inhibitor. (P and Q) Viability of 231.sgCtrl, 231.sgCTBP1_1 and 231.sgCTBP1_2 cells grown in anchorage-independent conditions for 7 days, quantified by WST-1 assay (P) together with their fluorescence microscopy images with 10× magnification (Q). (R) CTBP1 expression in parental MDA-MB-231 cells, its single-cell-derived progenies (SCPs) and *in vivo* isolated sub-lines with different lung metastatic capabilities from GSE2603 (*n* = 19). (S) Representative images of lungs collected from nude mice injected intravenously with 231.sgCtrl, 231.sgCTBP1_1 or 231.sgCTBP1_2. Mice were sacrificed at week 7 and lungs were fixed in Bouin's Solution. (T) Hematoxylin and eosin staining of metastatic nodules in lungs from (S). (U) Luciferase signal coming from metastatic nodules in lungs of (S) as quantified by a luciferase assay.

of pro-apoptotic genes Noxa and Bax [34]. Therefore, we examined the expression of these in both MDA-MB-231 and MCF-7 cells upon miR-644a overexpression. While the expression of p21 was only upregulated in p53-*wt* MCF-7 cells, Noxa, but neither Bax nor Puma, was significantly upregulated in p53-*mut* MDA-MB-231 cells at mRNA and protein level upon miR-644a transfection (Figure 7D and 7E; Supplementary Figure S6D and S6E). Analysis of commonly upregulated genes by *wt*-p53 in breast cancer patients from Troester *et al.* [32] and loss of CTBP1 in MCF-7 cells from Di *et al.* [35] also identified only p21 and BTG2 (data not shown) which further supported that miR-644a mediated upregulation of *wt*-p53 and p21 is *via* CTBP1 downregulation.

We then asked whether we can induce apoptosis upon miR-644a expression or CTBP1 knockdown in p53-*wt* MCF-7 cells if *mut*-p53 was co-expressed. After confirming successful overexpression of *mut*-p53 with western blot (Figure 7F and 7I), we performed an apoptosis assay followed by qRT-PCR of Noxa. Overexpression of *mut*-p53 alone in p53-*wt* MCF-7 cells induced a certain level of apoptosis and Noxa expression, which is further enhanced either by overexpression of miR-644a or by knockdown of CTBP1 (Figure 7G–7K). This confirms that miR-644a mediates apoptosis in the presence of *mut*-p53 mainly by the pro-apoptotic gene Noxa. Furthermore, presence of *mut*-p53 did not affect downregulation of CTBP1 (Figure 7F and 7I; Supplementary Figure S6F and S6H) suggesting that there is no feedback regulation of CTBP1 by p53 in the miR-644a/CTBP1/p53 axis. Lastly, to validate that p53 serves as a switch in miR-644a/CTBP1/p53 axis in breast cancer, we checked the downstream effects of miR-644a in 6 different breast cancer cell lines (three p53-*wt* and three p53-*mut*) after forced miR-644a expression using mimics. We demonstrated that depending on the p53 status, miR-644a/CTBP1/p53 axis leads to either p21 upregulation (in

case of p53-*wt* cells) or Noxa upregulation (in case of p53-*mut* cells) explaining the observed G1 arrest or apoptosis induction, respectively (Figure 7L).

p53 mutant patients with high CTBP1 level are predicted to have a worse survival

It has been known that p53-*wt* patients have better survival as compared to p53-*mut* ones [36–38]. We confirmed this by analyzing published patient data and showed that p53-*mut* patients survive less than p53-*wt* patients (Supplementary Figure S7A and 7B). Unlike the prognostic relevance of miR-644a signature, no correlation of CTBP1 mRNA levels with the survival of breast cancer patients from GSE58644 and GSE19536 [39] datasets was found (Supplementary Figure S7C and S7D). Therefore, we asked if the expression level of CTBP1 can be associated with the survival of p53-*mut* patients in these datasets. This was indeed the case for both of the datasets as well as an online survival analysis tool, Kaplan-Meier (KM) Plotter [40] (Figure 7M–7O). However, in p53-*wt* patients CTBP1 expression level did not have any significant effect on survival of the patients (Supplementary Figure S7E–S7G). We observed a similar pattern in ovarian cancer where p53-*mut* patients with high CTBP1 levels are less likely to survive compared to patients with low CTBP1 (Figure 7P; Supplementary Figure S7H). Overall, these data suggest that CTBP1 expression may be associated with survival of p53-*mut* breast and ovarian cancer patients.

DISCUSSION

Little is known about the deregulation of miR-644a in cancer. It has been shown that high expression of miR-644a (previously known as miR-644 according to

miRBase release 21 [41]) is correlated with shorter overall survival in Acute myeloid leukemia (AML) [42], and miR-644a is found to be upregulated in bladder cancer [43]. On the contrary, miR-644a overexpression was shown to downregulate an isoform of the androgen receptor, and decrease viability in prostate cancer cell lines [44]. However, there is no functional study on miR-644a, and its deregulation in breast cancer has not been reported. Here, we demonstrated that miR-644a acts as a tumor suppressor in breast cancer by regulating tumor progression, metastasis, and drug resistance affecting the patient survival (Figure 7Q). We identified the transcriptional co-repressor CTBP1 as the major functional target of miR-644a phenocopying all its effects on cell proliferation, apoptosis, EMT and drug resistance. Its loss upon miR-644a

overexpression on one hand leads to G1 arrest or apoptosis by increasing p53 expression and on the other hand leads to increased E-Cadherin to inhibit EMT and metastasis.

It has been known that activated p53 can either lead to apoptosis or cell cycle arrest in a highly context dependent manner. As reviewed by Haupt *et al.* [45] and Fridman *et al.* [46], cell type, strength and nature of the stimulus as well as the presence of collateral signals can determine cell fate in the presence of a stress stimulus. Importance of the latter has been shown in case of DNA damage during which Myc shifts the balance of cell fate from cell cycle arrest to apoptosis by blocking p21 induction *via* recruiting Miz-1 to p21 promoter site, and thereby preventing p53-mediated transcription [47, 48]. Therefore, we also checked changes in Myc expression

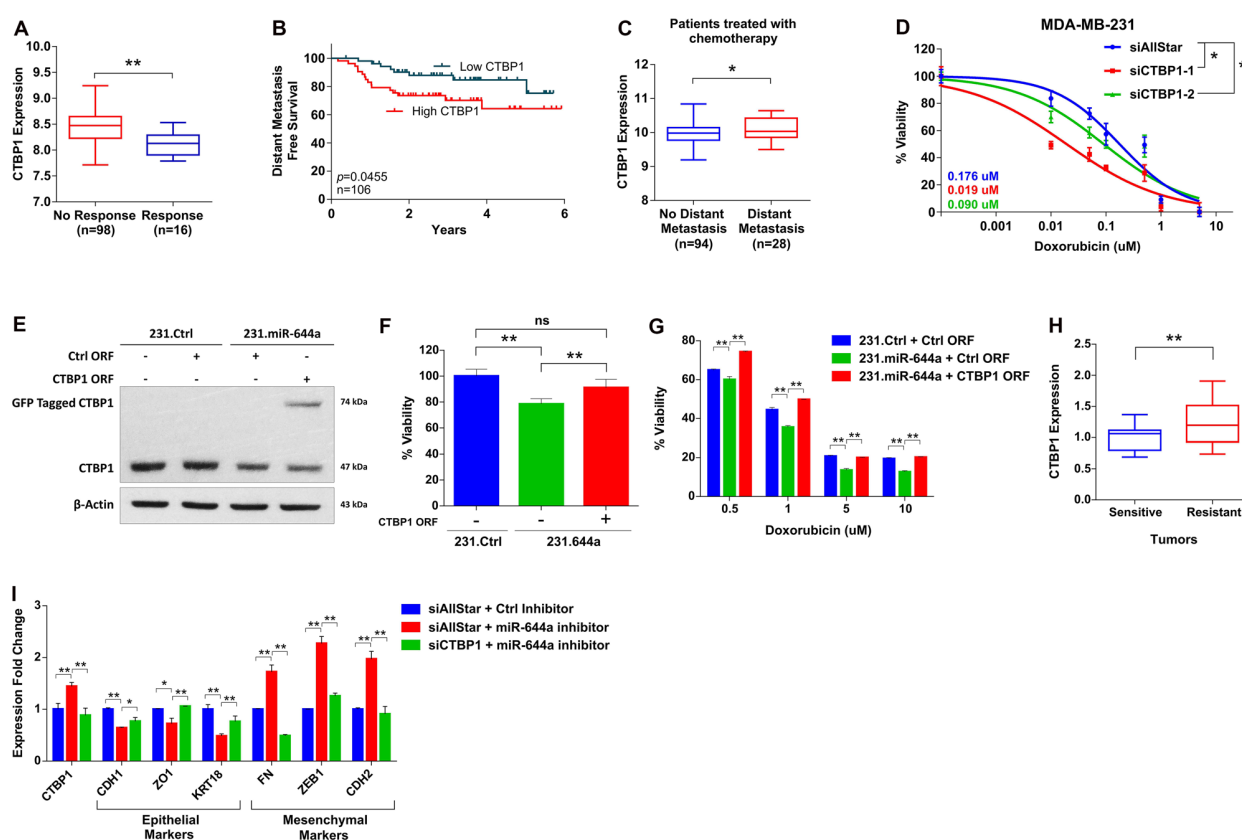


Figure 6: CTBP1 expression correlates with response to chemotherapy, and its loss sensitizes to chemotherapy and enhances epithelial-like state. (A) CTBP1 expression in anthracycline treated breast cancer patients from GSE16446 with no response to treatment ($n = 98$) or pathologic complete response ($n = 16$). (B) Kaplan Meier survival curve representing the percentage distant metastasis-free survival in breast cancer patients treated with anthracyclines based on CTBP1 median expression levels in GSE16446 dataset ($n = 106$). (C) CTBP1 expression in patients with no distant metastasis ($n = 94$) or with distant metastasis ($n = 28$) among breast cancer patients treated with chemotherapy from GSE58644. (D) Effect of CTBP1 knockdown on the response of MDA-MB-231 cells to doxorubicin. Cells were transfected with siAllStar, siCTBP1-1 or siCTBP1-2 and treated with increasing concentrations of doxorubicin. $n = 4$. (E and F) 231.Ctrl or 231.miR644a cells were transfected with Ctrl open reading frame (ORF) or CTBP1 ORF in mentioned combination to rescue the CTBP1 expression. Western Blot analysis showing rescue of CTBP1 expression in MDA-MB-231 (E). Effect of CTBP1 rescue on viability of MDA-MB-231 cells (F). (G) Bar graph showing the effect of CTBP1 rescue as shown in (F) on the response of MDA-MB-231 to 4 different doses of doxorubicin. $n = 4$. (H) qRT-PCR analysis of CTBP1 expression in xenografts sensitive or resistant to doxorubicin that were previously used to test the changes in miR-644a levels upon drug resistance (Figure 3C). $n = 3$. (I) qRT-PCR analysis of epithelial and mesenchymal marker gene expression in MCF-12A cells upon CTBP1 rescue by miR-644a inhibitor and further knockdown by siCTBP1.

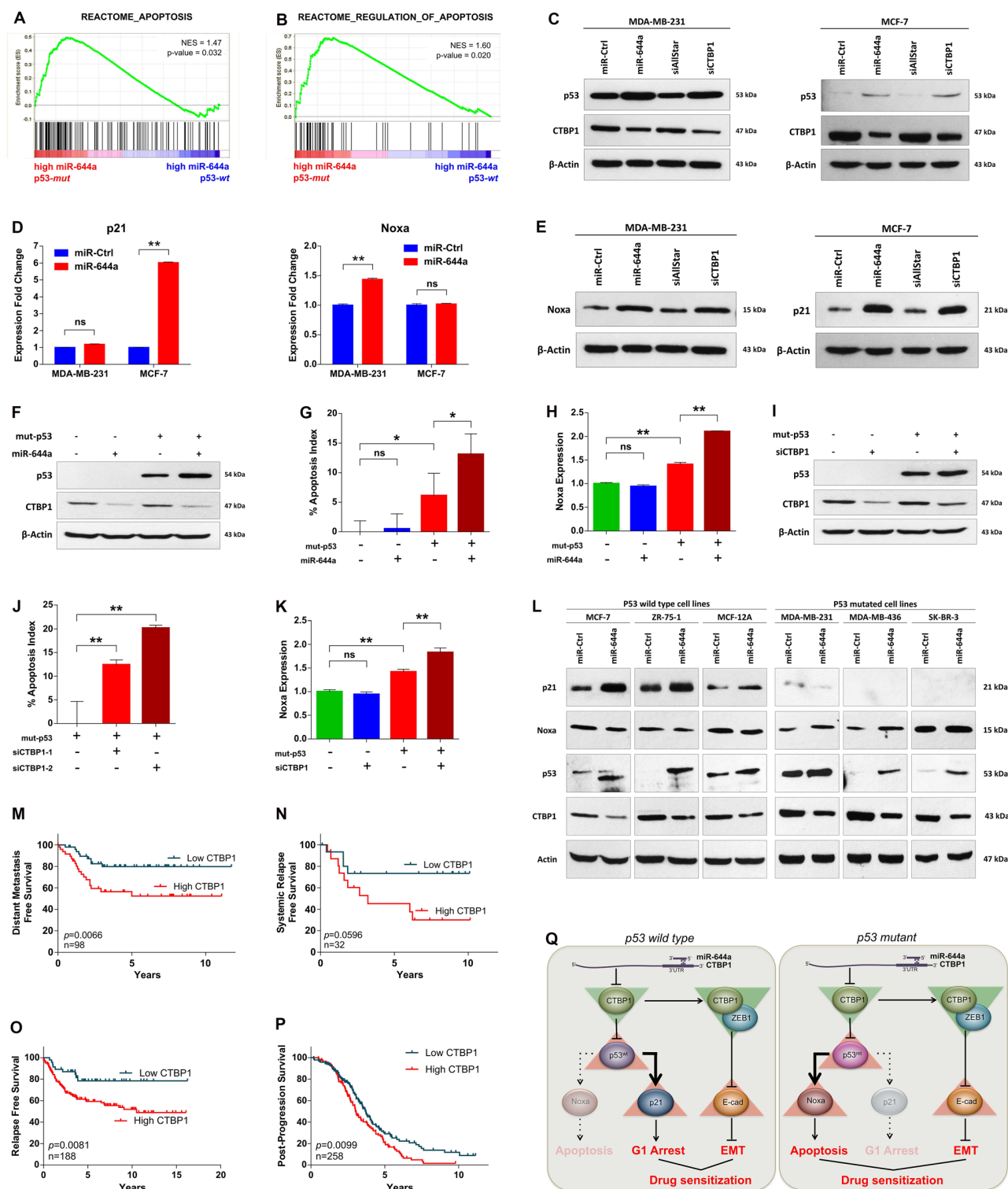


Figure 7: miR-644a/CTBP1-mediated wild type or mutant p53 upregulation acts as a switch on G1-arrest or apoptosis, and CTBP1 expression predicts survival of patients with p53 mutation. (A and B) Enrichment plots of patients from GSE22220 with high miR-644a levels ($n = 105$). Among patients with high miR-644a, genes annotated to Apoptosis (A) and Regulation of Apoptosis (B) pathways in Reactome were significantly enriched in p53-mut group as compared to p53-wt group. (C) Western Blot analysis showing the regulation of p53 in MDA-MB-231 (left) and MCF-7 cells (right) upon miR-644a overexpression or CTBP1 knockdown. (D and E) qRT-PCR (D) and western blot (E) analysis of p21 and Noxa gene expression in MDA-MB-231 and MCF-7 cells upon miR-644a overexpression or CTBP1 knockdown. (F–K) Changes in the apoptotic index based on Caspase-3 cleavage in p53-wt MCF-7 cells transfected with miR-644a (G) or siCTBP1-1, siCTBP1-2 (J) together with *mut*-p53 ORF. Regulation of Noxa expression upon miR-644a overexpression (H) or

CTBP1 knockdown (K) in the presence of *mut*-p53 was shown with qRT-PCR analysis. Overexpression of *mut*-p53 in p53-*wt* MCF-7 cells was confirmed with Western Blot analysis (F and I). (L) Western blot analysis showing CTBP1, p53, Noxa and p21 expression upon miR-644a upregulation using mimics in 6 different breast cancer cell lines. (M and N) Kaplan Meier survival curves of breast cancer patients with p53 mutation based on CTBP1 median expression levels in datasets GSE58644 ($n = 98$) representing percentage distant metastasis-free survival (M) and in GSE19536 ($n = 32$) representing percentage systemic relapse-free survival (N). (O and P) Kaplan Meier survival curve of breast (O) and ovarian (P) cancer patients with p53 mutation based on 'best cut-off' for CTBP1 expression levels in KM Plotter ($n = 188$ for O, $n = 258$ for P) representing percentage relapse-free (O) and post-progression (P) survival. (Q) Schematic description of miR-644a/CTBP1/p53 axis-mediated drug resistance by simultaneous modulation of cell survival and EMT in p53-*wt* (left) and p53-*mut* (right) cells.

with miR-644a overexpression, but did not observe an induction in Myc levels (data not shown), which might be due to the presence of a stimulus other than DNA damage in our system. Here, we propose that the mutation status of p53 is yet another factor that is important for the decision of undergoing either to cell cycle arrest or to apoptosis. Our results demonstrated that increase in p53 levels upon miR-644a overexpression or CTBP1 knockdown increases p21 which protects cells from p53-dependent apoptosis [17], and causes cell cycle arrest in p53-*wt* cells whereas it induces apoptosis *via* increasing the expression of pro-apoptotic gene Noxa in p53-*mut* cells. We saw that overexpression of *mut*-p53 in p53-*wt* MCF-7 cells was enough to shift the balance between cell cycle arrest and apoptosis in favor of apoptosis through upregulation of an established pro-apoptotic BH3-only protein, Noxa, even though p21 was still induced (Supplementary Figure S6G and S6I).

p53 is mutated in 30% of breast cancer which causes several defects in p53 functioning like altered DNA binding affinity or loss of transcriptional activity [49, 50]. However, there is substantial evidence showing that mutant p53 is still able to induce apoptosis through different mechanisms. It has been shown that a transcriptionally inactive mutant p53 can still activate the pro-apoptotic gene Bax upon DNA damage [51], and some transcription-defective mutants retain significant apoptotic activity independent of Bax induction [52, 53]. These suggest that in case of p53 mutation, a "gain of pathway" phenomenon occurs which may involve either transcription-dependent or independent activation of a different set of pro-apoptotic genes [54]. Our results support these findings with regard to induction of apoptosis by miR-644a in p53-mutant breast cancer cells by activation of Noxa. Notably, although we found no correlation of CTBP1 levels with the survival of breast cancer patients (Supplementary Figure S7C–S7H), in all datasets that we analyzed, we confirm that p53-*mut* patients with breast or ovarian cancer that show high CTBP1 level are associated with a worse survival as compared to the patients with low CTBP1 group (Figure 7M–7P). This suggests that CTBP1 could be a potential prognostic factor for breast cancer patients with p53 mutations which may be due to the fact that apoptosis induction is more effective on prolonging overall survival of patients than p53-dependent growth arrest.

As EMT and cell survival are closely related with drug resistance, we examined the effects of miR-644a

and its target CTBP1 on drug sensitization, and observed that overexpression of miR-644a sensitized different breast cancer cells representing different subtypes to both chemotherapy and targeted therapy agents e.g. tamoxifen and gefitinib. In addition, low CTBP1 correlated with better response (Figure 6A) and longer distant metastasis-free survival of breast cancer patients treated with chemotherapy (Figure 6B). We have previously shown that miR-375 blocks EMT and sensitizes MCF-7 cells to tamoxifen [55]. Similarly, it has been shown that miR-147 blocks EMT and sensitizes colon cancer cells to gefitinib [56]. Although these drugs have different targets and mechanisms of action, we reveal miR-644a as a pleotropic sensitizer, which suggests that the inhibition of EMT might be a common nominator for sensitization to all drugs tested. However, as CTBP1 can increase the expression of MDR1 gene transcriptionally [57], we cannot rule out the alternative of possible downregulation of MDR1 upon miR-644a expression, which leads to inhibition of multi-drug resistance. Nevertheless, our results suggest that miR-644a or its target CTBP1 could be a potential drug candidate which can simultaneously block primary tumor growth, metastasis, and finally sensitize cancer cells to several different drugs. A recent study reported a small molecule, NSC95397, which inhibits the interaction between CTBP1 and its binding partners and blocks the CTBP1-mediated transcriptional repression [58]. Although using miRNAs as potential drugs could need longer time, small molecules targeting CTBP1 could act as potential drugs for cancer therapy in the near future. In conclusion, the miR-644a/CTBP1/p53 axis acts not only as biomarker of progression and drug response, but also could be targeted for cancer therapy.

MATERIALS AND METHODS

Cell culture and reagents

Human breast cancer cell lines MDA-MB-231, MCF-7, BT474, SK-BR-3, ZR-75-1 together with normal breast epithelial cell lines MCF-10A and MCF-12A were obtained from ATCC (Manassas, VA, USA). MDA-MB-231 and SK-BR-3 cell lines were cultured with Dulbecco Modified Eagle Medium (Lonza, NJ, USA) while MCF-7, BT474 and ZR-75-1 cell lines were cultured with DMEM supplemented with 0.1% insulin. MCF10A and MCF-12A cell line was cultured

with DMEM (Lonza, NJ, USA) supplemented with 0.1% insulin (0.01 mg/ml), 0.002% EGF (20 ng/ml) (both from Sigma-Aldrich, Saint-Louis, MO, USA). All media were supplemented with 50 U/ml penicillin/streptomycin, 1% non-essential amino acids and 10% fetal bovine serum (Lonza, NJ, USA). All cell lines were tested for mycoplasma contamination regularly using MycoAlert mycoplasma detection kit (Lonza, NJ, USA).

Transient transfection with miRNA mimics, hairpin inhibitors, siRNAs and expression constructs

Transfections were carried out as previously described [59]. Sequence of miR-644a mimic was AGUGUGGCUUUCUUAGAGC. For miRNA mimic viability screen, miRNA mimics were transfected at a concentration of 20 nM for 48 hours. For other experiments, miR-644a and all siRNA transfections were done at a concentration of 40 nM whereas hairpin inhibitors were transfected at a concentration of 100 nM for either 48 hours or 72 hours. 50 ng (for 96-well experiments) or 500 ng (for 6-well experiments) per well of GFP tagged CTBP1 (NM_001012614; Cat. No. RG208594) human ORF Clone and Myc-DDK tagged TP53 (NM_000546; Cat. No. RC200003) human mutant ORF Clone vector were purchased from Origene and used for overexpression and rescue experiments. Latter one expresses transcript variant 1 of Homo sapiens protein p53 having an R175H mutation.

Plasmid construction and site-directed mutagenesis

The construction of plasmid carrying 3'-UTR sequence of CTBP1 gene was done as previously described [9]. The 967 bp length 3'-UTR which contained last exon sequence common to two transcript variants of CTBP1 and annotated CCDS in NCBI Consensus Coding Sequence database [60] was used to design the primers. Primers used were 5'-GGCACTCGAGCTGCTGTGGAAGGTAT-3' and 5'-ATACAAGCGGCCGAGTCACAAACATGATTTTAAAC-3'. For site-directed mutagenesis, the predicted hsa-miR-644a target site of the previously described psiCheck2/CTBP1-3'UTRwt construct were disrupted by four point mutations in the seed region (Supplementary Figure S3A).

Quantitative RT-PCR analysis of mRNAs and miRNAs

Total RNA was isolated using TRIsure (Bioline, Luckenwalde, Germany). cDNA synthesis was performed using RevertAid RT Reverse Transcription Kit (Life Technologies), following manufacturer's instructions. Each real-time PCR assay was carried out in triplicates

using LightCycler 480 SYBR Green I Master kit (Roche). Sequences of the primers were provided elsewhere (Supplementary Table S5). ACTB and HPRT were used as housekeeping genes. Data were analyzed according to $\Delta\Delta C_T$ method [61]. Quantitative RT-PCR analysis for miRNAs was done as previously described [9]. RNU44 was used as the housekeeping gene.

Western blot

Whole-cell lysates were prepared using RIPA lysis buffer containing protease inhibitor Complete Mini (Roche, Basel), anti-phosphatase PhosSTOP (Roche, Basel), 10 mM NaF and 1 mM Na4VO3. Protein concentrations were determined with BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL). Proteins were denatured with 4× loading dye containing 10% SDS and 50% glycerol at 95°C for 5 minutes, and 20 µg proteins were loaded in each lane. Protein samples were separated by SDS-PAGE and incubated with primary antibodies (Supplementary Table S6). Horseradish peroxidase conjugated anti-mouse or anti-rabbit antibodies (Cell signaling Technology, USA) were used as secondary antibodies, and signals were detected by enhanced chemiluminescence (Thermo Scientific, Rockford, IL).

Cell cycle, viability and apoptosis assays

Cell cycle analysis was performed as previously described [62]. Transfections were done in 6-well plates (2.5×10^5 cells per well) in triplicates. For drug sensitization assays, drug treatments were done one day after miRNA mimic or siRNA transfections with doxorubicin (0.05–10 µM), cisplatin (0.001–30 µM), tamoxifen (2.5–40 µM) or gefitinib (0.1–20 µM) for 72 hours. Cell viability (including viability of miRNA mimic screen and drug sensitization assays) and apoptosis were assessed by Cell Titer Glo Luminescent Cell Viability and Caspase-Glo 3/7 assays (Promega, WI, USA), respectively according to the manufacturer's instructions. Cell viability after polyHEMA assay was assessed with WST-1 assay (Roche) according to the manufacturer's instructions.

RTCA proliferation and migration assays

Real time growth and migration of MDA-MB-231 and MDA-MB-436 cells were monitored using xCelligence Real-time Cell Analyzer system (Acea Biosciences, San Diego, CA, USA) as previously described [9, 59]. For invasion assay, cells were transfected as described above and seeded in Matrigel invasion chambers (BD Pharmingen) in 1% FBS DMEM. After 48 hours, number of invaded cells were counted by flow cytometry (FACS Calibur) and analyzed by Cell Quest Pro software (BD Bioscience). In wound healing assay, 1×10^5 cells were seeded on 24-well plates, and

pictures were taken with Axiovert 25 light microscope (Hund, Wetzlar, Germany). PolyHEMA assay was performed as previously described [55].

Dual luciferase reporter assay

Dual-luciferase reporter assay was performed as previously described [9]. Luciferase activity was measured in Synergy HT microplate reader machine (BioTek, Vermont, US) after 24 hours of transfection, and values were normalized to firefly luciferase activity.

Immunofluorescence staining and microscopy

Nuclear staining of cells were done with 4, 6-diamidino-2-phenylindole (DAPI) and filamentous actin were stained with Alexa Fluor 488 phalloidin (1:40; Invitrogen) as previously described [9]. Coverslips were mounted with Shandon Immu-Mount reagent. Images were taken using a Zeiss microscopy.

Lentiviral vector constructs and infection

SMARTchoice human lentiviral hsa-miR-644a shMIMIC hCMV-turboGFP, GIPZ human CTBP1 shRNA vectors with clone IDs V3LHS_380132, V3LHS_398420 and V3LHS_113279 encoding different shRNAs sequences and GIPZ non-silencing lentiviral shRNA control were purchased from Dharmacon (Lafayette, CO). MDA-MB-231.luc cells (a kind gift from Dr. Dihua Yu (MD Anderson, TX)) were transduced with miR-644a viral particles in 24-wells plate, and 96 hours post-transduction, selection with 1 µg/ml of puromycin was started. To produce viral particles with CTBP1 shRNAs and non-silencing shRNA control, 6 µg of each of these vectors and 4.3 µl of trans-lentiviral packaging mix (Dharmacon) were used to co-transfect HEK293FT cells in 6-wells plate with CaCl₂ reagent (Dharmacon). 48 hours post-transfection, first viral particles were harvested. 96 hours post-transduction, selection with 1 µg/ml of puromycin was started. LentiCRISPRv2 vector was a kind gift from Dr. Feng Zhang (MIT, Boston, MA) [63]. For sgRNA design (Supplementary Figure S3B and S3C), candidate target sequences were determined using E-CRISPR tool [64]. For the packaging of pLentiCRISPR/CTBP1 sgRNA1 and pLentiCRISPR/CTBP1 sgRNA2 vectors, 30% confluent HEK293FT cells in 100 mm plates have been co-transfected using 42 µg of these vectors, 31.5 µg of pMD2.G (Addgene) and 21 µg psPAX2 (Addgene). Transfection, transduction and selection were performed as described previously for GIPZ vectors series.

In vivo experiments

All animal experiments have been approved by the animal ethics committee of Bilkent University. For primary tumors, 6–8 week old female athymic Nu/nu

mice were injected with 2×10^6 cells into both left and right mammary fat pads (MFP) subcutaneously without incision, with 5–6 mice per group. Tumor growth was monitored by measuring the tumor volume twice a week with a caliper after tumors become palpable. Tumor volumes were calculated as $\text{length} \times \text{width}^2/2$. All the mice were sacrificed once one of the mice reached threshold of 1500 mm³ volume, and tumors were collected and weighed. For doxorubicin response experiments, primary tumors were developed using above mentioned protocol, and mice were treated with a dose of 5 mg/kg body weight of mice weekly through intravenous injection when tumors became palpable. In the beginning, all tumors showed sensitivity towards treatment and showed decrease in volume. Some of these sensitive tumors were collected after 8–10 weeks of treatment. Later, remaining tumors started to increase in size, and showed resistance towards treatment, and these resistant tumors were collected after 12–15 weeks of treatment. For tail-vein metastasis assay, 1.5×10^6 cells were injected into tail vein of each 6–8 weeks old female athymic Nu/nu mice, with 3–5 mice per group. The mice were sacrificed, and lung metastasis was evaluated once one of the mice became moribund with a luciferase assay. Due to heterogeneous distribution of nodules, three different parts were randomly collected from each lung to make a tissue pool and weighed for normalization. Lung tissues were ground in cold PBS by tissue homogenizer and treated with lysis buffer (Promega). 500 µl lysis buffer was added to 140–180 mg weight. After 15 min incubation, 100 µl of luciferase substrate (Promega) was added to 20 µl of the lysed sample, and luminescence was measured with a luminometer. For Bouin's fixation, harvested lungs were cleaned with PBS and placed into Bouin's solution on a shaker for overnight. After fixation they were kept in 70% alcohol. The lung samples used for Bouin's fixation were excluded from luciferase assay and H&E staining.

miRNA target prediction

Targetscan release 6.2 (<http://www.targetscan.org>), PITA miRNA target recognition from Segal Lab of Computational Biology (<http://genie.weizmann.ac.il>) and miRDB (<http://mirdb.org/miRDB/index.html>) was used as target prediction algorithms for identification of miR-644a targets. Common predicted targets between all three databases and downregulated genes by miR-644a (microarray results) were represented in a Venn diagram using Venny 2.0.

Microarray analysis

Expression profiling data were normalized with quantile normalization. Quality control and differential gene expression analysis was conducted using *limma* in Bioconductor [65]. Microarray data can be retrieved from

NCBI Gene Expression Omnibus (GEO) database with the accession number GSE82058.

Patient data and statistical analysis

Cell line and patient data were retrieved from the NCBI GEO database (GSE4922, GSE2603, GSE16446, GSE19536, GSE22220, GSE38167, GSE40059, GSE45666, GSE58606 and GSE58644) and from the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) project with data deposited in EMBL European Genome-Phenome Archive (<http://www.ebi.ac.uk/ega/>), with an accession number EGAS00000000122. A brief summary of these datasets is provided in Supplementary Table S4. For GSE19536, patient data were retrieved from GSE3985 which is a former study done with the same patient samples and which is providing more detailed patient data. Significance scores of genes up and down-regulated by miR-644a were calculated as previously described [66]. miR-644a signature score was defined as the ratio of the significance scores of genes up and down-regulated by miR-644a. For the GSEA analysis, patients in GSE58644 dataset were grouped based on their miR-644a significance score ratios, which we defined as the miR-644a signature score. For the GSEA analysis with GSE22220 dataset, patients expressing high levels of miR-644a were separated into two groups as p53-*mut* and p53-*wt*. Survival curves were generated using Kaplan-Meier method. Patients without any available survival time or event were excluded from the corresponding patient groups. All separations were done from median. For KM Plotter curves, ‘best cut-off’ option was selected for the separation of patients. Significance of the differences in survival between two groups was calculated by Log-rank (Mantel-Cox) test. For the separation of patients as p53-*wt* or p53-*mut*, data provided by the publishers of the studies were utilized if available. In GSE58644 and GSE22220, the separation was done by clustering patients according to their expression levels of a gene signature associated with p53 status [32]. The accuracy of p53 status prediction with this gene signature was tested by using data from GSE19536 in which p53 status of the patients was provided. 85% and 77% of the p53-*mut* and p53-*wt* patients, respectively could be predicted from their expression levels of the p53 status signature. The generated heatmap is provided as Supplementary Figure S8. Heatmaps for the other datasets with accession numbers GSE58644 and GSE22220 are provided as Supplementary Figure S9. Gene ontology analyses were done with DAVID bioinformatics tool [67]. For the microarray datasets that contain negative expression values, a small number was added such that all values became positive [68]. Comparisons between two groups were made by two-tailed student *t*-test. Significance cut-off were taken as $P = 0.05$. For the real-time cell growth experiments, data were normalized to time of transfection and statistical significance was determined with a paired, two-tailed

student *t*-test. Statistical significance between two dose response curves was also determined with a paired, two-tailed student *t*-test.

ACKNOWLEDGMENTS AND GRANT SUPPORT

This work was supported by EMBO Installation Grant 2791 (ÖŞ), FP7 Marie Curie Career Integration Grant PCIG14-GA-2013-631149 (ÖŞ), TÜBİTAK 2232 Grant 114C060 (ÖŞ), TÜBA GEBİP 2014 (ÖŞ) and the scholarship from Higher Education Commission of Pakistan (UR).

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin*. 2013; 63:11–30.
2. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, et al. Molecular portraits of human breast tumours. *Nature*. 2000; 406:747–752.
3. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011; 144:646–674.
4. Lujambio A, Lowe SW. The microcosmos of cancer. *Nature*. 2012; 482:347–355.
5. Ambros V. microRNAs: tiny regulators with great potential. *Cell*. 2001; 107:823–826.
6. Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor suppressors. *Dev Biol*. 2007; 302:1–12.
7. Raza U, Zhang JD, Sahin O. MicroRNAs: master regulators of drug resistance, stemness, and metastasis. *J Mol Med (Berl)*. 2014; 92:321–336.
8. Wang L, Wang J. MicroRNA-mediated breast cancer metastasis: from primary site to distant organs. *Oncogene*. 2012; 31:2499–2511.
9. Jurmeister S, Baumann M, Balwierz A, Keklikoglou I, Ward A, Uhlmann S, Zhang JD, Wiemann S, Sahin O. MicroRNA-200c represses migration and invasion of breast cancer cells by targeting actin-regulatory proteins FHOD1 and PPM1F. *Mol Cell Biol*. 2012; 32:633–651.
10. Kopp F, Oak PS, Wagner E, Roidl A. miR-200c sensitizes breast cancer cells to doxorubicin treatment by decreasing TrkB and Bmi1 expression. *Plos One*. 2012; 7:e50469.
11. Liu L, Qiu M, Tan G, Liang Z, Qin Y, Chen L, Chen H, Liu J. miR-200c Inhibits invasion, migration and proliferation of bladder cancer cells through down-regulation of BMI-1 and E2F3. *J Transl Med*. 2014; 12:305.

12. Jong HL, Mustafa MR, Vanhoutte PM, AbuBakar S, Wong PF. MicroRNA 299–3p modulates replicative senescence in endothelial cells. *Physiol Genomics*. 2013; 45:256–267.
13. Willers IM, Martinez-Reyes I, Martinez-Diez M, Cuezva JM. miR-127–5p targets the 3'UTR of human beta-F1-ATPase mRNA and inhibits its translation. *Biochim Biophys Acta*. 2012; 1817:838–848.
14. Lee CH, Kuo WH, Lin CC, Oyang YJ, Huang HC, Juan HF. MicroRNA-regulated protein-protein interaction networks and their functions in breast cancer. *Int J Mol Sci*. 2013; 14:11560–11606.
15. Matamala N, Vargas MT, Gonzalez-Campora R, Minambres R, Arias JI, Menendez P, Andres-Leon E, Gomez-Lopez G, Yanowsky K, Calvete-Candenas J, Inglada-Perez L, Martinez-Delgado B, Benitez J. Tumor microRNA expression profiling identifies circulating microRNAs for early breast cancer detection. *Clin Chem*. 2015; 61:1098–1106.
16. Tofigh A, Suderman M, Paquet ER, Livingstone J, Bertos N, Saleh SM, Zhao H, Souleimanova M, Cory S, Lesurf R, Shahalizadeh S, Garcia Lopez N, Riazalhosseini Y, et al. The prognostic ease and difficulty of invasive breast carcinoma. *Cell Rep*. 2014; 9:129–142.
17. Avery-Kiejda KA, Braye SG, Mathe A, Forbes JF, Scott RJ. Decreased expression of key tumour suppressor microRNAs is associated with lymph node metastases in triple negative breast cancer. *BMC cancer*. 2014; 14:51.
18. Jordan VC. Tamoxifen as the first targeted long-term adjuvant therapy for breast cancer. *Endocr Relat Cancer*. 2014; 21:R235–246.
19. Kobayashi S, Shimamura T, Monti S, Steidl U, Hetherington CJ, Lowell AM, Golub T, Meyerson M, Tenen DG, Shapiro GI, Halmos B. Transcriptional profiling identifies cyclin D1 as a critical downstream effector of mutant epidermal growth factor receptor signaling. *Cancer Res*. 2006; 66:11389–11398.
20. Bergman LM, Birts CN, Darley M, Gabrielli B, Blaydes JP. CtBPs promote cell survival through the maintenance of mitotic fidelity. *Mol Cell Biol*. 2009; 29:4539–4551.
21. Birts CN, Harding R, Soosaipillai G, Halder T, Azim-Araghi A, Darley M, Cutress RI, Bateman AC, Blaydes JP. Expression of CtBP family protein isoforms in breast cancer and their role in chemoresistance. *Biol Cell*. 2010; 103:1–19.
22. Pena C, Garcia JM, Garcia V, Silva J, Dominguez G, Rodriguez R, Maximiano C, Garcia de Herreros A, Munoz A, Bonilla F. The expression levels of the transcriptional regulators p300 and CtBP modulate the correlations between SNAIL, ZEB1, E-cadherin and vitamin D receptor in human colon carcinomas. *Int J Cancer*. 2006; 119:2098–2104.
23. Luo D, Wilson JM, Harvel N, Liu J, Pei L, Huang S, Hawthorn L, Shi H. A systematic evaluation of miRNA:mRNA interactions involved in the migration and invasion of breast cancer cells. *J Transl Med*. 2013; 11:57.
24. Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu WP, Giri DD, Viale A, Olshen AB, Gerald WL, Massague J. Genes that mediate breast cancer metastasis to lung. *Nature*. 2005; 436:518–524.
25. Ivshina AV, George J, Senko O, Mow B, Putti TC, Smeds J, Lindahl T, Pawitan Y, Hall P, Nordgren H, Wong JEL, Liu ET, Bergh J, et al. Genetic reclassification of histologic grade delineates new clinical subtypes of breast cancer. *Cancer Res*. 2006; 66:10292–10301.
26. Dvinge H, Git A, Graf S, Salmon-Divon M, Curtis C, Sottoriva A, Zhao YJ, Hirst M, Armisen J, Miska EA, Chin SF, Provenzano E, Turashvili G, et al. The shaping and functional consequences of the microRNA landscape in breast cancer. *Nature*. 2013; 497:378–382.
27. Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A. (2010). eds.: *AJCC cancer staging manual*: New York, NY: Springer).
28. Desmedt C, Di Leo A, de Azambuja E, Larsimont D, Haibe-Kains B, Selleslags J, Delaloge S, Duhem C, Kains JP, Carly B, Maerevoet M, Vindevoghel A, Rouas G, et al. Multifactorial Approach to Predicting Resistance to Anthracyclines. *J Clin Oncol*. 2011; 29:1578–1586.
29. Juul N, Szallasi Z, Eklund AC, Li QY, Burrell RA, Gerlinger M, Valero V, Andreopoulou E, Esteva FJ, Symmans WF, Desmedt C, Haibe-Kains B, Sotiriou C, et al. Assessment of an RNA interference screen-derived mitotic and ceramide pathway metagene as a predictor of response to neoadjuvant paclitaxel for primary triple-negative breast cancer: a retrospective analysis of five clinical trials. *Lancet Oncol*. 2010; 11:358–365.
30. Li Y, Zou LH, Li QY, Haibe-Kains B, Tian RY, Li Y, Desmedt C, Sotiriou C, Szallasi Z, Iglehart JD, Richardson AL, Wang ZC. Amplification of LAPTM4B and YWHAZ contributes to chemotherapy resistance and recurrence of breast cancer. *Nat Med*. 2010; 16:214–U121.
31. Buffa FM, Camps C, Winchester L, Snell CE, Gee HE, Sheldon H, Taylor M, Harris AL, Ragoussis J. microRNA-associated progression pathways and potential therapeutic targets identified by integrated mRNA and microRNA expression profiling in breast cancer. *Cancer Res*. 2011; 71:5635–5645.
32. Troester MA, Herschkowitz JI, Oh DS, He X, Hoadley KA, Barbier CS, Perou CM. Gene expression patterns associated with p53 status in breast cancer. *BMC cancer*. 2006; 6:276.
33. Vousden KH, Lu X. Live or let die: the cell's response to p53. *Nat Rev Cancer*. 2002; 2:594–604.
34. Grooteclaes M, Deveraux Q, Hildebrand J, Zhang Q, Goodman RH, Frisch SM. C-terminal-binding protein corepresses epithelial and proapoptotic gene expression programs. *PNAS*. 2003; 100:4568–4573.
35. Di LJ, Byun JS, Wong MM, Wakano C, Taylor T, Bilke S, Baek S, Hunter K, Yang H, Lee M, Zvosec C, Khramtsova G, Cheng F, et al. Genome-wide profiles of

- CtBP link metabolism with genome stability and epithelial reprogramming in breast cancer. *Nat Commun.* 2013; 4:1449.
36. Kovach JS, Hartmann A, Blaszyk H, Cunningham J, Schaid D, Sommer SS. Mutation detection by highly sensitive methods indicates that p53 gene mutations in breast cancer can have important prognostic value. *Proc Natl Acad Sci U S A.* 1996; 93:1093–1096.
 37. Murakami I, Hiyama K, Ishioka S, Yamakido M, Kasagi F, Yokosaki Y. p53 gene mutations are associated with shortened survival in patients with advanced non-small cell lung cancer: an analysis of medically managed patients. *Clin Cancer Res.* 2000; 6:526–530.
 38. Yang P, Du CW, Kwan M, Liang SX, Zhang GJ. The impact of p53 in predicting clinical outcome of breast cancer patients with visceral metastasis. *Sci Rep.* 2013; 3:2246.
 39. Enerly E, Steinfeld I, Kleivi K, Leivonen SK, Aure MR, Russnes HG, Ronneberg JA, Johnsen H, Navon R, Rodland E, Makela R, Naume B, Perala M, et al. miRNA-mRNA Integrated Analysis Reveals Roles for miRNAs in Primary Breast Tumors. *Plos One.* 2011; 6.
 40. Gyorffy B, Surowiak P, Budczies J, Lanczky A. Online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data in non-small-cell lung cancer. *Plos One.* 2013; 8:e82241.
 41. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res.* 2014; 42:D68–73.
 42. Diaz-Beya M, Brunet S, Nomdedeu J, Tejero R, Diaz T, Pratcorona M, Tormo M, Ribera JM, Escoda L, Duarte R, Gallardo D, Heras I, Queipo de Llano MP, et al. MicroRNA expression at diagnosis adds relevant prognostic information to molecular categorization in patients with intermediate-risk cytogenetic acute myeloid leukemia. *Leukemia.* 2014; 28:804–812.
 43. Scheffer AR, Holdenrieder S, Kristiansen G, von Ruecker A, Muller SC, Ellinger J. Circulating microRNAs in serum: novel biomarkers for patients with bladder cancer? *World J Urol.* 2014; 32:353–358.
 44. Ostling P, Leivonen SK, Aakula A, Kohonen P, Makela R, Hagman Z, Edsjo A, Kangaspeska S, Edgren H, Nicorici D, Bjartell A, Ceder Y, Perala M, et al. Systematic analysis of microRNAs targeting the androgen receptor in prostate cancer cells. *Cancer Res.* 2011; 71:1956–1967.
 45. Haupt S, Berger M, Goldberg Z, Haupt Y. Apoptosis - the p53 network. *J Cell Sci.* 2003; 116:4077–4085.
 46. Fridman JS, Lowe SW. Control of apoptosis by p53. *Oncogene.* 2003; 22:9030–9040.
 47. Herold S, Wanzel M, Beuger V, Frohme C, Beul D, Hillukkala T, Syvaaja J, Saluz HP, Haenel F, Eilers M. Negative regulation of the mammalian UV response by Myc through association with Miz-1. *Mol Cell.* 2002; 10:509–521.
 48. Seoane J, Le HV, Massague J. Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature.* 2002; 419:729–734.
 49. Raycroft L, Schmidt JR, Yoas K, Hao MM, Lozano G. Analysis of p53 mutants for transcriptional activity. *Mol Cell Biol.* 1991; 11:6067–6074.
 50. Wright JD, Lim C. Mechanism of DNA-binding loss upon single-point mutation in p53. *J Biosci.* 2007; 32:827–839.
 51. Speidel D, Helmbold H, Deppert W. Dissection of transcriptional and non-transcriptional p53 activities in the response to genotoxic stress. *Oncogene.* 2006; 25:940–953.
 52. Ryan KM, Vousden KH. Characterization of structural p53 mutants which show selective defects in apoptosis but not cell cycle arrest. *Mol Cell Biol.* 1998; 18:3692–3698.
 53. Haupt Y, Rowan S, Shaulian E, Vousden KH, Oren M. Induction of apoptosis in HeLa cells by trans-activation-deficient p53. *Genes Dev.* 1995; 9:2170–2183.
 54. He M, Rennie PS, Dragowska V, Nelson CC, Jia W. A mutant P53 can activate apoptosis through a mechanism distinct from those induced by wild type P53. *FEBS letters.* 2002; 517:151–154.
 55. Ward A, Balwierz A, Zhang JD, Kublbeck M, Pawitan Y, Hielscher T, Wiemann S, Sahin O. Re-expression of microRNA-375 reverses both tamoxifen resistance and accompanying EMT-like properties in breast cancer. *Oncogene.* 2013; 32:1173–1182.
 56. Lee CG, McCarthy S, Gruidl M, Timme C, Yeatman TJ. MicroRNA-147 induces a mesenchymal-to-epithelial transition (MET) and reverses EGFR inhibitor resistance. *Plos One.* 2014; 9:e84597.
 57. Jin W, Scotto KW, Hait WN, Yang JM. Involvement of CtBP1 in the transcriptional activation of the MDR1 gene in human multidrug resistant cancer cells. *Biochem Pharmacol.* 2007; 74:851–859.
 58. Blevins MA, Kouznetsova J, Krueger AB, King R, Griner LM, Hu X, Southall N, Marugan JJ, Zhang Q, Ferrer M, Zhao R. Small Molecule, NSC95397, Inhibits the CtBP1-Protein Partner Interaction and CtBP1-Mediated Transcriptional Repression. *J Biomol Screen.* 2015; 20:663–672.
 59. Sahin O, Frohlich H, Lobke C, Korf U, Burmester S, Majety M, Mattern J, Schupp I, Chaouiya C, Thieffry D, Poustka A, Wiemann S, Beissbarth T, et al. Modeling ERBB receptor-regulated G1/S transition to find novel targets for de novo trastuzumab resistance. *BMC Syst Biol.* 2009; 3:1.
 60. Pruitt KD, Harrow J, Harte RA, Wallin C, Diekhans M, Maglott DR, Searle S, Farrell CM, Loveland JE, Ruef BJ, Hart E, Suner MM, Landrum MJ, et al. The consensus coding sequence (CCDS) project: Identifying a common protein-coding gene set for the human and mouse genomes. *Genome Res.* 2009; 19:1316–1323.
 61. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001; 25:402–408.
 62. Uhlmann S, Mannsperger H, Zhang JD, Horvat EA, Schmidt C, Kublbeck M, Henjes F, Ward A, Tschulena U, Zweig K, Korf U, Wiemann S, Sahin O. Global microRNA

- level regulation of EGFR-driven cell-cycle protein network in breast cancer. *Mol Syst Biol.* 2012; 8:570.
63. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods.* 2014; 11:783–784.
64. Heigwer F, Kerr G, Boutros M. E-CRISP: fast CRISPR target site identification. *Nature methods.* 2014; 11:122–123.
65. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Molec Biol.* 2004; 3:Article3.
66. Xu J, Acharya S, Sahin O, Zhang Q, Saito Y, Yao J, Wang H, Li P, Zhang L, Lowery FJ, Kuo WL, Xiao Y, Ensor J, et al. 14–3–3zeta turns TGF-beta's function from tumor suppressor to metastasis promoter in breast cancer by contextual changes of Smad partners from p53 to Gli2. *Cancer Cell.* 2015; 27:177–192.
67. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc.* 2009; 4:44–57.
68. Drăghici S. (2012). *Statistics and data analysis for microarrays using R and Bioconductor.* (Boca Raton, FL: CRC Press).



REVIEW

miR-200c: a versatile watchdog in cancer progression, EMT, and drug resistance

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Received: 21 February 2016 / Revised: 5 April 2016 / Accepted: 11 April 2016 / Published online: 20 April 2016
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Abstract MicroRNAs (miRNAs) are 20–22-nucleotide small endogenous non-coding RNAs which regulate gene expression at post-transcriptional level. In the last two decades, identification of almost 2600 miRNAs in human and their potential to be modulated opened a new avenue to target almost all hallmarks of cancer. miRNAs have been classified as tumor suppressors or oncogenes depending on the phenotype they induce, the targets they modulate, and the tissue where they function. miR-200c, an illustrious tumor suppressor, is one of the highly studied miRNAs in terms of development, stemness, proliferation, epithelial-mesenchymal transition (EMT), therapy resistance, and metastasis. In this review, we first focus on the regulation of miR-200c expression and its role in regulating EMT in a ZEB1/E-cadherin axis-dependent and ZEB1/E-cadherin axis-independent manner. We then describe the role of miR-200c in therapy resistance in terms of multidrug resistance, chemoresistance, targeted therapy resistance, and radiotherapy resistance in various cancer types. We highlight the importance of miR-200c at the intersection of EMT and chemoresistance. Furthermore, we show how miR-200c coordinates several important signaling cascades such as TGF- β signaling, PI3K/Akt signaling, Notch signaling, VEGF signaling, and NF- κ B signaling. Finally, we discuss miR-200c as a potential prognostic/diagnostic biomarker in several diseases, but mainly focusing on cancer and its potential application in future therapeutics.

Keywords miR-200c · Epithelial-mesenchymal transition (EMT) · Drug resistance · TGF- β · ZEB1/2 · Cell signaling · Biomarker

Introduction

MicroRNAs (miRNAs), small non-coding RNAs that control gene expression at post-transcriptional level, were discovered only two decades ago, and their importance in cell biology is exponentially increasing since then [1, 2]. Lin-4 gene, rather than being translated into a protein, transcribed into a pair of small RNAs (later termed as miRNAs) which imperfectly base-paired to complementary sequences in the 3'-UTR of different messenger RNAs (mRNAs), thus found to be involved in post-transcriptional gene regulation. Later, the discovery of the second miRNA, let-7 [3], and its sequence conservation across species from flies to human triggered seminal follow-up works to unveil the basic concepts of miRNA biogenesis and function [4–6]. Over the period of the last 15 years, a total of more than 24,000 miRNA loci have been discovered in 206 different species highlighting the potential key regulatory functions of miRNAs in several organisms [7]. To date, miRNAs have been estimated to regulate more than 60 % of human genome [8] and dysregulated miRNA expression has been implicated in regulating various diseases and developmental disorders. Moreover, the packed structure of miRNAs led to their less vulnerability to degradation even in serum environment [9] ensuring their suitability for diagnostic and therapeutic purposes [10–12]. miR-200c was first shown to be dysregulated in several cancer cell lines [13]. Since then, miRNAs from miR-200 family, in general, but miR-200c, in particular, have been explored to have an impact on a large variety of biological processes and have been demonstrated to play crucial roles in epithelial-mesenchymal

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transition (EMT), cell invasion, proliferation, metastasis, apoptosis, autophagy, and therapy resistance in several cancer types [14, 15]. There are more than 400 publications related to miR-200c depicting the importance of this miRNA; thus, the vast information about miR-200c deserves a compilation. In this review, we focus on the molecular functioning of miR-200c with special emphasis on regulation of EMT, cell invasion, therapy resistance, role in signaling pathways, and its potential as a biomarker in diverse diseases including cancer.

Regulation of miR-200c expression

miR-200 family is composed of two clusters: the miR-200a/b/429 cluster containing miR-200a, miR-200b, and miR-429 on chromosome 1p36 and the miR-200c/141 cluster containing miR-200c and miR-141 on chromosome 12p13 (Fig. 1). In addition to four enhancer boxes (E-box 1–4, CAGGTG), miR-200c promoter has two specific sites (Z-box 1 and 2, CAGGTA) restricted for the binding of ZEB transcription factors [16]. ZEB1 alone or bound to different co-repressors such as CtBPs and BRG1 suppresses miR-200c expression [17, 18]. On the other hand, P300/PCAF complex can activate miR-200c promoter via lysine acetylation of ZEB1 resulting in the release of ZEB1 suppression on miR-200c/141 transcription [19]. A study conducted on vMH neural progenitor cells revealed Sox2 and E2F3 as additional transcription factors regulating transcription from the miR-200c/141 gene cluster. Specific binding sites conserved across species (human, mouse, and rat) have been reported for Sox2 and E2F at the distal (in the –1.4 to –1.3 kb region with respect to the

transcriptional start +1 of miR-200c/141 cluster) and proximal promoter regions (–500 to –400 bp), respectively [20]. c-Myb transcription factor also activates miR-200c gene through the binding sites located in –1653 to –1647 bp and –988 to –982 bp regions of the promoter [21]. Furthermore, the p53/p63/p73 family of transcription factors has been reported to positively regulate miR-200c expression in ovarian cancer cells [22]. In mouse embryonic fibroblasts, stemness-related transcription factor, Oct4, in combination with Sox2, has been implicated to serve as an activator of miR-200c gene expression [23].

Epigenetic gene regulatory mechanisms, such as DNA methylation, are also capable of playing a predominant role in regulating the expression of miR-200c (Fig. 1). For instance, in breast cancer cells, miR-200c/141 cluster often undergoes aberrant silencing due to methylation of CpG island located in its promoter region [24]. In advanced stage bladder cancer and poorly differentiated breast cancer cell lines, miR-200 expression, along with miR-205, is found to be silenced either due to promoter hypermethylation or due to direct binding of Twist transcriptional repressor on the promoter [25]. Interestingly, miR-200c, itself, can indirectly act as an epigenetic regulator. For example, ectopic expression of miR-200b or miR-200c in mesenchymal breast cancer cells resulted in partial upregulation of E-cadherin level due to the degradation of ZEB1-histone deacetylase repressor complexes and increase in the acetylation of histone H3 at the E-cadherin promoter [26]. Overall, in addition to transcriptional regulation of miR-200c, epigenetic mechanisms also play a critical role in tuning miR-200c expression and in regulating its downstream effects.

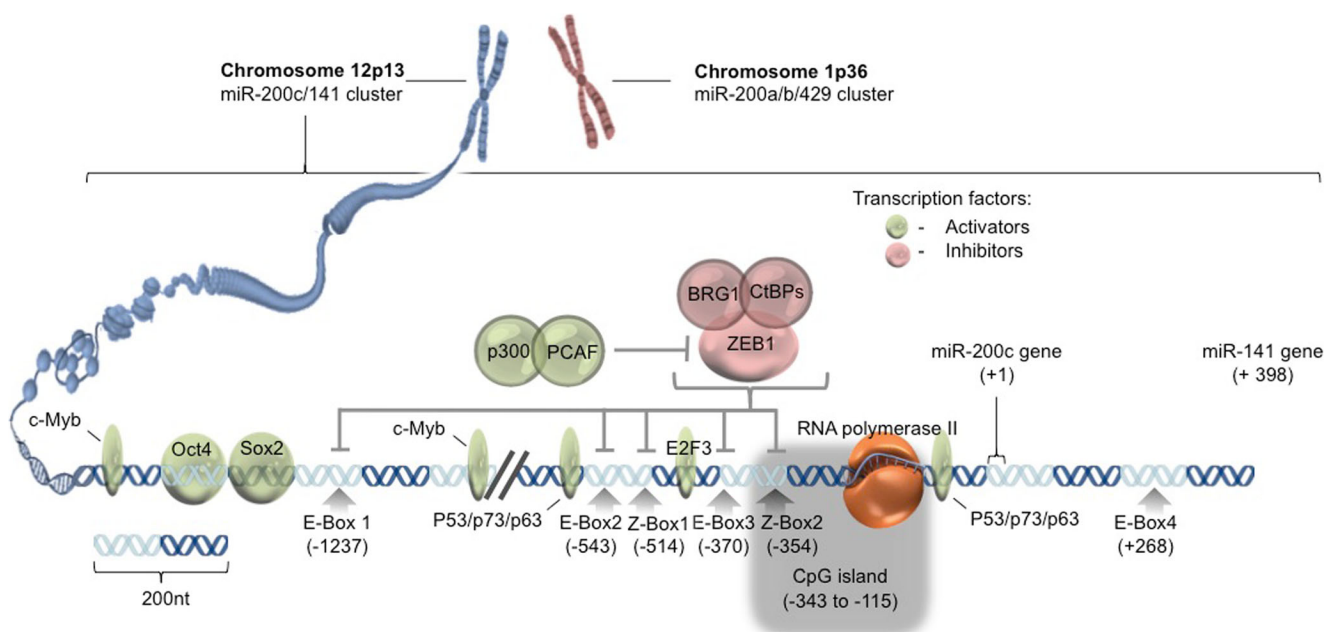


Fig. 1 Regulation of miR-200c expression. Graphical depiction of chromosomal position of miR-200c/141 gene cluster, promoter and enhancer sequences, and transcription factor binding sites

Role of miR-200c in epithelial-mesenchymal transition

EMT, defined as transformation of immotile epithelial cells to motile and invasive mesenchymal cells by losing their cell-to-cell adhesion properties [27], is a crucial step in metastatic cascade. One of the hallmarks of EMT is functional loss of cell surface marker E-cadherin and gain of mesenchymal characteristics via elevating the levels of mesenchymal proteins such as vimentin, fibronectin 1 (FN1), and N-cadherin [28]. Transcription factors associated with EMT, notably ZEB1 and ZEB2, suppress E-cadherin expression and thus trigger the transformation of epithelial cells into mesenchymal state and subsequently initiate the early steps of metastasis [29, 30].

miR-200c inhibits EMT via regulating ZEB1/2/E-cadherin axis

miR-200 family members including miR-200c directly target and repress ZEB1 and ZEB2 expression [31] (Fig. 2), and their frequent loss with concomitant increase in ZEB1 and ZEB2 expression has been observed in different cancers promoting EMT by downregulating E-

cadherin [16, 32, 33]. Conversely, ectopic expression of E-cadherin in mesenchymal cells promotes the reverse process and mesenchymal-epithelial transition, confirming bona fide effects [31, 34]. For instance, via regulating miR-200c in a feedback loop, ZEB1 contributes to harbor stabilized EMT in invading cancer cells [16, 35]. Interestingly, miR-200c-ZEB1 interaction-mediated EMT is also associated with acquisition of cancer stemness as miR-200c suppresses cancer stem cell (CSC)-like features by directly targeting ZEB1 as well as stem cell factors like Bmi1, Sox2, and Klf4 [33, 36]. ZEB1 expression has been shown to be high in embryonic stem (ES) cells, and the expression of miR-200 family members is positively correlated with ESC differentiation [33, 35, 37].

Furthermore, the other factors involved in regulation of miR-200c also hold significant importance in initiating metastatic cascade. For instance, tumor suppressor transcription factor, p53, has been demonstrated to suppress EMT and EMT-related cancer stemness via provoking transcriptional activation of miR-200c and associated downregulation of ZEB transcription factors [38, 39]. In addition, the C subunit of oncogenic mucin 1 (MUC1-C) protein, which induces ZEB1 expression in a nuclear factor kappa B (NF- κ B) p65-dependent manner, can

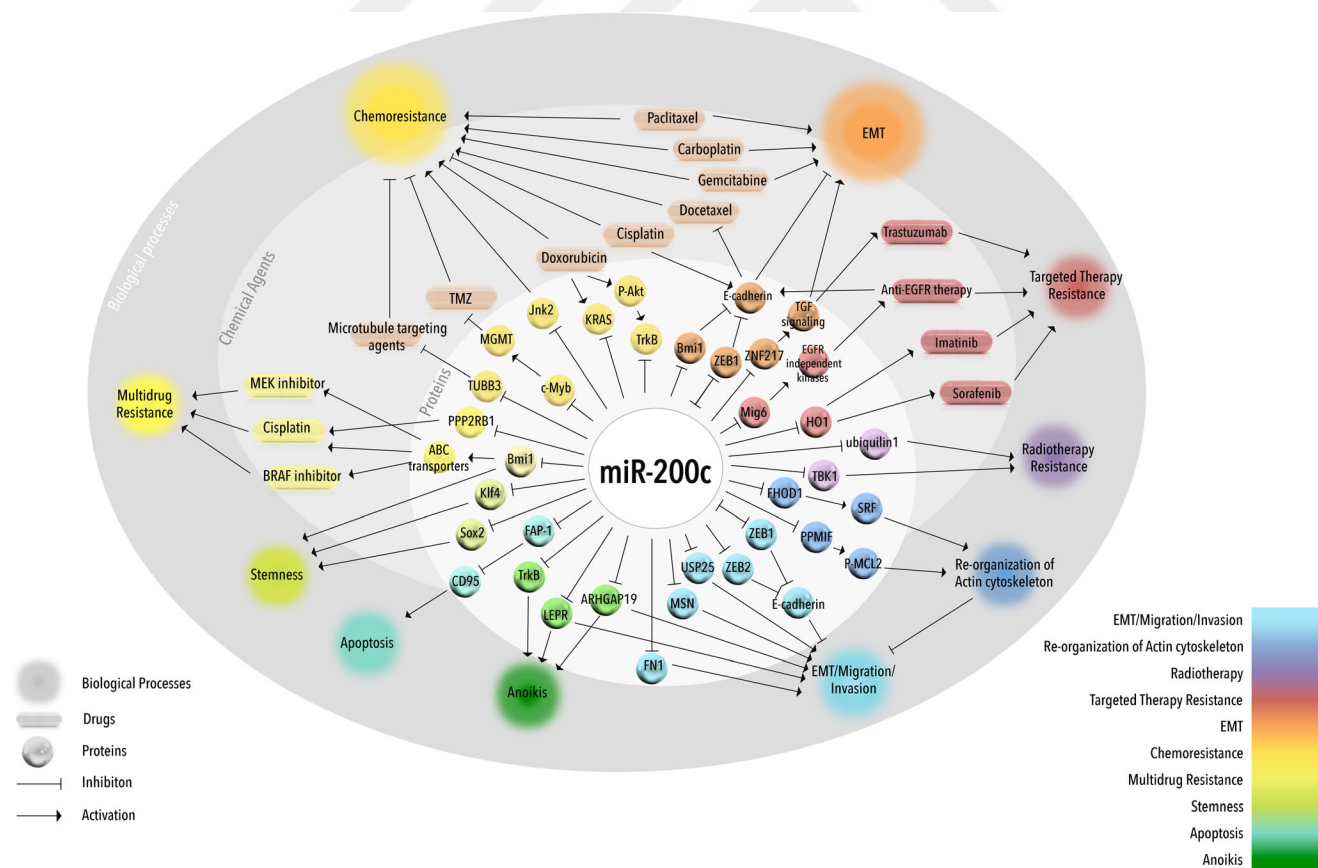


Fig. 2 miR-200c regulates EMT and drug resistance. Graphical representation of miR-200c-mediated regulation of EMT and drug resistance via direct or indirect targeting of several proteins involved in EMT and multiple drug resistance mechanisms

transcriptionally suppress miR-200c by forming a repression complex with ZEB1 and thus can regulate subsequent induction of EMT [40] (Fig. 2).

miR-200c inhibits EMT via regulating targets other than ZEB1/2

Despite its importance, E-cadherin restoration is not always enough for the inhibition of migration and invasion potential of cancer cells; however, it has been shown that miR-200c expression, alone, is enough to inhibit EMT, migration, and invasion which suggests the involvement of other direct targets of miR-200c as EMT regulators [41]. A recent study has suggested miR-200c-mediated repression of protein kinase A subunits as the underlying mechanism of miR-200c-mediated inhibition of migration in breast cancer cells [42]. This effect of miR-200c has also been linked to its targeting of ubiquitin-specific peptidase 25 (USP25) in non-small-cell lung cancer (NSCLC) [43]. In addition, we have demonstrated that miR-200c directly targets actin cytoskeleton regulatory proteins, FHOD1 and PPM1F, and represses migration and invasion of breast cancer cells in a ZEB1/2/E-cadherin-independent manner [41]. Importantly, several molecules linked to cell motility, such as FN1, moesin, neurotrophic tyrosine receptor kinase type 2 (NTRK2 or TrkB), leptin receptor (LEPR), and Rho GTPase-activating protein 19 (ARHGAP19), are among the direct targets of miR-200c, and in aggressive breast and endometrial cancer cells which lack miR-200c, aberrant expression of these targets contributes to the EMT phenotype [44] (Fig. 2). Moreover, not only ZEB2 but also other proteins in ZEB2 and Snail1 transcriptional repressor complexes such as Crtap, FHOD1, Smad2, Map3k1, Tob1, Ywhag/14-3-3 γ , Ywhab/14-3-3 β , Smad5, Zfp36, Xbp1 and Mapk12 are also among the direct targets of miR-200c [45].

Cyclin-dependent kinase inhibitor, p21, is also capable of blocking EMT [46] whereas loss of p21 induces EMT and downregulates several miRNAs, including miR-200c, in a colorectal cancer cell line. Interestingly, overexpression of miR-200c, alone, in these p21-deficient cells inhibited EMT, migration, and invasion [47]. miR-200 family miRNAs are usually repressed in cells subjected to EMT progression. For example, cells, which underwent EMT in response to transforming growth factor beta (TGF- β) or to ectopic expression of protein tyrosine phosphatase, *Pez*, show decreased levels of miR-200 family miRNAs [48]. Using a mouse model system, another study has also shown that TGF- β -mediated EMT progression is indeed correlated with downregulation of miR-200 family miRNAs in mammary epithelial cells [34]. Overall, these studies suggest that both the regulation and regulatory effects of miR-200c are critical determinants in initiating EMT and pursuing the subsequent steps in metastatic cascade.

Role of miR-200c in therapy resistance

In addition to regulation of EMT and metastasis, miR-200 family, in general, and miR-200c, in particular, also take part in regulating therapy resistance. For instance, a recent study with tissue samples from esophageal cancer patients receiving neoadjuvant chemotherapy has clearly shown an inverse correlation between miR-200c expression and sensitivity toward chemotherapeutic agents. The underlying mechanism is proposed to be enhanced activation of the PI3K/Akt pathway, which is preceded by downregulation of a subunit of protein phosphatase 2A, PPP2R1B, a negative regulator of Akt phosphorylation. PPP2R1B is a direct target of miR-200c; therefore, its downregulation through upregulated miR-200c results in the activation of the PI3K/Akt pathway and subsequent resistance against cisplatin [49] (Fig. 2). In addition, higher levels of circulating miR-200c have been observed in patients of castration-resistant prostate cancer (CRPC), particularly, in those showing resistance to docetaxel. Despite this seemingly negative correlation between miR-200c levels and drug sensitivity in patients, most of the *in vitro* studies suggest an opposite link and present miR-200c as an inducer of sensitivity against anti-cancer agents. This discrepancy may be due to the influence of non-tumor cells on the levels of circulating miR-200c as well as the regulatory effects of the tumor microenvironment on cancer cells at the primary tumor site [50]. Several targets of miR-200c are known to play important roles in mediating multidrug resistance, chemo-sensitivity and chemo-resistance, and targeted therapy resistance as well as response to radiotherapy.

miR-200c and multidrug resistance

The role of miR-200c in multidrug resistance was first demonstrated in relation to ATP-binding cassette (ABC) transporters (Fig. 2), the largest transmembrane protein family, responsible for resistance against multiple drugs. In general, overexpression of ABC transporters creates increased drug efflux, resulting in a decreased intracellular drug deposition and a subsequent acquisition of resistance against the given therapy [51]. miR-200c has been shown to play an active role in limiting ABC transporter-mediated multidrug resistance in melanoma as its ectopic expression in these cells was able to downregulate ABC transporters: ABCG2, ABCG5, and multi-drug resistance gene (MDR1). The underlying effect is attributed to direct targeting of *Bmi1* by miR-200c, and knocking down *Bmi1* in these cells results in lower ABC transporter levels and higher sensitivity to a DNA-damaging agent cisplatin, as well as to a BRAF and a MEK inhibitor (PLX4720 and U0126, respectively) [52]. Being a member of polycomb group proteins, *Bmi1* is reported to be upregulated in various cancers and is associated with a more aggressive

disease progression and cancer stemness [53]. From these findings, miR-200c seems to be an important player during multidrug resistance considering the pivotal role of ABC transporters in mediating chemoresistance in a wide variety of cancers.

miR-200c and chemotherapy resistance

Overexpression of miR-200c has been shown to enhance the response to several chemotherapeutic agents in various cancer types. In melanoma cells, miR-200c overexpression results in increased sensitivity to cisplatin [52] (Fig. 2). In endometrial and ovarian cancers, miR-200c increases taxane sensitivity by targeting class III B-tubulin gene (TUBB3), which is known to be a mediator of resistance against microtubule-targeting agents [54, 55]. A recent study, encompassing a panel of ovarian adenocarcinoma cell lines, has also shown the inhibitory action of miR-200c on TUBB3 expression, which also depends on the nuclear localization of an RNA-binding protein, HuR [56]. Another study revealed that decitabine treatment-dependent upregulation of miR-200c and miR-141 promotes epithelial-like characteristics in highly invasive oxaliplatin-resistant colorectal cancer cells [57].

On the other hand, loss of miR-200c has been shown to be associated with acquisition of resistance against various chemotherapeutic agents in different cancer types. Doxorubicin treatment of a breast cancer cell line, BT474, results in decreased miR-200c level, which ultimately leads to an increase in KRAS, a direct target of miR-200c. This study suggests miR-200c as an important checkpoint for the development of chemoresistance mediated through activation of an important oncogenic pathway [58]. In recurrent and metastatic colorectal cancer, miR-200c expression is suppressed, and its overexpression attenuates the JNK signaling pathway resulting in an increased sensitivity of resistant cells to several chemotherapeutic agents and a subsequent decrease in their metastatic potential in both in vitro and in vivo models. Furthermore, miR-200c level is inversely correlated with JNK2, ABCB1, and MMP-9, which are the downstream targets of the JNK pathway, and this negative correlation may be used as a predictive marker of therapeutic outcome in these patients [59]. In two different breast cancer cell lines, resistance against doxorubicin is correlated with marked inhibition of miR-200c where TrkB has been suggested as the key target of miR-200c in regulating this resistance [60]. TrkB plays important roles in differentiation, in proliferation, and mostly, in cell survival mediated through PLC, MAPK, or PI3K/Akt signaling in neurons [61]. Notably, TrkB has also been found to be associated with drug resistance in neuroblastoma [62, 63] and head and neck squamous cell carcinoma [64]. Interestingly, TrkB downregulation after ectopic expression of miR-200c in doxorubicin-resistant breast cancer cells sensitizes the cells toward doxorubicin treatment. Furthermore,

TrkB expression has been correlated with the phosphorylation status of Akt in miR-200c-treated doxorubicin-resistant cells where low p-Akt level has been observed with a concomitant decrease in TrkB protein level [60]. All in all, these studies propose possible mechanisms to explain the multi-factorial nature of a given miRNA (here: miR-200c) in mediating the response of cancer cells against different types of chemotherapy.

miR-200c and targeted therapy resistance

Besides regulating chemotherapy resistance, miR-200c also takes part in mediating targeted therapy resistance (Fig. 2). In a microarray-based study on tamoxifen-sensitive MCF-7 and tamoxifen-resistant LY2 breast cancer cell lines, several novel miRNAs including miR-200c were found to be differentially expressed between sensitive and resistant cells [65]. In a later study by the same group, it has been shown that ectopic expression of miR-200c in endocrine-resistant LY2 cells has significant effect on enhanced sensitivity to fulvestrant, but not to tamoxifen [66]. Targeting the MAPK pathway is a current treatment option for metastatic melanoma patients; however, nearly 50 % of the patients receiving BRAF and MEK inhibitors develop resistance within 6–7 months following treatment initiation. Overexpression of miR-200c also significantly enhanced the anti-proliferative effects of both PLX4720, a specific BRAF inhibitor, and trametinib, a selective inhibitor of MEK1/2 kinase, in melanoma cells [67] suggesting a potential anti-MAPK therapy sensitizer role for miR-200c.

Reduced miR-200c and subsequent increase in its target genes such as Bmi1, ZEB2, TUBB3, ABCG5, and MDR1 have also been shown to promote acquisition of BRAF inhibitor resistance in melanoma. Therefore, these genes could be potential therapeutic targets against development of BRAF inhibitor resistance [68]. In sorafenib- and imatinib-resistant renal cell carcinoma cells, heme oxygenase 1 (HO1) has been found to be a direct target of miR-200c. This enzyme is induced during oxidative stress, and its overexpression following a decrease in miR-200c level in resistant cells is found to be partially responsible for drug resistance by inhibiting apoptosis and autophagy [69]. Exploring druggable targets of miR-200c responsible for conferring drug resistance may help in shaping the strategies to improve the efficacy of chemotherapy and targeted therapy.

miR-200c and radiotherapy resistance

The first line of evidence regarding association between miR-200c and radiotherapy resistance came from a study on breast cancer cells where low levels of miR-200c expression were correlated with radio-tolerance, and ectopic miR-200c expression enhanced the (IR)-induced apoptosis by directly targeting

TANK-binding kinase 1 (TBK1) [70] (Fig. 2). Recently, another target of miR-200c, ubiquitin 1, has been shown to positively regulate radio-resistance by enhancing IR-induced autophagy in MDA-MB-231 breast cancer cells. Ectopic expression of miR-200c reversed the phenotype and sensitized the cells to radiation [71]. Furthermore, nanoparticle-assisted delivery of miR-200c has been shown to inhibit CSC-like properties and to augment radiosensitivity in three different gastric cancer cell lines [72]. Altogether, miR-200c-assisted/conjugated therapies (chemotherapy, targeted therapy, and radiotherapy) may have greater potential to overcome radiotherapy resistance and to limit escape mechanisms in multiple cancer types.

miR-200c at the intersection of EMT and drug resistance

Over the last decade, several lines of evidence show that EMT might render the cells resistant against the implemented anti-cancer therapy supporting the notion that EMT and therapy resistance go hand in hand [73]. In addition, involvement of miRNAs in combined regulation of drug resistance and EMT is evident [74]. Likewise, miR-200c, a negative regulator of EMT, has shown great potential to overcome both chemotherapy and targeted therapy resistance through reversing the EMT process or promoting epithelial-like state (Fig. 2).

miR-200c, EMT, and chemotherapy resistance

Platinum-based chemotherapeutic agents, e.g., cisplatin, are being used as first line treatment for NSCLC, but resistance to these agents is inevitable. In highly aggressive cisplatin-resistant NSCLC cells, miR-200c is lost due to promoter hypermethylation, and forced introduction of miR-200c induces loss of mesenchymal features, which reverses the resistance against cisplatin [75]. In addition, upregulation of several EMT markers including ZEB1/ZEB2 and downregulation of epithelial marker, E-cadherin, are identified as the characteristic features of docetaxel-resistant prostate cancer cells, and the underlying mechanism again correlates with decreased miR-200c expression [76] (Fig. 2). Furthermore, gemcitabine-resistant pancreatic cancer cells showed EMT features along with low miR-200a, miR-200b, and miR-200c levels. Upregulation of these miRNAs upon treatment with two natural agents, 3,3'-diindolylmethane and isoflavone, reversed the EMT phenotype [77]. Moreover, the anti-cancer effects of an anti-diabetic drug, metformin, have been associated with downregulation of several CSC markers, such as CD44 and EPCAM in pancreatic cancer, which, in turn, linked with re-expression of several miRNAs including miR-200c in gemcitabine-sensitive and gemcitabine-resistant cells [78]. Similarly in pancreatic CSCs, decrease in

chemoresistance has been attributed to overexpression of miR-200c [79]. In addition, miR-200c together with other miRNAs from the miR-200 family has been shown to sensitize ovarian cancer cell lines with strong EMT phenotype to anti-cancer drugs paclitaxel and carboplatin [80].

Although miR-200c-mediated attenuation of chemoresistance is mainly associated with reversal of the EMT process, underlying mechanisms are not restricted to the canonical miR-200c/ZEB1/E-cadherin axis but involve other miR-200c targets as well. For instance, in glioblastoma cells, ectopic ZEB1 expression decreased the sensitivity to chemotherapeutic agent, temozolomide (TMZ), in vitro while ZEB1 knockdown resulted in opposite effects in both in vitro and in vivo. ZEB1 was found to be co-localizing with a major chemoresistance enzyme, MGMT, at the edges of glioblastoma xenograft tumors where cells are more invasive and chemoresistant. In principle, increased ZEB1 level in these cells blocks miR-200c expression and subsequently upregulates c-Myb transcription factor (a direct target of miR-200c) which, in turn, activates MGMT transcription resulting in attenuation of anti-proliferative effects of TMZ [81]. Interestingly, a recent study on breast cancer lung metastasis has shown that EMT is dispensable for lung metastasis, but miR-200-mediated reversal of EMT helps in sensitizing metastatic cells to chemotherapeutic drug cyclophosphamide [82]. These results indicate the importance of miR-200c as a key element in determining the fate of chemoresistant cancer cell via regulating EMT.

miR-200c, EMT, and targeted therapy resistance

Association of miR-200c-mediated inhibition of EMT with targeted therapy resistance has been established through several studies. In particular, resistance against anti-EGFR therapies (e.g., cetuximab, gefitinib, erlotinib, etc.) has been demonstrated to be tightly linked with acquisition of EMT phenotypes proceeded by the loss of miR-200c in various cancer types. For instance, in urothelial carcinoma cells, showing mesenchymal-like features and exhibiting resistance against anti-EGFR therapies, a complete reversal of resistance could be acquired with the introduction of miR-200c and subsequent downregulation of ZEB1/2 and upregulation of E-cadherin [83]. In gefitinib-resistant EGFR-mutant lung cancer cell lines, low miR-200c expression due to DNA hypermethylation was found to be correlated with EMT features characterized by increased ZEB1 and decreased E-cadherin levels in these cells [84]. Likewise, reversal of cetuximab resistance by miR-200c is accompanied by the loss of EMT features in highly invasive and aggressive NSCLC cells [75]. Similarly, reversal of erlotinib resistance in EGFR-mutant NSCLC xenografts treated with a potent natural agent, silibinin, is associated with the loss of EMT-related features including low miR-200c and E-cadherin and high ZEB1, Snail, and N-

cadherin expression [85]. However, in a similar study with erlotinib-resistant lung adenocarcinoma cells, overexpressed miR-200c regulates expression of EMT network proteins resulting in a slight increase in erlotinib sensitivity. Furthermore, a TGF- β 1-responsive element is identified at the promoter region of miR-200c, which suggests the suppression of miR-200c in a Smad3/4-dependent manner at the downstream of activated TGF- β signaling. Interestingly, TGF- β 1 is also not able to fully induce EMT and to cause significant changes in erlotinib response in these cells [86]. Therefore, further understanding of the effects of TGF- β signaling on miR-200c-mediated EMT and anti-EGFR resistance could provide more details about the importance of the tumor microenvironment in controlling miRNA expression and response to targeted therapies.

Recently, a study in the context of the TGF- β /miR200c axis explored that prolonged exposure to TGF- β not only facilitates EMT but also makes cells undergo EMT-associated kinase switch, which results in EGFR inhibitor resistance characterized by decrease in miR-200c levels and upregulation of one of its direct targets, Mig6/ERRFI-1. Mig6 is a negative regulator of the EGFR family, and its overexpression increases the internalization of EGFR and thereby reduces its localization to the surface. Despite having lower EGFR activation, cells overexpressing Mig6 show higher Akt phosphorylation through activation of EGFR-independent tyrosine kinases and thereby increase their survival. Furthermore, elevated Mig6/miR-200c ratio is found to be associated with erlotinib resistance in a panel of 25 tumor cell lines and in patient-derived xenografts (PDXs) of NSCLC and pancreatic cancer [87]. Introduction of miR-200c to bladder cancer cells results in complete reversal of resistance against anti-EGFR therapies mainly through Mig6 downregulation, which in turn leads to increased surface levels of EGF receptors, thus making the anti-EGFR therapy more effective in inhibiting the signal [83]. Recently, a feedback loop between miR-200c and TGF- β pathway was discovered in which miR-200c can regulate TGF- β signaling by directly targeting ZNF217, a transcriptional activator of autocrine TGF- β . Elevated TGF- β signaling leads to increased invasiveness as well as trastuzumab resistance (Fig. 2). In trastuzumab-resistant breast cancer cells, miR-200c was found to be the most significantly downregulated miRNA, and its ectopic expression decreases invasiveness and increases trastuzumab sensitivity through downregulation of ZNF217. Besides ZNF217, ZEB1 expression was also shown to be significantly upregulated in trastuzumab-resistant cells. Double knock-down of ZEB1 and ZNF217 decreased invasiveness and partially restored trastuzumab sensitivity. Furthermore, miR-200c treatment of xenografts of a trastuzumab-resistant breast cancer cell line leads to re-gain of trastuzumab sensitivity [88]. In the same scenario, another level of regulation comes from a long non-coding RNA, Lnc-ATB, which is expressed at the

downstream of TGF- β signaling and competitively binds to miR-200c conferring ZEB1 and ZNF217 (upregulation)-mediated trastuzumab resistance [89]. In summary, these studies clearly provide the cue that miR-200c could efficiently control the response of cancer cells to anti-cancer therapies through EMT-dependent and EMT-independent pathways.

miR-200c regulation of signaling pathways

Over the years, it has been well established that miR-200c plays critical roles in regulating multiple biological processes and signaling cascades varying from MAPK signaling, to Notch signaling, to TLR signaling (Fig. 3). In the context of developmental program, miR-200c has been found to accumulate in epithelial buds in developing submandibular glands and control FGFR-mediated epithelial proliferation by targeting vldlr and its ligand reelin which regulate FGFR signaling in these cells [90]. Furthermore, miR-200c-mediated activation of BMP signaling via upregulation of amelogenin and E-cadherin and via downregulation of noggins triggers tooth development and renewal through dental epithelial cell differentiation in mice [91].

IGF-induced PI3K/Akt and MAPK signaling can enhance EMT and metastasis by downregulating miR-200c levels in gastric cancer, which can be restored by Cbl-b-mediated ubiquitination of IGF-IR [92] (Fig. 3). Recent data showed that miR-200c negatively regulates KRAS as well, resulting in inhibition of both PI3K/Akt and MAPK pathways in breast and lung cancer cells [58, 93]. TGF- β -induced expression of ZEB transcription factors downregulates miR-200c resulting in reduced EGFR activity due to upregulation of Mig6 and confers EMT [87]. On the contrary, TGF- β -induced upregulation of miR-200c has been shown in diabetic nephropathy where miR-200c targets FOG2, which is a negative regulator of the PI3K/Akt pathway [94]. miR-200c also suppresses TGF- β signaling by targeting ZNF217 in breast cancer cells, an indirect mechanism to target ZEB-induced metastasis [88].

In addition to the well-characterized role of miR-200 family miRNAs in the regulation of EMT, miR-200c has been widely reported to take part in controlling tumor progression either by regulating or being regulated by different signaling mechanisms. Regarding cell death signaling, miR-200c sensitizes breast cancer cells to anoikis by targeting NF- κ B-induced TrkB and NTF3 [44, 95] and enhances Fas-associated apoptosis by targeting FAP-1 in different cancer cell types [96] (Fig. 3). Additionally, in triple negative breast cancer (TNBC), miR-200c has been shown to enhance apoptosis by directly targeting X-linked inhibitor of apoptosis (XIAP) which serves as E3 ubiquitin ligase and degrades caspases essential for apoptosis [97]. In undifferentiated human ESCs, miR-200c is upregulated, and its knock-down downregulates Nanog expression and enhances

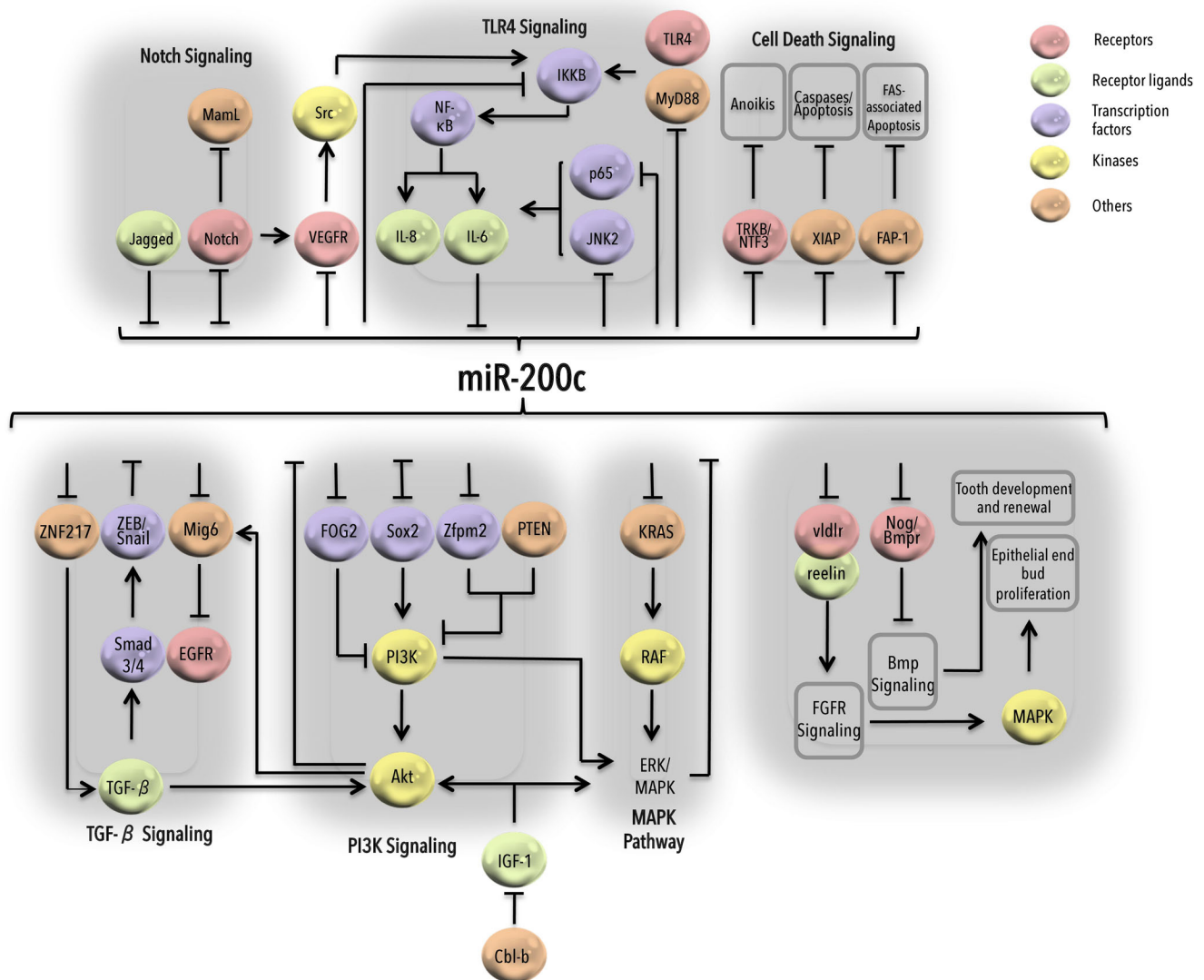


Fig. 3 miR-200c in signaling pathways. Graphical representation of involvement of miR-200c in multiple signaling cascades and their cross-talk

GATA4 levels resulting in apoptosis [98]. Furthermore, Oct4 and Sox2 can bind to miR-200c promoter and enhance MET and induce pluripotent stem cell development by suppressing ZEB2 [23]. miR-200c was found to be downregulated in normal mammary progenitor cells and breast CSCs. When overexpressed, it targets Bmi1, a known regulator of stem cell self-renewal, and suppresses the mammary duct formation ability of mammary stem cells and tumor growth driven by breast CSC [36]. In colorectal cancer, miR-200c regulates stemness by targeting Sox2 in a double negative feedback loop, and loss of miR-200c resulted in enhanced expression of stem cell markers (CD166, CD133, and beta-catenin) downstream of Sox2. In the same study, miR-200c-Sox2 feedback loop has also been shown to regulate the PI3K/Akt pathway and control the proliferative and metastatic potential of colorectal cancer via phosphorylation of PI3K by Sox2 [99]. On the contrary, in pancreatic and ovarian cancer, miR-200c

has been shown to be directly targeting Zfp2 and indirectly reducing Pten levels, both of which are negative regulators of the PI3K/Akt pathway [100, 101].

Notch signaling determines accurate growth and development of various tissues and organs, and its aberrant activation may lead to cancer. Overexpressing miR-200c in metastatic prostate cancer impedes proliferative potential of cancer cells by directly targeting Jagged1 receptors involved in Notch signaling [102] whereas overexpression of Notch1 decreases miR-200c levels in pancreatic cancer [103] (Fig. 3). Notch also activates truncated isoform of Flt1/VEGFR-1 (which is downregulated in the presence of miR-200c [104] and of importance in angiogenesis-associated VEGF signaling) leading to activation of proto-oncogene Src, which cross talks with and downregulates miR-200c in an NF- κ B/IL-6-dependent manner [105]. miR-200c regulates TLR4/MyD88-dependent activation of NF- κ B by directly targeting MyD88.

Interestingly, TAB2, TRAF6, and IRAK1, at the upstream of NF- κ B activation in TL4/MyD88 and TNF- α signaling, are also among the potential targets of miR-200c depicting multi-step targeting of signaling components by miRNAs [106]. In breast cancer cells, MUC1-C induces EMT by upregulating ZEB1 in an NF- κ B/p65-dependent manner and downregulating miR-200c by making a complex with upregulated ZEB1 [40]. IL-6-mediated suppression of miR-200c led towards activation of its downstream targets JNK2 and p65 (required for constitutive activation of IL-6) whereas loss of IL-6 in a mouse model impaired tumorigenicity due to activation of miR-200c [107]. Overexpressing miR-200c also controls inflammation-associated IL-8 in leiomyoma (smooth muscle tumor) by targeting IKKB at the upstream of NF- κ B, thus decreasing p65 binding at IL-8 promoter [108]. Overall, in addition to playing important roles in EMT and metastasis, miR-200c has been adapted to regulate multiple normal growth, development, cancer, and other diseases.

miR-200c as a biomarker

Similar to proteins, expression level of individual or group of miRNAs has been suggested to be utilized as biomarkers to predict disease progression, diagnosis, and treatment response. In this scenario, dysregulated expression of miR-200c in multiple cancer-related mechanisms, which we have discussed here, is enough to support its use as a biomarker, at least in cancer. miR-200c can both be (i) an intracellular marker in tissues (cancer and normal) and (ii) an extracellular marker in body fluids (blood, urine) as free molecules or secreted in exosomes (Table 1).

As an intracellular marker, significantly downregulated miR-200c together with downregulation of miR-335 and miR-218 and upregulation of miR-122, miR-155, and miR-210 has been anticipated to be used as a potential biomarker of pathogenesis for clear cell renal cell cancer [120]. In addition, miR-200c has been suggested as predictor of survival and biomarker of relapse in stage I epithelial ovarian cancer patients [119]. In Caucasian and Asian intrahepatic cholangiocellular carcinoma (ICC) patients, miR-200c downregulation resulted in enhanced EMT which led towards increased cell motility and invasion capacity of ICC as well as induced hepatic stem cell-like gene expression pattern, such as NCAM1 upregulation [111]. In bladder cancer, low levels of miR-200c along with miR-141 and miR-30b are associated with poor prognosis [123]. In TNBC, reduced expression of miR-200c along with miR-205 has been found to be significantly associated with lymph node metastasis [124]. Recently, it has been revealed that miR-200c levels are inversely correlated with advanced clinical stages and lymph node metastasis of ovarian cancer [47]. In intraductal papillary mucinous neoplasm (IPMN), the high-grade tumors were demonstrated to

have EMT characteristics such as low E-cadherin and high vimentin and ZEB1 levels. Furthermore, miRNA array analysis identified miR-200c and miR-141 to be downregulated in malignant tumors as compared to low-grade IPMN tumors. This underpins the importance of the miR-200c-ZEB1 pathway in the progression of IPMN and presents it not only as a prognostic biomarker but also as a potential target for IPMN therapy [113].

In addition to EMT and metastasis, miR-200c expression has potential to predict therapy resistance as well. Ovarian and breast cancer patients showing incomplete response to paclitaxel treatment expressed low levels of miR-200c along with significant increase in beta-tubulin and ZEB1 expression when compared with patients showing complete response to paclitaxel [109, 118]. Furthermore, in NSCLC patients, high expression of miR-200c has been found to be correlated with bigger tumor size and worse overall survival suggesting an oncogenic activity of miR-200c [114, 115]. In terms of tumor subtype classification, oncocytoma and chromophobe structures in renal tumors can be distinguished according to miR-200c and miR-139-5p expression patterns [121]. Moreover, miR-200c together with differential expression of other miRNAs is a candidate to distinguish hepatocellular carcinoma (HCC) from intrahepatic cholangiocarcinoma [112].

Besides its potential to be used as a biomarker in tissues, miR-200c level in serum as circulating miRNA has also been proven to be a promising prognostic and diagnostic factor for several diseases including various cancer types. For instance, increased levels of circulating miR-200 family members in serum have been shown to be associated with serous epithelial ovarian cancer (SEOC) [131] (Table 1). High level of miR-200c in sera of ovarian [134] and esophageal cancer patients [135] is also correlated with poor prognosis. Furthermore, presence of miR-200c in patients' sera as circulating miRNA has been validated as a diagnostic marker for gastric cancer [127]. miR-200c has been shown as a significant predictor of tumor stage in colorectal cancer as high level of this miRNA in serum is able to significantly discriminate stage IV from stage I–III colorectal cancer patients. In addition, these high serum levels are positively correlated with lymph node metastasis, distant metastasis, poor prognosis, and tumor recurrence in colorectal cancer [125]. Similarly, in prostate cancer, together with four other circulating miRNAs, elevated levels of miR-200c have been shown to be higher in sera taken from patients with metastatic castration-resistant prostate cancer as compared to healthy controls [132]. Dysregulated level of miR-200c in serum has also been observed in non-cancer diseases such as Crohn's disease [126] and Kawasaki disease [128] which further proves its fate as a diagnostic biomarker.

Circulating tumor cells (CTCs), on their own, are shown to be indicators of cancer metastasis, but are

Table 1 miR-200c as a biomarker

Biomarker	Disease	Status	Reference
Intracellular marker			
Tissues	<i>Breast cancer</i>	Paclitaxel treatment response	[109]
	<i>Colorectal cancer</i>	Metastasis	[110]
	<i>ICC</i>	Diagnosis, subtype Classification, EMT	[111, 112]
	<i>IPMN</i>	Malignancy, EMT	[113]
	<i>Non-small cell lung cancer</i>	EMT, metastasis, Gemcitabine drug response	[114–117]
	<i>Ovarian cancer</i>	Paclitaxel treatment response	[118]
	<i>Epithelial ovarian cancer</i>	Patient survival	[119]
	<i>Renal cell carcinoma</i>	Diagnosis, prognosis, metastasis, subtype classification	[120–122]
CTCs	<i>Bladder cancer</i>	Prognosis	[123]
	<i>Breast cancer</i>	Metastasis	[124]
Extracellular marker			
Serum	<i>Breast cancer</i>	Metastasis (via CTC)	[50]
	<i>Colorectal cancer</i>	Prognosis, metastasis	[125]
	<i>Crohn's disease</i>	Diagnosis	[126]
	<i>Gastric cancer</i>	Diagnosis, prognosis	[127]
	<i>Kawasaki disease</i>	Diagnosis	[128]
	<i>Lung cancer</i>	Prognosis	[129]
	<i>Ovarian cancer</i>	Diagnosis (via exosomes)	[130]
	<i>SEOC</i>	Diagnosis	[131]
	<i>Prostate cancer</i>	Metastasis	[132]
	<i>Chronic kidney diseases</i>	Diagnosis (via exosomes)	[133]

Diseases and their status related to miR-200c expression are listed with the location where significant miR-200c expression has been detected to be used as a biomarker

ICC intrahepatic cholangiocarcinoma, *IPMN* intraductal papillary mucinous neoplasm, *CTC* circulating tumor cells, *SEOC* serous epithelial ovarian cancer

difficult to detect [136]. Deep miRNA-array analysis of circulating miRNAs in the sera of metastatic breast cancer patients and healthy controls has demonstrated that miR-200c levels were significantly higher in CTC-positive patients compared to CTC-negative patients and healthy controls suggesting a role for circulating miRNAs in predicting CTC status and associated metastatic disease [50].

miRNAs are not only circulating in extracellular fluids, but they are also secreted from tissues to their extracellular environment in exosomes. For instance, levels of miR-200c and seven other miRNAs in tumor-secreted exosomes were found to be similar to those in ovarian tumor cells confirming diagnostic potential of profiling circulating tumor exosomes [130]. Furthermore, exosomal secretion of miR-200c in urine has been observed in various chronic kidney diseases [133] and in systemic diseases such as lupus [137]. As a whole, miR-200c has a huge potential to be used as a biomarker of diagnosis, disease progression, and predicting treatment response.

Future perspectives

The role of miR-200 family, especially miR-200c, in regulating cancer metastasis and acquisition of drug resistance, two major interlinked hallmarks of cancer, has been very well established. However, a new insight came from a recent study in breast cancer where miR-200 overexpression has been shown not to affect onset of lung metastasis but to increase post-metastatic chemosensitivity suggesting the potential of miR-200-based EMT-targeting strategy in enhancing chemosensitivity [82]. Further understanding of underlying mechanisms will help improve and re-shape future EMT-targeting strategies.

With the advances in the field of non-coding RNAs over the last decade with the help of technological advancements in deep sequencing, researchers have now started focusing on the interactions among various kinds of non-coding RNAs, e.g., long intergenic RNAs, circular RNAs and miRNAs, etc., to understand the developmental and disease-associated mechanisms. Recently, a long non-coding RNA named ATB

has been shown to be activated downstream of TGF- β , which competitively binds to miR-200 family in HCC cells, upregulates ZEB1/2 transcription factors, and induces EMT [138]. Therefore, future research on miR-200c function should also include lncRNAs, in addition to proteins, to have a better understanding of its mechanism of action.

Importantly, cell-cell interaction via exosome transport within the tumor microenvironment is of great importance in maintaining tumor state and developing drug resistance. In this line, pre-treatment of A549 cells with exosomes collected from cisplatin-treated counterparts decreased miR-200c expression along with altering the expression of different miRNAs [139]. Untangling the puzzles of the tumor microenvironment will further improve our knowledge about involvement of miR-200c and its trafficking in regulating tumor state.

Tumor suppressor miRNAs, like miR-200c, have potential to be used as therapeutic agents, but their delivery and stability in body fluids is of great concern. Major challenges include (1) poor penetration of miRNAs in tumor tissues, (2) miRNA degradation in body fluids, (3) miRNA-associated immunotoxicity and neurotoxicity, (4) inefficient gene silencing due to poor intracellular delivery of miRNAs, (5) off-target effects, and (6) unavailability of sufficient miRNA-processing enzymes. Multiple miRNA modification techniques have been described to better overcome these barriers (reviewed in [140]). For example, targeted delivery of aptamer-conjugated let-7g in a xenograft model of lung adenocarcinoma has shown promising results in reducing tumor growth [141]. In addition, combining miRNAs with other therapy agents is another option to better utilize miRNAs as therapeutic tool. For instance, miR-200c has been found to increase the radiosensitivity by directly regulating oxidative stress response genes PRDX2, GAPB/Nrf2, and SESN1 which led to the inhibition of DNA double-strand break repair, increased levels of reactive oxygen species, and upregulated p21 [142].

Recently, miR-34, a well-known tumor suppressor miRNA that is in a double-negative feedback loop with Snail, like miR-200c and ZEB1, has been entered in a phase-I clinical trial (NCT01829971) for the treatment of HCC [143]. Furthermore, regarding the control of multiple oncogenes in different tumor tissues, miR-200c can be considered as a promising targeted therapy molecule similar to miR-34a. Therefore, a broad range of tumor suppressor functions conducted by miR-200c reviewed here also need to be assessed in clinical trials for the benefit of patients using miR-200c-based therapeutics.

Concluding remarks

Regulation of gene expression by miRNAs is not to serve just as a switch—turning protein production on or off—but to fine-tune the overall gene expression output providing

robustness to cell-specific programs. In this article, we reviewed miR-200c, a well-established, exceptional miRNA, which fine-tunes gene regulation in various diseases, especially in cancer. Notably, miR-200c simultaneously regulates major elements of cancer progression, i.e., proliferation, EMT, and drug resistance, in a context-dependent manner. Commonly known mesenchymal transcription factors ZEB1 and ZEB2 are among the direct targets of miR-200c; thus, it has established a strong link with EMT regulation. In addition, miR-200c is also correlated with and regulates chemoresistance, multidrug resistance, targeted therapy resistance, radiotolerance, and stemness through targeting and modulating the activities of key molecules involved in these processes. Overall, miR-200c functions at the crossroad of EMT and drug resistance reversal and has great potential to be used as therapy sensitizer and biomarker for several cancer tissues due to its presence in both intracellular as well as extracellular environment.

Acknowledgments and funding information We would like to thank Suhail Ansari and Rasmi Mishra for their critical reading of the manuscript and valuable suggestions. This work is, in part, supported by the European Molecular Biology Organization (EMBO) Installation Grant (Grant No. 2791) and TUBITAK (Grant No. 214S104).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116:281–297
2. Lee RC, Feinbaum RL, Ambros V (1993) The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* 75:843–854
3. Vella MC, Choi EY, Lin SY, Reinert K, Slack FJ (2004) The *C. elegans* microRNA *let-7* binds to imperfect *let-7* complementary sites from the *lin-41* 3'UTR. *Genes Dev* 18:132–137
4. Kaufman EJ, Miska EA (2010) The microRNAs of *Caenorhabditis elegans*. *Semin Cell Dev Biol* 21:728–737
5. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001) Identification of novel genes coding for small expressed RNAs. *Science* 294:853–858
6. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, Hayward DC, Ball EE, Degnan B, Muller P et al (2000) Conservation of the sequence and temporal expression of *let-7* heterochronic regulatory RNA. *Nature* 408:86–89
7. Kozomara A, Griffiths-Jones S (2014) miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res* 42:D68–73
8. Friedman RC, Farh KK, Burge CB, Bartel DP (2009) Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 19:92–105
9. Trang P, Weidhaas JB, Slack FJ (2008) MicroRNAs as potential cancer therapeutics. *Oncogene* 27(Suppl 2):S52–57

10. Fasanaro P, Greco S, Ivan M, Capogrossi MC, Martelli F (2010) microRNA: emerging therapeutic targets in acute ischemic diseases. *Pharmacol Ther* 125:92–104
11. Hydbring P, Badalian-Very G (2013) Clinical applications of microRNAs. *F1000Research* 2:136
12. Li C, Feng Y, Coukos G, Zhang L (2009) Therapeutic microRNA strategies in human cancer. *AAPS J* 11:747–757
13. Hurteau GJ, Spivack SD, Brock GJ (2006) Potential mRNA degradation targets of hsa-miR-200c, identified using informatics and qRT-PCR. *Cell Cycle* 5:1951–1956
14. Humphries B, Yang C (2015) The microRNA-200 family: small molecules with novel roles in cancer development, progression and therapy. *Oncotarget* 6:6472–6498
15. Mutlu M, Saatci Ö, Raza U, Eyüpoglu E, Yurdusev E, Sahin Ö (2015) MIR200C (microRNA 200c). *Atlas Genet Cytogenet Oncol Haematol* 19:270–285
16. Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spaderna S, Brabletz T (2008) A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. *EMBO Rep* 9:582–589
17. Browne G, Sayan AE, Tulchinsky E (2010) ZEB proteins link cell motility with cell cycle control and cell survival in cancer. *Cell Cycle* 9:886–891
18. Sanchez-Tillo E, Lazaro A, Torrent R, Cuatrecasas M, Vaquero EC, Castells A, Engel P, Postigo A (2010) ZEB1 represses E-cadherin and induces an EMT by recruiting the SWI/SNF chromatin-remodeling protein BRG1. *Oncogene* 29:3490–3500
19. Mizuguchi Y, Specht S, Lunz JG 3rd, Isse K, Corbitt N, Takizawa T, Demetris AJ (2012) Cooperation of p300 and PCAF in the control of microRNA 200c/141 transcription and epithelial characteristics. *PLoS One* 7, e32449. doi:[10.1371/journal.pone.0032449](https://doi.org/10.1371/journal.pone.0032449)
20. Peng C, Li N, Ng YK, Zhang J, Meier F, Theis FJ, Merckenschlager M, Chen W, Wurst W, Prakash N (2012) A unilateral negative feedback loop between miR-200 microRNAs and Sox2/E2F3 controls neural progenitor cell-cycle exit and differentiation. *J Neurosci Off J Soc Neurosci* 32:13292–13308
21. Pieraccioli M, Imbastari F, Antonov A, Melino G, Raschella G (2013) Activation of miR200 by c-Myb depends on ZEB1 expression and miR200 promoter methylation. *Cell Cycle* 12:2309–2320
22. Knouf EC, Garg K, Arroyo JD, Correa Y, Sarkar D, Parkin RK, Wurz K, O'Brian KC, Godwin AK, Urban ND et al (2012) An integrative genomic approach identifies p73 and p63 as activators of miR-200 microRNA family transcription. *Nucleic Acids Res* 40:499–510
23. Wang G, Guo X, Hong W, Liu Q, Wei T, Lu C, Gao L, Ye D, Zhou Y, Chen J et al (2013) Critical regulation of miR-200/ZEB2 pathway in Oct4/Sox2-induced mesenchymal-to-epithelial transition and induced pluripotent stem cell generation. *Proc Natl Acad Sci U S A* 110:2858–2863
24. Vrba L, Jensen TJ, Garbe JC, Heimark RL, Cress AE, Dickinson S, Stampfer MR, Futscher BW (2010) Role for DNA methylation in the regulation of miR-200c and miR-141 expression in normal and cancer cells. *PLoS One* 5, e8697. doi:[10.1371/journal.pone.0008697](https://doi.org/10.1371/journal.pone.0008697)
25. Wiklund ED, Bramsen JB, Hulf T, Dyrskjot L, Ramanathan R, Hansen TB, Villadsen SB, Gao S, Ostensfeld MS, Borre M et al (2011) Coordinated epigenetic repression of the miR-200 family and miR-205 in invasive bladder cancer. *Int J Cancer J Int du Cancer* 128:1327–1334
26. Tryndyak VP, Beland FA, Pogribny IP (2010) E-cadherin transcriptional down-regulation by epigenetic and microRNA-200 family alterations is related to mesenchymal and drug-resistant phenotypes in human breast cancer cells. *Int J Cancer J Int du Cancer* 126:2575–2583
27. Lamouille S, Xu J, Derynck R (2014) Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* 15:178–196
28. De Craene B, Berx G (2013) Regulatory networks defining EMT during cancer initiation and progression. *Nat Rev Cancer* 13:97–110
29. Peinado H, Olmeda D, Cano A (2007) Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer* 7:415–428
30. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, Savagner P, Gitelman I, Richardson A, Weinberg RA (2004) Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* 117:927–939
31. Park SM, Gaur AB, Lengyel E, Peter ME (2008) The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev* 22:894–907
32. Li L, Li B, Chen D, Liu L, Huang C, Lu Z, Lun L, Wan X (2015) miR-139 and miR-200c regulate pancreatic cancer endothelial cell migration and angiogenesis. *Oncol Rep* 34:51–58
33. Wellner U, Schubert J, Burk UC, Schmalhofer O, Zhu F, Sonntag A, Waldvogel B, Vannier C, Darling D, zur Hausen A et al (2009) The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. *Nat Cell Biol* 11:1487–1495
34. Korpai M, Lee ES, Hu G, Kang Y (2008) The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *J Biol Chem* 283:14910–14914
35. Brabletz S, Brabletz T (2010) The ZEB/miR-200 feedback loop—a motor of cellular plasticity in development and cancer? *EMBO Rep* 11:670–677
36. Shimono Y, Zabala M, Cho RW, Lobo N, Dalerba P, Qian D, Diehn M, Liu H, Panula SP, Chiao E et al (2009) Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. *Cell* 138:592–603
37. Bar M, Wyman SK, Fritz BR, Qi J, Garg KS, Parkin RK, Kroh EM, Bendoraite A, Mitchell PS, Nelson AM et al (2008) MicroRNA discovery and profiling in human embryonic stem cells by deep sequencing of small RNA libraries. *Stem Cells* 26:2496–2505
38. Chang CJ, Chao CH, Xia W, Yang JY, Xiong Y, Li CW, Yu WH, Rehman SK, Hsu JL, Lee HH et al (2011) p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. *Nat Cell Biol* 13:317–323
39. Kim T, Veronese A, Pichiorri F, Lee TJ, Jeon YJ, Volinia S, Pineau P, Marchio A, Palatini J, Suh SS et al (2011) p53 regulates epithelial-mesenchymal transition through microRNAs targeting ZEB1 and ZEB2. *J Exp Med* 208:875–883
40. Rajabi H, Alam M, Takahashi H, Kharbanda A, Guha M, Ahmad R, Kufe D (2014) MUC1-C oncoprotein activates the ZEB1/miR-200c regulatory loop and epithelial-mesenchymal transition. *Oncogene* 33:1680–1689
41. Jurmeister S, Baumann M, Balwierz A, Keklikoglou I, Ward A, Uhlmann S, Zhang JD, Wiemann S, Sahin O (2012) MicroRNA-200c represses migration and invasion of breast cancer cells by targeting actin-regulatory proteins FHOD1 and PPM1F. *Mol Cell Biol* 32:633–651
42. Sigloch FC, Burk UC, Biniotsek ML, Brabletz T, Schilling O (2015) miR-200c dampens cancer cell migration via regulation of protein kinase A subunits. *Oncotarget* 6:23874–23889
43. Li J, Tan Q, Yan M, Liu L, Lin H, Zhao F, Bao G, Kong H, Ge C, Zhang F et al (2014) miRNA-200c inhibits invasion and metastasis of human non-small cell lung cancer by directly targeting ubiquitin specific peptidase 25. *Mol Cancer* 13:166

44. Howe EN, Cochrane DR, Richer JK (2011) Targets of miR-200c mediate suppression of cell motility and anoikis resistance. *Breast Cancer Res: BCR* 13:R45
45. Perdigao-Henriques R, Petrocchi F, Altschuler G, Thomas MP, Le MT, Tan SM, Hide W, Lieberman J (2015) miR-200 promotes the mesenchymal to epithelial transition by suppressing multiple members of the Zeb2 and Snail1 transcriptional repressor complexes. *Oncogene*. doi:10.1038/onc.2015.69
46. Liu M, Casimiro MC, Wang C, Shirley LA, Jiao X, Katiyar S, Ju X, Li Z, Yu Z, Zhou J et al (2009) p21CIP1 attenuates Ras- and c-Myc-dependent breast tumor epithelial mesenchymal transition and cancer stem cell-like gene expression in vivo. *Proc Natl Acad Sci U S A* 106:19035–19039
47. Li XL, Hara T, Choi Y, Subramanian M, Francis P, Bilke S, Walker RL, Pineda M, Zhu Y, Yang Y et al (2014) A p21-ZEB1 complex inhibits epithelial-mesenchymal transition through the microRNA 183-96-182 cluster. *Mol Cell Biol* 34:533–550
48. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ (2008) The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* 10:593–601
49. Hamano R, Miyata H, Yamasaki M, Kurokawa Y, Hara J, Moon JH, Nakajima K, Takiguchi S, Fujiwara Y, Mori M et al (2011) Overexpression of miR-200c induces chemoresistance in esophageal cancers mediated through activation of the Akt signaling pathway. *Clin Cancer Res: Off J Am Assoc Cancer Res* 17:3029–3038
50. Madhavan D, Zucknick M, Wallwiener M, Cuk K, Modugno C, Scharpf M, Schott S, Heil J, Turchinovich A, Yang R et al (2012) Circulating miRNAs as surrogate markers for circulating tumor cells and prognostic markers in metastatic breast cancer. *Clin Cancer Res: Off J Am Assoc Cancer Res* 18:5972–5982
51. Gottesman MM (2002) Mechanisms of cancer drug resistance. *Annu Rev Med* 53:615–627
52. Liu S, Tetzlaff MT, Cui R, Xu X (2012) miR-200c inhibits melanoma progression and drug resistance through down-regulation of BMI-1. *Am J Pathol* 181:1823–1835
53. Park IK, Morrison SJ, Clarke MF (2004) Bmi1, stem cells, and senescence regulation. *J Clin Invest* 113:175–179
54. Cittelly DM, Dimitrova I, Howe EN, Cochrane DR, Jean A, Spoelstra NS, Post MD, Lu X, Broadus RR, Spillman MA et al (2012) Restoration of miR-200c to ovarian cancer reduces tumor burden and increases sensitivity to paclitaxel. *Mol Cancer Ther* 11:2556–2565
55. Cochrane DR, Spoelstra NS, Howe EN, Nordeen SK, Richer JK (2009) MicroRNA-200c mitigates invasiveness and restores sensitivity to microtubule-targeting chemotherapeutic agents. *Mol Cancer Ther* 8:1055–1066
56. Prislei S, Martinelli E, Mariani M, Raspaglio G, Sieber S, Ferrandina G, Shahabi S, Scambia G, Ferlini C (2013) MiR-200c and HuR in ovarian cancer. *BMC Cancer* 13:72
57. Tanaka S, Hosokawa M, Ueda K, Iwakawa S (2015) Effects of decitabine on invasion and exosomal expression of miR-200c and miR-141 in oxaliplatin-resistant colorectal cancer cells. *Biol Pharm Bull* 38:1272–1279
58. Kopp F, Wagner E, Roidl A (2014) The proto-oncogene KRAS is targeted by miR-200c. *Oncotarget* 5:185–195
59. Sui H, Cai GX, Pan SF, Deng WL, Wang Y, Chen ZS, Cai SJ, Zhu HR, Li Q (2014) miR-200c attenuates P-gp mediated MDR and metastasis by targeting JNK2/c-Jun signaling pathway in colorectal cancer. *Mol Cancer Ther*. doi:10.1158/1535-7163.MCT-14-0167
60. Kopp F, Oak PS, Wagner E, Roidl A (2012) miR-200c sensitizes breast cancer cells to doxorubicin treatment by decreasing TrkB and Bmi1 expression. *PLoS One* 7:e50469
61. Segal RA (2003) Selectivity in neurotrophin signaling: theme and variations. *Annu Rev Neurosci* 26:299–330
62. Brodeur GM, Mintum JE, Ho R, Simpson AM, Iyer R, Varela CR, Light JE, Kolla V, Evans AE (2009) Trk receptor expression and inhibition in neuroblastomas. *Clin Cancer Res: Off J Am Assoc Cancer Res* 15:3244–3250
63. Ho R, Eggert A, Hishiki T, Mintum JE, Ikegaki N, Foster P, Camoratto AM, Evans AE, Brodeur GM (2002) Resistance to chemotherapy mediated by TrkB in neuroblastomas. *Cancer Res* 62:6462–6466
64. Lee J, Jiffar T, Kupferman ME (2012) A novel role for BDNF-TrkB in the regulation of chemotherapy resistance in head and neck squamous cell carcinoma. *PLoS One* 7, e30246. doi:10.1371/journal.pone.0030246
65. Manavalan TT, Teng Y, Appana SN, Datta S, Kalbfleisch TS, Li Y, Klinge CM (2011) Differential expression of microRNA expression in tamoxifen-sensitive MCF-7 versus tamoxifen-resistant LY2 human breast cancer cells. *Cancer Lett* 313:26–43
66. Manavalan TT, Teng Y, Litchfield LM, Muluhngwi P, Al-Rayyan N, Klinge CM (2013) Reduced expression of miR-200 family members contributes to antiestrogen resistance in LY2 human breast cancer cells. *PLoS One* 8, e62334. doi:10.1371/journal.pone.0062334
67. Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J, Hamid O, Schuchter L, Cebon J, Ibrahim N et al (2012) Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med* 367:1694–1703
68. Liu S, Tetzlaff MT, Wang T, Yang R, Xie L, Zhang G, Krepler C, Xiao M, Beqiri M, Xu W et al (2015) miR-200c/Bmi1 axis and epithelial-mesenchymal transition contribute to acquired resistance to BRAF inhibitor treatment. *Pigment Cell Melanoma Res* 28:431–441
69. Gao C, Peng FH, Peng LK (2014) MiR-200c sensitizes clear-cell renal cell carcinoma cells to sorafenib and imatinib by targeting heme oxygenase-1. *Neoplasia*. doi:10.4149/neo_2014_083
70. Lin J, Liu C, Gao F, Mitchel RE, Zhao L, Yang Y, Lei J, Cai J (2013) miR-200c enhances radiosensitivity of human breast cancer cells. *J Cell Biochem* 114:606–615
71. Sun Q, Liu T, Yuan Y, Guo Z, Xie G, Du S, Lin X, Xu Z, Liu M, Wang W et al (2015) MiR-200c inhibits autophagy and enhances radiosensitivity in breast cancer cells by targeting UBQLN1. *Int J Cancer J Int du Cancer* 136:1003–1012
72. Cui FB, Liu Q, Li RT, Shen J, Wu PY, Yu LX, Hu WJ, Wu FL, Jiang CP, Yue GF et al (2014) Enhancement of radiotherapy efficacy by miR-200c-loaded gelatinase-stimuli PEG-Pep-PCL nanoparticles in gastric cancer cells. *Int J Nanomedicine* 9:2345–2358
73. Singh A, Settleman J (2010) EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene* 29:4741–4751
74. Raza U, Zhang JD, Sahin O (2014) MicroRNAs: master regulators of drug resistance, stemness, and metastasis. *J Mol Med (Berl)* 92:321–336
75. Ceppi P, Mudduluru G, Kumaraswamy R, Rapa I, Scagliotti GV, Papotti M, Allgayer H (2010) Loss of miR-200c expression induces an aggressive, invasive, and chemoresistant phenotype in non-small cell lung cancer. *Mol Cancer Res* 18:1207–1216
76. Puh R, Hoefer J, Schafer G, Erb HH, Oh SJ, Klocker H, Heidegger I, Neuwirt H, Culig Z (2012) Epithelial-to-mesenchymal transition leads to docetaxel resistance in prostate cancer and is mediated by reduced expression of miR-200c and miR-205. *Am J Pathol* 181:2188–2201
77. Li Y, VandenBoom TG 2nd, Kong D, Wang Z, Ali S, Philip PA, Sarkar FH (2009) Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. *Cancer Res* 69:6704–6712

78. Bao B, Wang Z, Ali S, Ahmad A, Azmi AS, Sarkar SH, Banerjee S, Kong D, Li Y, Thakur S et al (2012) Metformin inhibits cell proliferation, migration and invasion by attenuating CSC function mediated by deregulating miRNAs in pancreatic cancer cells. *Cancer Prev Res* 5:355–364
79. Ma C, Huang T, Ding YC, Yu W, Wang Q, Meng B, Luo SX (2015) microRNA-200c overexpression inhibits chemoresistance, invasion and colony formation of human pancreatic cancer stem cells. *Int J Clin Exp Pathol* 8:6533–6539
80. Brozovic A, Duran GE, Wang YC, Francisco EB, Sikic BI (2015) The miR-200 family differentially regulates sensitivity to paclitaxel and carboplatin in human ovarian carcinoma OVCAR-3 and MES-OV cells. *Mol Oncol* 9:1678–1693
81. Siebzehnubel FA, Silver DJ, Tugertimur B, Deleyrolle LP, Siebzehnubel D, Sarkisian MR, Devers KG, Yachnis AT, Kupper MD, Neal D et al (2013) The ZEB1 pathway links glioblastoma initiation, invasion and chemoresistance. *EMBO Mol Med* 5:1196–1212
82. Fischer KR, Durrans A, Lee S, Sheng J, Li F, Wong ST, Choi H, El Rayes T, Ryu S, Troeger J et al (2015) Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance. *Nature* 527:472–476
83. Adam L, Zhong M, Choi W, Qi W, Nicoloso M, Arora A, Calin G, Wang H, Siefker-Radtke A, McConkey D et al (2009) miR-200 expression regulates epithelial-to-mesenchymal transition in bladder cancer cells and reverses resistance to epidermal growth factor receptor therapy. *Clin Cancer Res: Off J Am Assoc Cancer Res* 15:5060–5072
84. Shien K, Toyooka S, Yamamoto H, Soh J, Jida M, Thu KL, Hashida S, Maki Y, Ichihara E, Asano H et al (2013) Acquired resistance to EGFR inhibitors is associated with a manifestation of stem cell-like properties in cancer cells. *Cancer Res* 73:3051–3061
85. Cufi S, Bonavia R, Vazquez-Martin A, Oliveras-Ferraro C, Corominas-Faja B, Cuyas E, Martin-Castillo B, Barralon-Catalan E, Visa J, Segura-Carretero A et al (2013) Silibinin suppresses EMT-driven erlotinib resistance by reversing the high miR-21/low miR-200c signature in vivo. *Sci Rep* 3:2459
86. Bryant JL, Britson J, Balko JM, William M, Timmons R, Frolov A, Black EP (2012) A microRNA gene expression signature predicts response to erlotinib in epithelial cancer cell lines and targets EMT. *Br J Cancer* 106:148–156
87. Izumchenko EG, Chang X, Michailidi C, Kagohara LT, Ravi R, Paz K, Brait M, Hoque MO, Ling S, Bedi A et al (2014) The TGFbeta-miR200-Mig6 pathway orchestrates the EMT-associated kinase switch that induces resistance to EGFR inhibitors. *Cancer Res*. doi:10.1158/0008-5472.CAN-14-0110
88. Bai WD, Ye XM, Zhang MY, Zhu HY, Xi WJ, Huang X, Zhao J, Gu B, Zheng GX, Yang AG et al (2014) MiR-200c suppresses TGF-beta signaling and counteracts trastuzumab resistance and metastasis by targeting ZNF217 and ZEB1 in breast cancer. *Int J Cancer J Int du Cancer*. doi:10.1002/ijc.28782
89. Shi SJ, Wang LJ, Yu B, Li YH, Jin Y, Bai XZ (2015) LncRNA-ATB promotes trastuzumab resistance and invasion-metastasis cascade in breast cancer. *Oncotarget* 6:11652–11663
90. Rebutini IT, Hayashi T, Reynolds AD, Dillard ML, Carpenter EM, Hoffman MP (2012) miR-200c regulates FGFR-dependent epithelial proliferation via Vldlr during submandibular gland branching morphogenesis. *Development* 139:191–202
91. Cao H, Jheon A, Li X, Sun Z, Wang J, Florez S, Zhang Z, McManus MT, Klein OD, Amendt BA (2013) The Pitx2:miR-200c/141:noggin pathway regulates Bmp signaling and ameblast differentiation. *Development* 140:3348–3359
92. Li H, Xu L, Li C, Zhao L, Ma Y, Zheng H, Li Z, Zhang Y, Wang R, Liu Y et al (2014) Ubiquitin ligase Cbl-b represses IGF-I-induced epithelial mesenchymal transition via ZEB2 and microRNA-200c regulation in gastric cancer cells. *Mol Cancer* 13:136
93. Song C, Liu LZ, Pei XQ, Liu X, Yang L, Ye F, Xie X, Chen J, Tang H, Xie X (2015) miR-200c inhibits breast cancer proliferation by targeting KRAS. *Oncotarget*
94. Park JT, Kato M, Yuan H, Castro N, Lanting L, Wang M, Natarajan R (2013) FOG2 protein down-regulation by transforming growth factor-beta1-induced microRNA-200b/c leads to Akt kinase activation and glomerular mesangial hypertrophy related to diabetic nephropathy. *J Biol Chem* 288:22469–22480
95. Howe EN, Cochrane DR, Citty DM, Richer JK (2012) miR-200c targets a NF-kappaB up-regulated TrkB/NTF3 autocrine signaling loop to enhance anoikis sensitivity in triple negative breast cancer. *PLoS One* 7:e49987
96. Schickel R, Park SM, Murmann AE, Peter ME (2010) miR-200c regulates induction of apoptosis through CD95 by targeting FAP-1. *Mol Cell* 38:908–915
97. Ren Y, Han X, Yu K, Sun S, Zhen L, Li Z, Wang S (2014) microRNA-200c downregulates XIAP expression to suppress proliferation and promote apoptosis of triple-negative breast cancer cells. *Mol Med Rep* 10:315–321
98. Huang HN, Chen SY, Hwang SM, Yu CC, Su MW, Mai W, Wang HW, Cheng WC, Schuyler SC, Ma N et al (2014) miR-200c and GATA binding protein 4 regulate human embryonic stem cell renewal and differentiation. *Stem Cell Res* 12:338–353
99. Lu YX, Yuan L, Xue XL, Zhou M, Liu Y, Zhang C, Li JP, Zheng L, Hong M, Li XN (2014) Regulation of colorectal carcinoma stemness, growth, and metastasis by a miR-200c-Sox2-negative feedback loop mechanism. *Clin Cancer Res: Off J Am Assoc Cancer Res* 20:2631–2642
100. Klein D, Misawa R, Bravo-Egana V, Vargas N, Rosero S, Piroso J, Ichii H, Umland O, Zhijie J, Tsinoremas N et al (2013) MicroRNA expression in alpha and beta cells of human pancreatic islets. *PLoS One* 8, e55064. doi:10.1371/journal.pone.0055064
101. Luo X, Dong Z, Chen Y, Yang L, Lai D (2013) Enrichment of ovarian cancer stem-like cells is associated with epithelial to mesenchymal transition through an miRNA-activated AKT pathway. *Cell Prolif* 46:436–446
102. Vallejo DM, Caparros E, Dominguez M (2011) Targeting Notch signalling by the conserved miR-8/200 microRNA family in development and cancer cells. *EMBO J* 30:756–769
103. Bao B, Wang Z, Ali S, Kong D, Li Y, Ahmad A, Banerjee S, Azmi AS, Miele L, Sarkar FH (2011) Notch-1 induces epithelial-mesenchymal transition consistent with cancer stem cell phenotype in pancreatic cancer cells. *Cancer Lett* 307:26–36
104. Mezquita B, Mezquita J, Barrot C, Carvajal S, Pau M, Mezquita P, Mezquita C (2014) A truncated-Flt1 isoform of breast cancer cells is upregulated by Notch and downregulated by retinoic acid. *J Cell Biochem* 115:52–61
105. Mezquita B, Mezquita P, Pau M, Mezquita J, Mezquita C (2014) Unlocking doors without keys: activation of Src by truncated C-terminal intracellular receptor tyrosine kinases lacking tyrosine kinase activity. *Cells* 3:92–111
106. Wendlandt EB, Graff JW, Giannini TL, McCaffrey AP, Wilson ME (2012) The role of microRNAs miR-200b and miR-200c in TLR4 signaling and NF-kappaB activation. *Innate Immun* 18:846–855
107. Rokavec M, Wu W, Luo JL (2012) IL6-mediated suppression of miR-200c directs constitutive activation of inflammatory signaling circuit driving transformation and tumorigenesis. *Mol Cell* 45:777–789
108. Chuang TD, Khorram O (2014) miR-200c regulates IL8 expression by targeting IKBKB: a potential mediator of inflammation in leiomyoma pathogenesis. *PLoS One* 9:e95370

109. Lobert S, Graichen ME, Morris K (2013) Coordinated regulation of beta-tubulin isoforms and epithelial-to-mesenchymal transition protein ZEB1 in breast cancer cells. *Biochemistry* 52:5482–5490
110. Hur K, Toiyama Y, Takahashi M, Balaguer F, Nagasaka T, Koike J, Hemmi H, Koi M, Boland CR, Goel A (2013) MicroRNA-200c modulates epithelial-to-mesenchymal transition (EMT) in human colorectal cancer metastasis. *Gut* 62:1315–1326
111. Oishi N, Kumar MR, Roessler S, Ji J, Forgues M, Budhu A, Zhao X, Andersen JB, Ye QH, Jia HL et al (2012) Transcriptomic profiling reveals hepatic stem-like gene signatures and interplay of miR-200c and epithelial-mesenchymal transition in intrahepatic cholangiocarcinoma. *Hepatology* 56:1792–1803
112. Karakatsani A, Papaconstantinou I, Gazouli M, Lyberopoulou A, Polymeneas G, Voros D (2013) Expression of microRNAs, miR-21, miR-31, miR-122, miR-145, miR-146a, miR-200c, miR-221, miR-222, and miR-223 in patients with hepatocellular carcinoma or intrahepatic cholangiocarcinoma and its prognostic significance. *Mol Carcinog* 52:297–303
113. Lahat G, Lubezky N, Loewenstein S, Nizri E, Gan S, Pasmanik-Chor M, Hayman L, Barazowsky E, Ben-Haim M, Klausner JM (2014) Epithelial-to-Mesenchymal Transition (EMT) in Intraductal Papillary Mucinous Neoplasm (IPMN) is associated with high tumor grade and adverse outcomes. *Ann Surg Oncol*. doi:10.1245/s10434-014-3946-5
114. Kim MK, Jung SB, Kim JS, Roh MS, Lee JH, Lee EH, Lee HW (2014) Expression of microRNA miR-126 and miR-200c is associated with prognosis in patients with non-small cell lung cancer. *Virchows Archiv: Int J Pathol*. doi:10.1007/s00428-014-1640-4
115. Tejero R, Navarro A, Campayo M, Vinolas N, Marrades RM, Cordeiro A, Ruiz-Martinez M, Santasusagna S, Molins L, Ramirez J et al (2014) miR-141 and miR-200c as markers of overall survival in early stage non-small cell lung cancer adenocarcinoma. *PLoS One* 9:e101899
116. Pacurari M, Addison JB, Bondalapati N, Wan YW, Luo D, Qian Y, Castranova V, Ivanov AV, Guo NL (2013) The microRNA-200 family targets multiple non-small cell lung cancer prognostic markers in H1299 cells and BEAS-2B cells. *Int J Oncol* 43:548–560
117. Zhang HH, Zhang ZY, Che CL, Mei YF, Shi YZ (2013) Array analysis for potential biomarker of gemcitabine identification in non-small cell lung cancer cell lines. *Int J Clin Exp Pathol* 6:1734–1746
118. Leskela S, Leandro-Garcia LJ, Mendiola M, Barriuso J, Inglada-Perez L, Munoz I, Martinez-Delgado B, Redondo A, de Santiago J, Robledo M et al (2011) The miR-200 family controls beta-tubulin III expression and is associated with paclitaxel-based treatment response and progression-free survival in ovarian cancer patients. *Endocrine-Related Cancer* 18:85–95
119. Marchini S, Cavalieri D, Fruscio R, Calura E, Garavaglia D, Nerini IF, Mangioni C, Cattoretti G, Clivio L, Beltrame L et al (2011) Association between miR-200c and the survival of patients with stage I epithelial ovarian cancer: a retrospective study of two independent tumour tissue collections. *Lancet Oncol* 12:273–285
120. White NM, Bao TT, Grigull J, Youssef YM, Girgis A, Diamandis M, Fatoohi E, Metias M, Honey RJ, Stewart R et al (2011) miRNA profiling for clear cell renal cell carcinoma: biomarker discovery and identification of potential controls and consequences of miRNA dysregulation. *J Urol* 186:1077–1083
121. Fridman E, Dotan Z, Barshack I, David MB, Dov A, Tabak S, Zion O, Benjamin S, Benjamin H, Kuker H et al (2010) Accurate molecular classification of renal tumors using microRNA expression. *J Mol Diagn: JMD* 12:687–696
122. Wotschovsky Z, Busch J, Jung M, Kempkensteffen C, Weikert S, Schaser KD, Melcher I, Kilic E, Miller K, Kristiansen G et al (2013) Diagnostic and prognostic potential of differentially expressed miRNAs between metastatic and non-metastatic renal cell carcinoma at the time of nephrectomy. *Clin Chim Acta* 416:5–10
123. Mahdavi-zhad A, Mousavi-Bahar SH, Poorolajal J, Yadegarazari R, Jafari M, Shabab N, Saidijam M (2015) Evaluation of miR-141, miR-200c, miR-30b expression and clinicopathological features of bladder cancer. *Int J Mol Cell Med* 4:32–39
124. Berber U, Yilmaz I, Narli G, Haholu A, Kucukodaci Z, Demirel D (2014) miR-205 and miR-200c: predictive micro RNAs for lymph node metastasis in triple negative breast cancer. *J Breast Cancer* 17:143–148
125. Toiyama Y, Hur K, Tanaka K, Inoue Y, Kusunoki M, Boland CR, Goel A (2014) Serum miR-200c is a novel prognostic and metastasis-predictive biomarker in patients with colorectal cancer. *Ann Surg* 259:735–743
126. Paraskevi A, Theodoropoulos G, Papaconstantinou I, Mantzaris G, Nikiteas N, Gazouli M (2012) Circulating MicroRNA in inflammatory bowel disease. *J Crohn's Colitis* 6:900–904
127. Valladares-Ayerbes M, Reboredo M, Medina-Villaamil V, Iglesias-Diaz P, Lorenzo-Patino MJ, Haz M, Santamarina I, Blanco M, Fernandez-Tajes J, Quindos M et al (2012) Circulating miR-200c as a diagnostic and prognostic biomarker for gastric cancer. *J Transl Med* 10:186
128. Yun KW, Lee JY, Yun SW, Lim IS, Choi ES (2014) Elevated serum level of microRNA (miRNA)-200c and miRNA-371-5p in children with Kawasaki disease. *Pediatr Cardiol* 35:745–752
129. Liu XG, Zhu WY, Huang YY, Ma LN, Zhou SQ, Wang YK, Zeng F, Zhou JH, Zhang YK (2012) High expression of serum miR-21 and tumor miR-200c associated with poor prognosis in patients with lung cancer. *Med Oncol* 29:618–626
130. Taylor DD, Gercel-Taylor C (2008) MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecol Oncol* 110:13–21
131. Kan CW, Hahn MA, Gard GB, Maidens J, Huh JY, Marsh DJ, Howell VM (2012) Elevated levels of circulating microRNA-200 family members correlate with serous epithelial ovarian cancer. *BMC Cancer* 12:627
132. Cheng HH, Mitchell PS, Kroh EM, Dowell AE, Chery L, Siddiqui J, Nelson PS, Vessella RL, Knudsen BS, Chinnaiyan AM et al (2013) Circulating microRNA profiling identifies a subset of metastatic prostate cancer patients with evidence of cancer-associated hypoxia. *PLoS One* 8, e69239. doi:10.1371/journal.pone.0069239
133. Lv LL, Cao Y, Liu D, Xu M, Liu H, Tang RN, Ma KL, Liu BC (2013) Isolation and quantification of microRNAs from urinary exosomes/microvesicles for biomarker discovery. *Int J Biol Sci* 9:1021–1031
134. Zuberi M, Mir R, Das J, Ahmad I, Javid J, Yadav P, Masroor M, Ahmad S, Ray PC, Saxena A (2015) Expression of serum miR-200a, miR-200b, and miR-200c as candidate biomarkers in epithelial ovarian cancer and their association with clinicopathological features. *Clin Transl Oncol* 17:779–787
135. Hong L, Han Y, Zhang H, Fan D (2015) Prognostic markers in esophageal cancer: from basic research to clinical use. *Expert Rev Gastroenterol Hepatol* 9:887–889
136. Pantel K, Alix-Panabieres C (2010) Circulating tumour cells in cancer patients: challenges and perspectives. *Trends Mol Med* 16:398–406
137. Perez-Hernandez J, Forner MJ, Pinto C, Chaves FJ, Cortes R, Redon J (2015) Increased urinary exosomal microRNAs in patients with systemic lupus erythematosus. *PLoS One* 10, e0138618. doi:10.1371/journal.pone.0138618
138. Yuan JH, Yang F, Wang F, Ma JZ, Guo YJ, Tao QF, Liu F, Pan W, Wang TT, Zhou CC et al (2014) A long noncoding RNA activated

- by TGF-beta promotes the invasion-metastasis cascade in hepatocellular carcinoma. *Cancer Cell* 25:666–681
139. Xiao X, Yu S, Li S, Wu J, Ma R, Cao H, Zhu Y, Feng J (2014) Exosomes: decreased sensitivity of lung cancer A549 cells to cisplatin. *PLoS One* 9, e89534. doi:[10.1371/journal.pone.0089534](https://doi.org/10.1371/journal.pone.0089534)
140. Chen Y, Gao DY, Huang L (2015) In vivo delivery of miRNAs for cancer therapy: challenges and strategies. *Adv Drug Deliv Rev* 81: 128–141
141. Esposito CL, Cerchia L, Catuogno S, De Vita G, Dassie JP, Santamaria G, Swiderski P, Condorelli G, Giangrande PH, de Franciscis V (2014) Multifunctional aptamer-miRNA conjugates for targeted cancer therapy. *Mol Ther: J Am Soc Gene Ther* 22: 1151–1163
142. Cortez MA, Valdecanas D, Zhang X, Zhan Y, Bhardwaj V, Calin GA, Komaki R, Giri DK, Quini CC, Wolfe T et al (2014) Therapeutic delivery of miR-200c enhances radiosensitivity in lung cancer. *Mol Ther: J Am Soc Gene Ther*. doi:[10.1038/mt.2014.79](https://doi.org/10.1038/mt.2014.79)
143. Li XJ, Ren ZJ, Tang JH (2014) MicroRNA-34a: a potential therapeutic target in human cancer. *Cell Death Dis* 5, e1327. doi:[10.1038/cddis.2014.270](https://doi.org/10.1038/cddis.2014.270)

Heterozygous Deep-Intronic Variants and Deletions in *ABCA4* in Persons with Retinal Dystrophies and One Exonic *ABCA4* Variant

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Communicated by Andreas Gal

Received 30 July 2014; accepted revised manuscript 18 October 2014.

Published online 3 November 2014 in Wiley Online Library (www.wiley.com/humanmutation). DOI: 10.1002/humu.22717

ABSTRACT: Variants in *ABCA4* are responsible for autosomal-recessive Stargardt disease and cone-rod dystrophy. Sequence analysis of *ABCA4* exons previously revealed one causative variant in each of 45 probands. To identify the “missing” variants in these cases, we performed multiplex ligation-dependent probe amplification-based deletion scanning of *ABCA4*. In addition, we sequenced the promoter region, fragments containing five deep-intronic splice variants, and 15 deep-intronic regions containing weak splice sites. Heterozygous deletions spanning *ABCA4* exon 5 or exons 20–22 were found in two probands, heterozygous deep-intronic variants were identified in six probands, and a deep-intronic variant was found together with an exon 20–22 deletion in one proband. Based on ophthalmologic findings and characteristics of the identified exonic variants present in *trans*, the deep-intronic variants V1 and V4 were predicted to be relatively mild and severe, respectively. These findings are important for proper genetic counseling and for the development of variant-specific therapies.

Hum Mutat 36:43–47, 2015. © 2014 Wiley Periodicals, Inc.

KEY WORDS: *ABCA4*; Stargardt; STGD1; retinal dystrophies; splicing; deep-intronic variants; mutation

The ATP-binding cassette, subfamily A, member 4 (*ABCA4*) protein is located at the rim of the outer segment discs in rods and in the cell membrane of cones. Variants in *ABCA4* (MIM #601691) were associated with autosomal-recessive Stargardt disease (STGD1) [Allikmets et al., 1997b]. Subsequently, different combinations of *ABCA4* variants have been linked to a spectrum of phenotypes ranging from mild macular dystrophy to severe early-onset panretinal dystrophy [Allikmets et al., 1997a; Cremers et al., 1998; Maugeri et al., 1999; Klevering et al., 2004]. In the proposed genotype–phenotype correlation model, a combination of a mild and a severe *ABCA4* variant causes STGD1 [Maugeri et al., 1999], the most frequent associated disease, whereas a moderate and a severe variant gives rise to a more severe phenotype, cone-rod dystrophy (CRD) [Cremers et al., 1998]. Two severe variants in *ABCA4* result in a panretinal CRD [Cremers et al., 1998; Klevering et al., 2004].

A significant subset of individuals with classic STGD1 however carries only one or no *ABCA4* variant [Maugeri et al., 1999]. A proportion of these cases may be explained by the identification of variants in genes that are associated with retinal dystrophies resembling STGD1. *ELOVL4* [Edwards et al., 2001] and *PRPH2* [Poloschek et al., 2010] contain variants that are responsible for autosomal-dominant STGD. Furthermore, the “missing” variants in STGD1 patients may be the result of the limitations of the employed molecular genetic analysis. Homozygous *ABCA4* deletions disrupting the open reading frame are not expected to account for the “missing” variants in STGD1 as they would result in a panretinal dystrophy. Heterozygous deletions, however, can have been missed as PCR-based mutation scanning and Sanger sequencing of *ABCA4* exons would not detect small heterozygous deletions. Southern blot analysis revealed heterozygous deletions of exon 18 [Yatsenko et al., 2003] and exons 20–22 [Maugeri et al., 1999].

Deep sequencing of the *ABCA4* mRNA of healthy retinal tissues revealed several low-expressed alternative transcripts of *ABCA4* [Braun et al., 2013]. Sequence analysis of rarely used deep-intronic splice sites in STGD1 individuals identified variants at five splice junctions (i.e., V1 through V5), causing intron retention or premature termination of an exon [Braun et al., 2013]. The variants V6 and V7 reported in the same study are not deep-intronic and can be identified using regular exon analysis.

Additional Supporting Information may be found in the online version of this article.

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Contract grant sponsors: Foundation Fighting Blindness USA (grants BR-GE-0510-04890RAD, C-GE-0811-0545-RAD01); FP7-PEOPLE-2012-ITN Programme EyeTN (agreement 317472); The Macula Vision Research Foundation; The Nijmeegse Oogonderzoek Stichting; The Prof. Dr. H.J. Flieringa Foundation SW00; The Rotterdam Eye Hospital; The MD Fonds and the Stichting A.F. Deutman Researchfonds Oogheelkunde.

Previously, we employed a palette of genotyping methods to scan for exonic *ABCA4* variants and deletions [Maugeri et al., 1999; Jaakson et al., 2003]. In this study, we have expanded our genotyping studies to identify the second variant in maculopathy cases with one *ABCA4* allele. Forty-five probands with one heterozygous *ABCA4* variant (33 STGD1, four STGD1/CRD, five arCRD, three ar cone dystrophy [CD]; Supp. Table S1), a cohort of 81 STGD1 probands with two *ABCA4* variants and nine additional STGD1 probands without *ABCA4* variants, were ascertained in The Netherlands, the majority of which originates from the Southeast. One hundred and sixty-seven ethnically matched healthy control samples were collected in The Netherlands.

Clinical data were collected from the medical records. From the patients with an intronic variant, data were collected focused on the age at diagnosis, best corrected visual acuity (BCVA) using Snellen visual acuity, ophthalmoscopy, and full-field electroretinography (ffERG). ffERG was performed using Dawson–Trick–Litzkow electrodes and the RETI-port system (Roland Consults, Stasche & Finger GmbH, Brandenburg an der Havel, Germany), and the recordings were performed in accordance with the guidelines of the International Society for Clinical Electrophysiology of Vision (ISCEV) [Marmor et al., 2009]. The inclusion criteria for STGD1 were the presence of progressive retinal dystrophy with beaten-metal appearance and/or yellow-white pisciform flecks and/or autofluorescent depositions under the RPE. Inclusion criteria for CRD were a progressive decline of visual acuity, and on ERG a reduction of both cone and rod responses, with cone responses equally or more severely reduced. Exclusion criteria were an onset >40 years, extinguished scotopic or photopic responses on ERG, and congenital nystagmus. This study was approved by the local medical ethics committees and adhered to the tenets of the Declaration of Helsinki. All participants provided signed, informed consent for participation in the study, retrieval of medical records, and use of blood and DNA for research.

Blood samples were obtained of probands, their parents, and when possible, affected and unaffected siblings. DNA was isolated from peripheral blood lymphocytes by standard procedures. Multiplex ligation-dependent probe amplification (MLPA) reagents were obtained according to the manufacturer's instructions (SALSA MLPA kit P151-P152 *ABCA4*; MRC-Holland, Amsterdam, The Netherlands). Primers used to perform Sanger sequencing of all coding regions including the intron–exon boundaries and the putative promoter region (hg19 chr1:94,587,564–94,586,601; c.-963>c.1) of *ABCA4* are described in Supp. Table S2. Supp. Table S3 lists the primers employed to perform Sanger sequencing of the deep-intronic variants (V1–V5). Sanger sequencing of the 15 regions encompassing rarely used splice sites was performed using previously described primers [Braun et al., 2013]. The variants reported in this study were submitted to the locus-specific database for *ABCA4* at <http://www.lovd.nl/ABCA4>.

An overview of the genetic analyses performed previously and in this study is provided in Supp. Table S1. Sequence analysis of the promoter region did not yield any variants. An in-frame deletion of exons 20–22 (c.2919–?.3328+?del; p.S974Qfs*64) was identified previously by Southern blotting [Maugeri et al., 1999]. As part of a diagnostic deletion scanning protocol, DNAs of 27 patients with retinal dystrophies and one heterozygous *ABCA4* variant were fully analyzed for heterozygous *ABCA4* deletions using MLPA, which yielded two probands carrying the exon 20–22 deletion in a heterozygous state (A-IV:2 and F-II:1; Fig. 1; Table 1). A-IV:2 is a member of a previously described multiplex family segregating several monogenic and multifactorial retinal dystrophies [Klevering et al., 2004]. She was diagnosed with STGD1/CRD and carried the c.768G>T

variant on the other allele, which is a frequent causative *ABCA4* variant [Maugeri et al., 1999]. The exon 20–22 deletion was also found in a second cousin (A-IV:1) who carried the c.5461–10T>C variant in a heterozygous state. Finally, another family member with STGD1 (A-III:3) carried this deletion, but no second *ABCA4* variant was detected. As described previously, III:1 and III:3 also carry the same maternal haplotype containing *ABCA4* [Klevering et al., 2004]. Ophthalmological investigations were not carried out for III:1. In Family F, this deletion was found together with a heterozygous missense variant (p.C1488R) and a heterozygous deep-intronic splice variant (see below).

Using MLPA probes spanning exon 5, a novel heterozygous deletion with unknown boundaries (c.443–?.570+?del), resulting in a predicted truncated protein (p.G148Vfs*89), was detected in a STGD1 proband and an affected sibling of Family B (Fig. 1). Both also carried the c.768G>T variant in a heterozygous state. The identification of heterozygous *ABCA4* deletions of exon 5 and exons 20–22 explained six cases of three families (Fig. 1).

The 45 probands with one heterozygous *ABCA4* allele, as well as a group of nine STGD individuals without any pathogenic *ABCA4* variant, were analyzed for the presence of five deep-intronic variants (V1 through V5) and analyzed for sequence variants at the splice sites of 15 additional cryptic exons [Braun et al., 2013]. The deep-intronic variant c.5196+1137G>A (V1) was detected in isolated cases of two families (Families F and H; Fig. 1; Table 1). In Family F, the proband also carried a heterozygous missense variant (p.C1488R) and a heterozygous deletion of exons 20–22, but segregation analysis was not possible due to unavailability of parental DNAs. The second deep-intronic variant identified in our cohort was c.4539+2001G>A (V4), which was found in a heterozygous state in five probands (Families C–E, G, and I; Fig. 1; Table 1). In two STGD1 siblings of family C, V4 was found together with the heterozygous splice site variant c.5584+6T>C. The CRD phenotype in the sporadic patient in Family D is very likely caused by heterozygous variant V4 together with a heterozygous variant that is in linkage disequilibrium with the splice-site variant c.5461–10T>C. Individual II:1 of Family E carries the severe heterozygous missense variant p.A1794D and V4, together causing STGD1/CRD. Two siblings of Family G with STGD1/CRD carry the heterozygous missense variant p.L1631P, together with the heterozygous variant V4. The bull's eye phenotype in a sporadic case of Family I is caused by the heterozygous variant V4 and the heterozygous missense variant p.G1961E. The analysis of the remainder of the variants or alternatively spliced exons did not yield additional variants in our cohort. None of the deep-intronic variants were identified in the nine STGD cases without exonic *ABCA4* variants.

Using Sanger sequencing, we did not identify variants V1 and V4 in 167 ethnically matched controls. In the 45 retinal disease probands with one heterozygous exonic *ABCA4* allele and in nine STGD cases with no *ABCA4* variant, we did not find the deep-intronic variants V2, V3, and V5, nor any variants in the 15 regions encompassing weak splice sites [Braun et al., 2013]. For STGD cases with no *ABCA4* variant, we should bear in mind that the genetic defects can very well reside in other genes.

The clinical characteristics of five unrelated patients with intronic variants from Families E through I are shown in Supp. Table S4. They are all of Caucasian descent. These patients were diagnosed between the age of 12 and 23 years (mean: 18.4 years). The mean follow-up of disease was 25 years (range: 12–49 years). Patients from Families F and H showed the intronic variant V1. The proband from Family F carried an exon 20–22 deletion in *trans*, a protein-truncating (severe) allele [Maugeri et al., 1999]. She shows a STGD1

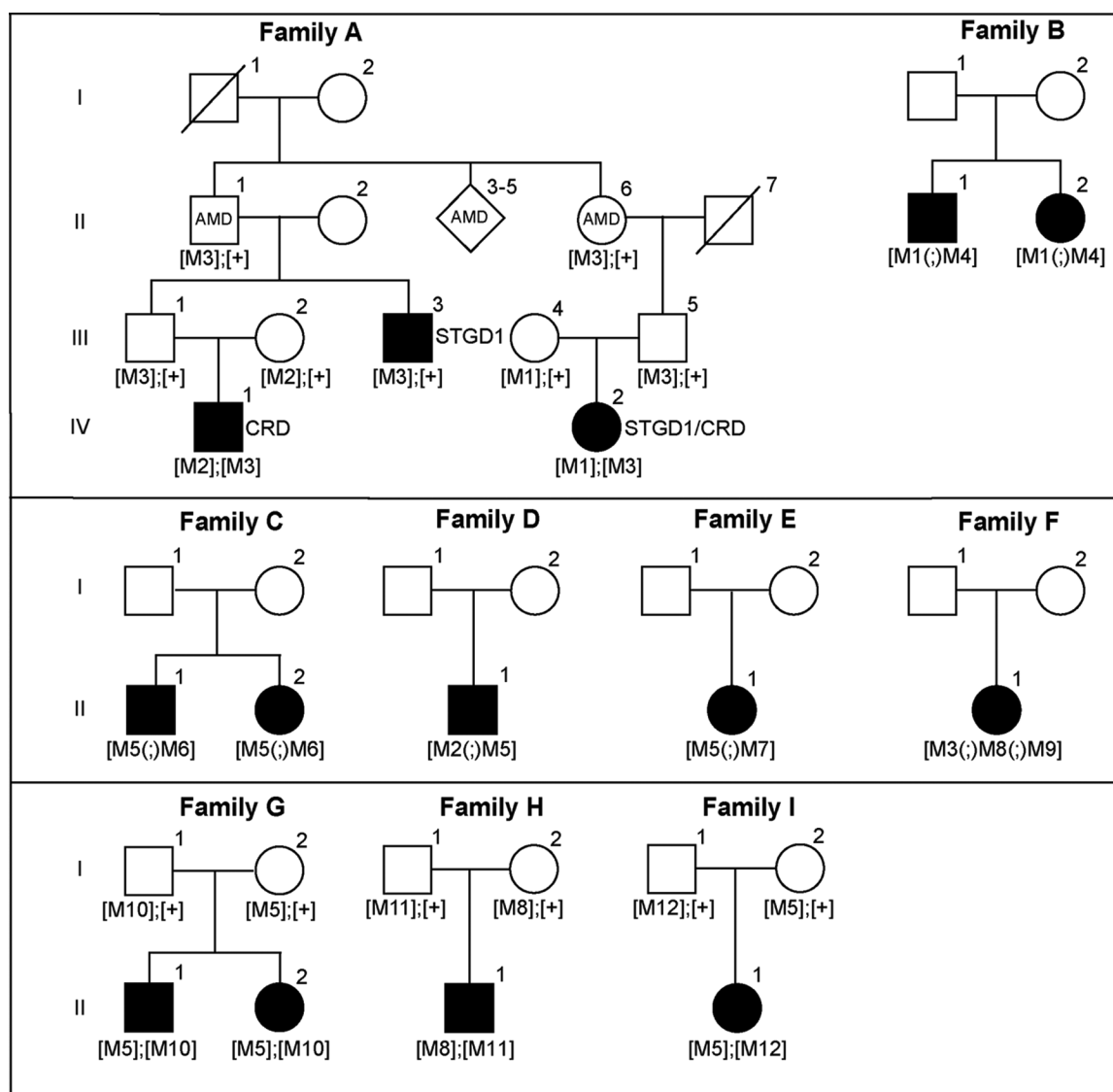


Figure 1. Pedigrees with retinal dystrophies and *ABCA4* variants. **A–B:** Families with retinal dystrophies due to compound heterozygous exonic *ABCA4* variants and intragenic deletions. Family A is adapted from Klevering et al. (2004), and contains several monogenic and multifactorial retinal dystrophies. A heterozygous deletion of exons 20–22 was found in A-IV:1, A-IV:2, and A-III:3. The latter patient does not carry a second *ABCA4* variant. In Family B, a heterozygous deletion spanning exon 5 was identified. **C–I:** Families with retinal dystrophies due to combinations of exonic *ABCA4* variants and deep-intronic variants. The exception is proband F-II:1, who carried a deletion spanning exon 20–22, together with a missense mutation, and a deep-intronic variant V1. Segregation analysis was not possible for Families B–F. *ABCA4* variants: M1: c.768G>T; M2: c.5461–10T>C; M3: p.(S974Qfs*64); M4: p.(G148Vfs*89); M5: c.4539+2001G>A (V4); M6: c.5584+6T>C; M7: p.(A1794D); M8: c.5196+1137G>A (V1); M9: p.(C1488R); M10: p.(L1631P); M11: p.(Q1292*); M12: p.(G1961E).

phenotype with a CRD pattern and a BCVA at last visit (age 35 years) of 20/600. The patient from Family H carried a nonsense variant in *trans*, and showed a typical STGD1 phenotype. His BCVA at last visit was 20/400 (age 32 years). Based on these results, V1 is deemed to have a mild effect on the function of *ABCA4*. Employing RNA extracted from keratinocytes derived from a STGD1 proband carrying V1 and p.T1526M (E.M.S., personal communication), Braun et al. (2013) identified intronic RNA insertions due to V1 in a significant fraction of RNA. Assuming that the *ABCA4* RNA splicing in keratinocytes is similar to that in the retina, this argues for a severe effect of V1, which is in contradiction with our assessment. V1 was very recently found as the most frequent deep-intronic variant (10/233) in STGD1 probands with one *ABCA4* variant [Zernant et al., 2014] and not present in 368 European controls. It was deemed

nonpathogenic as it was present in 8/200 (4%) African-American controls and only found in 1/46 (2%) African-American STGD1 cases with one *ABCA4* variant. However, the c.2588G>C variant in *ABCA4*, previously shown to be a mild allele, was found in a heterozygous state in 3% and 6% of Dutch and Swedish STGD1 cases, respectively [Maugeri et al., 1999; Maugeri et al., 2002]. The V1 variant c.5196+1137G>A is present at an orthologous position in *Macaca mulatta* as the major allele, and shown not to affect exon 36/exon 37 splicing [Zernant et al., 2014]. This result does not rule out pathogenicity of V1 in humans, however, as differences in RNA splicing have been reported between, for example, human patients and a “humanized” knock-in mouse that carries the same segment of human *CEP290* with a deep-intronic variant [Garanto et al., 2013].

Table 1. Retinal Dystrophies-Associated Variants Identified in *ABCA4*

Family	Diagnosis	<i>ABCA4</i> allele 1 (DNA)	<i>ABCA4</i> allele 1 (protein)	<i>ABCA4</i> allele 2 (DNA)	<i>ABCA4</i> allele 2 (protein)
A	STGD1/CRD	c.768G>T	p.(?)	c.2919-?-3328+?del	p.(S974Qfs*64)
B	STGD1	c.768G>T	p.(?)	c.443-?-570+?del	p.(G148Vfs*89)
C	STGD1	c.5584+6T>C	p.(?)	c.4539+2001G>A (V4)	p.(?)
D	CRD	c.5461-10T>C	p.(?)	c.4539+2001G>A (V4)	p.(?)
E	STGD1/CRD	c.5381C>A	p.(A1794D)	c.4539+2001G>A (V4)	p.(?)
F	STGD1/CRD	c.2919-?-3328+?del/c.4462T>C	p.(S974Qfs*64)/(p.C1488R)	c.5196+1137G>T (V1)	p.(?)
G	STGD1/CRD	c.4892T>C	p.(L1631P)	c.4539+2001G>A (V4)	p.(?)
H	STGD1	c.3874C>T	p.(Q1292*)	c.5196+1137G>T (V1)	p.(?)
I	CD	c.5882G>A	p.(G1961E)	c.4539+2001G>A (V4)	p.(?)

Nucleotide numbering uses +1 as the A of the ATG translation initiation codon in NM_000350.2, with the initiation codon as codon 1. To determine the correct mutation nomenclature, <http://www.LOVD.nl/mutalyzer>, Alamut Visual version 2.4 (Interactive Biosoftware, Rouen, France), and <http://www.hgvs.org/mutnomen/> were employed.

Patients from Families E, G, and I showed the intronic variant V4. Patients from Families E and I carried the p.A1794D and p.G1961E variants in *trans*, respectively, both of which were deemed to be mild variants in previous studies as they were identified in typical STGD1 cases that carried protein-truncating variants on the other allele [Maugeri et al., 1999; Burke et al., 2012]. The proband from Family E at her last visit had a BCVA of 20/1200 (age 67 years) and showed a STGD1/CRD picture. The proband from Family I showed a CD phenotype. Based on these data, it is proposed that V4 has a severe effect on *ABCA4* function.

As reverse-transcription polymerase chain reaction analysis using lymphoblast RNA from patients carrying V4 did not yield consistent results (data not shown), there is no experimental proof for this assumption. In the absence of detailed RNA analysis, we cannot be sure that V4 is disease-causing, and it is still possible that the causative variant is in linkage disequilibrium with V4. Although we did not perform haplotype analysis for V4, it is plausible that all probands carrying this variant share a common haplotype. Our findings, together with the frequent occurrence of V4 in *trans* with exonic *ABCA4* variants in a Belgian STGD1 cohort (E. de Baere, personal communication), strongly suggest that V4 is a founder mutation.

In contrast to the cohort from Iowa, the remaining deep-intronic splice variants V2, V3, and V5 were not identified in our cohort. We also did not identify variants in the 15 additional regions containing infrequently used deep-intronic splice sites as described elsewhere [Braun et al., 2013]. From the initial cohort of 127 probands with STGD or STGD/CRD, 88 cases (69%) are currently solved with two *ABCA4* alleles, which is a remarkable difference to the Iowa cohort in which 91.8% of the cases were explained.

Identification of a second *ABCA4* variant is of high significance for the relevant patients as this clearly implicates *ABCA4* as the underlying gene, and thereby autosomal-recessive inheritance of the gene defects. In view of the high frequency of heterozygous *ABCA4* variants in healthy individuals, that is, up to 1/20 individuals [Maugeri et al., 1999], the presence of one allele can be coincidental and the genetic cause situated in another gene. Affected persons with two *ABCA4* variants are also eligible for experimental treatments based on gene augmentation. Moreover, the nine persons from families C through I carrying heterozygous deep-intronic variants V1 or V4 in principle are eligible for a novel type of treatment based on correcting erroneously spliced RNAs, using antisense oligonucleotides. In fibroblasts and immortalized lymphocytes of patients with Leber congenital amaurosis, the potential of this approach was shown by reversing the effect of a deep-intronic splice defect in *CEP290* [Collin et al., 2012]. The “correction” of one of two alleles may be beneficial and the added advantage of this approach versus *ABCA4* gene augmentation is that overexpression of the *ABCA4* gene is not possible.

In conclusion, in this study we identified previously “hidden” *ABCA4* alleles in nine of 45 (20%) families with retinal dystrophies, leaving 36 of the 45 (80%) probands with one heterozygous exonic *ABCA4* allele unexplained. To identify additional putative intronic *ABCA4* variants in the remaining monoallelic cases, intronic DNA sequencing can be combined with mRNA analysis of differentiated retinal-like cells derived from patient’s somatic cells.

Disclosure statement: None of the authors have any financial interest or conflicting interest to disclose.

References

- Allikmets R, Shroyer NF, Singh N, Seddon JM, Lewis RA, Bernstein PS, Peiffer A, Zabriskie NA, Li Y, Hutchinson A, Dean M, Lupski JR, et al. 1997a. Mutation of the Stargardt disease gene (ABCR) in age-related macular degeneration. *Science* 277:1805–1807.
- Allikmets R, Singh N, Sun H, Shroyer NF, Hutchinson A, Chidambaram A, Gerrard B, Baird L, Stauffer D, Peiffer A, Rattner A, Smallwood P, et al. 1997b. A photoreceptor cell-specific ATP-binding transporter gene (ABCR) is mutated in recessive Stargardt macular dystrophy. *Nat Genet* 15:236–246.
- Braun TA, Mullins RF, Wagner AH, Andorf JL, Johnston RM, Bakall BB, Deluca AP, Fishman GA, Lam BL, Weleber RG, Cideciyan AV, Jacobson SG, et al. 2013. Non-exonic and synonymous variants in *ABCA4* are an important cause of Stargardt disease. *Hum Mol Genet* 22:5136–5145.
- Burke TR, Fishman GA, Zernant J, Schubert C, Tsang SH, Smith RT, Ayyagari R, Koenekoop RK, Umfress A, Ciccarelli ML, Baldi A, Iannaccone A, et al. 2012. Retinal phenotypes in patients homozygous for the G1961E mutation in the *ABCA4* gene. *Invest Ophthalmol Vis Sci* 53:4458–4467.
- Collin RW, den Hollander AI, van der Velde-Visser SD, Benniselli J, Bennett J, Cremers FP. 2012. Antisense oligonucleotide (AON)-based therapy for Leber congenital amaurosis caused by a frequent mutation in *CEP290*. *Mol Ther Nucleic Acids* 1:e14.
- Cremers FP, van de Pol DJ, van Driel M, den Hollander AI, van Haren FJ, Knoers NV, Tijmes N, Bergen AA, Rohrschneider K, Blankenagel A, Pinckers AJ, Deutman AF, et al. 1998. Autosomal recessive retinitis pigmentosa and cone-rod dystrophy caused by splice site mutations in the Stargardt’s disease gene ABCR. *Hum Mol Genet* 7:355–362.
- Edwards AO, Donoso LA, Ritter R 3rd. 2001. A novel gene for autosomal dominant Stargardt-like macular dystrophy with homology to the SUR4 protein family. *Invest Ophthalmol Vis Sci* 42:2652–2663.
- Garanto A, van Beersum SE, Peters TA, Roepman R, Cremers FP, Collin RW. 2013. Unexpected *CEP290* mRNA splicing in a humanized knock-in mouse model for Leber congenital amaurosis. *PLoS One* 8:e79369.
- Jaakson K, Zernant J, K  l  m M, Hutchinson A, Tonisson N, Glavac D, Ravnik-Glavac M, Hawlina M, Meltzer MR, Caruso RC, Testa F, Maugeri A, et al. 2003. Genotyping microarray (gene chip) for the ABCR (*ABCA4*) gene. *Hum Mutat* 22:395–403.
- Klevering BJ, Maugeri A, Wagner A, Go SL, Vink C, Cremers FPM, Hoyng CB. 2004. Three families displaying the combination of Stargardt’s disease with cone-rod dystrophy or retinitis pigmentosa. *Ophthalmology* 111:546–553.
- Marmor MF, Fulton AB, Holder GE, Miyake Y, Brigell M, Back M; International Society for Clinical Electrophysiology of Vision. 2009. ISCEV standard for full-field clinical electroretinography (2008 update). *Doc Ophthalmol* 118:69–77.
- Maugeri A, Flothmann K, Hemmrich N, Ingvas S, Jorge P, Paloma E, Patel R, Rozet JM, Tammur J, Testa F, Balcells S, Bird AC, et al. 2002. The *ABCA4* 2588G>C Stargardt mutation: single origin and increasing frequency from South-West to North-East Europe. *Eur J Hum Genet* 10:197–203.

- Maugeri A, van Driel MA, van de Pol DJ, Klevering BJ, van Haren FJ, Tijmes N, Bergen AA, Rohrschneider K, Blankenagel A, Pinckers AJ, Dahl N, Brunner HG, et al. 1999. The 2588G→C mutation in the ABCR gene is a mild frequent founder mutation in the Western European population and allows the classification of ABCR mutations in patients with Stargardt disease. *Am J Hum Genet* 64:1024–1035.
- Poloschek CM, Bach M, Lagreze WA, Glaus E, Lemke JR, Berger W, Neidhardt J. 2010. ABCA4 and ROM1: implications for modification of the PRPH2-associated macular dystrophy phenotype. *Invest Ophthalmol Vis Sci* 51:4253–4265.
- Yatsenko AN, Shroyer NF, Lewis RA, Lupski JR. 2003. An ABCA4 genomic deletion in patients with Stargardt disease. *Hum Mutat* 21:636–644.
- Zernant J, Xie YA, Ayuso C, Riveiro-Alvarez R, Lopez-Martinez MA, Simonelli F, Testa F, Gorin MB, Strom SP, Bertelsen M, Rosenberg T, Boone PM, et al. 2014. Analysis of the ABCA4 genomic locus in Stargardt disease. *Hum Mol Genet* 23: 6797–6806.





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MIR200C (microRNA 200c)

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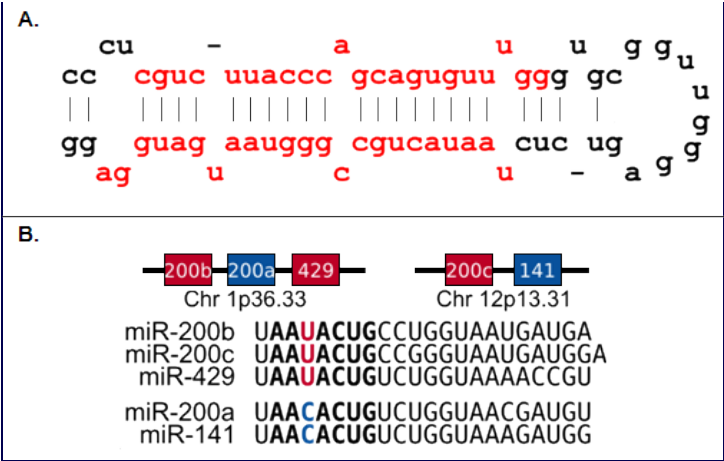
Abstract MicroRNAs (miRNAs) are 20-22 nucleotide long small non-coding RNAs and have a function of regulation of gene postranscriptionally via targeting mainly the 3'UTRs of the genes. miR-200c is a member of miR-200 family with 4 other family members (miR-200a, miR-200b, miR-429 and miR-141) located in chromosome 12 (12q13.31) together with miR-141. miRNAs can be classified as oncomiRs and tumor suppressors according to their target gene and which tissue they are expressed. miR-200c has been shown to be a tumor suppressor in various cancer types. miR-200c has been initially shown to regulate epithelial-mesenchymal transition (EMT) by downregulating ZEB1/2 and upregulating E-cadherin, known epithelial marker. Afterwards, it has been demonstrated that miR-200c also have other important functions in proliferation, cell cycle control, apoptosis, anoikis, invasion, and metastasis of cancer and also in other diseases. Furthermore, miR-200c is a well-established prognostic and diagnostic marker in different cancer types.

(Note : [for Links provided by Atlas : click](#))

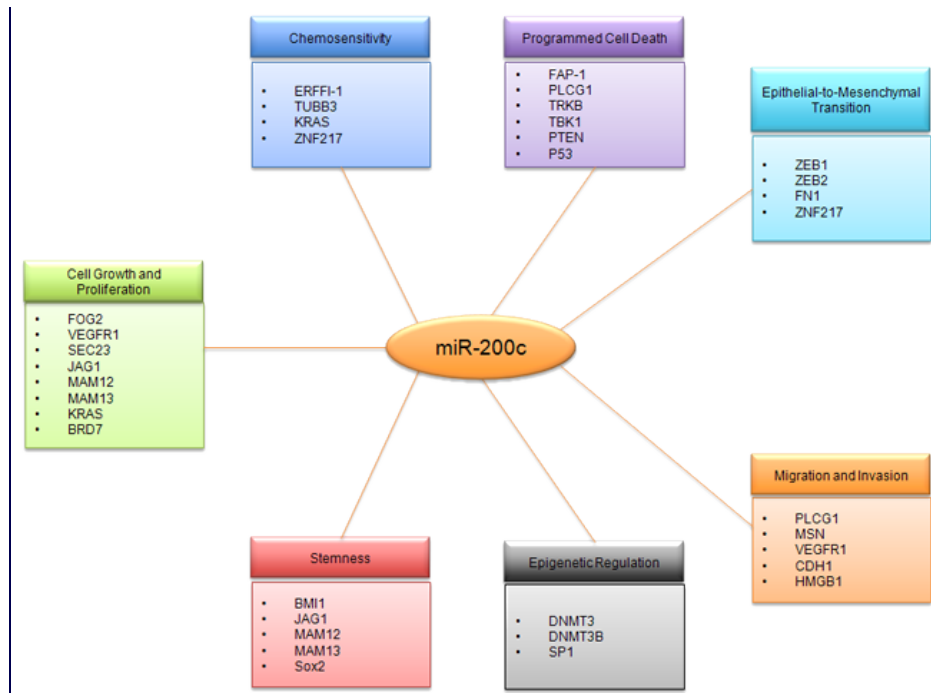
Identity

Other names	hsa-mir-200c MIRN200C mir-200c
HGNC (Hugo)	MIR200C
LocusID (NCBI)	406985
Atlas_Id	51054
Location	12p13.31 [Link to chromosome band 12p13]
Location_base_pair	Starts at 7072862 and ends at 7072929 bp from pter (according to hg19-Feb_2009) [Mapping MIR200C.png] Based on MapViewer Genes on Sequence, genes flanking MIRN200C oriented from centromere to telomere on 12q13.31 are: - ATN1; atrophin 1, 12q13.31 - U7; U7 small nuclear 1, 12q13.31 - C12orf57; chromosome 12 open reading frame 57, 12q13.31
Local_order	- PTPN6 ; protein tyrosine phosphatase, non-receptor type 6, 12q13.31 - MIRN200C ; microRNA 200c, 12q13.31 - MIRN141 ; microRNA 141, 12q13.31 - snoU89; small nucleolar RNA U89, 12q31.1 - PHB2 ; prohibitin 2, 12q31.1.

DNA/RNA



Description	miR-200c belongs to the miR-200 family, which consists of 5 members with two different chromosomal locations: miR-200c and miR-141 are located on chromosome 12p13 and miR-200a, miR-200b and miR-429 are located on 1p36. This family is frequently downregulated upon the progression of tumors and maps to fragile chromosomal regions. Members of this family are important regulators of epithelial-to-mesenchymal transition (EMT) and metastasis.
Transcription	miRNAs are generally transcribed by RNA polymerase II. hsa-mir-200c (precursor miRNA) Accession: MI0000650 Length: 68 bp Sequence: 5'- CCCUCGUCUUACCCAGCAGUGUUUGGGUGCGGUUGGGAGUCUCUAAUACUGCCGGGUAAGAUGGAGG-3' hsa-miR-200c* (-5p) (mature miRNA) Accession: MIMAT0004657 Length: 23 Sequence: 5'-CGUCUUACCCAGCAGUGUUUGG-3' hsa-miR-200c-3p (mature miRNA) Accession: MIMAT0000617 Length: 23 Sequence: 5'-UAAUACUGCCGGGUAAGAUGGA-3'
Pseudogene	No reported pseudogenes.
Protein	
Note	microRNAs are not translated into proteins.
Mutations	
Note	1) rs12904G>A single nucleotide polymorphism in the 3'UTR sequence of EFNA1, a target of miR-200c, results in susceptibility to gastric cancer (Li et al., 2014b). 2) rs1045385A>C SNP in the 3'UTR sequence of AP-2a mRNA increases AP-2a expression and results in cisplatin resistance in HEC-1A cell line of endometrial cancer (Wu et al., 2011).
Implicated in	
Note	
Entity	Cancer development



miR-200c targets several genes regulating numerous processes involved in cancer development and progression.

Entity

Prognosis

Bladder cancer

Loss of miR-200c expression was found to be associated with disease progression and poor outcome in 100 stage T1 bladder tumor patients (Wiklund et al., 2011a). Urinary miR-200 family levels are repressed in patients with bladder cancer (Wang et al., 2012).

Oncogenesis

Deep sequencing of nine [bladder urothelial carcinomas](#) (BUC) and matched normal urothelium revealed that the miR-200c/141 cluster is upregulated in bladder cancer (Han et al., 2011). Consistently, a study comparing miRNA expression patterns by microarray in 27 invasive and 30 superficial bladder tumors with 11 normal urothelia found that miR-200c was upregulated in bladder tumors compared to normal urothelium; however, expression of miR-200c was reduced in invasive compared to non-invasive tumors due to promoter hypermethylation (Wiklund et al., 2011a). Furthermore, microarray miRNA analysis of 43 primary tumors (10 colon, 10 bladder, 13 breast and 10 lung cancers) and matched lymph node metastases revealed that miR-200c and other miR-200 family members are downregulated in metastases compared to primary tumors (Baffa et al., 2009). Mechanistically, miR-200c has been implicated in the regulation of epithelial-to-mesenchymal transition (EMT) in bladder cancer cells. A comparison of nine bladder cancer cell lines revealed a correlation between high expression of miR-200c (and fellow miR-200 family member miR-200b) and epithelial phenotype (Adam et al., 2009). The same study also reported that miR-200c expression reverses resistance to anti-EGFR therapy in bladder cancer cell lines through targeting [ERRFI-1](#). However, contradictory to these results, in another study, it was found that the levels of miR-200c was upregulated in infiltrating BUC patients as compared with non-infiltrating BUC patients (Xie et al., 2012). These results were supported in a recent study, which again demonstrated that the levels of miR-200c were significantly higher in infiltrating carcinoma than in high grade bladder tumors (Lee et al., 2014).

Entity

Prognosis

Breast cancer

Diagnosis. Circulating tumor cell (CTC)-positive metastatic breast cancer patients had significantly higher levels of miR-200c than CTC-negative metastatic breast cancer patients and miR-200c along with some other miRNAs were suggested to be potential predictive markers for CTC status of metastatic breast cancer patients (Madhavan et al., 2012). miR-200c was found to be downregulated in breast cancer patients that are irresponsive to neoadjuvant chemotherapy than patients who respond (Chen et al., 2013c).

Oncogenesis

A double-negative feedback loop between ZEB family transcription factors and the miR-200 family was shown to regulate EMT in different cell systems, including breast cancer cells (Burk et al., 2008). Moreover, expression of miR-200c was revealed to be activated by [p53](#), resulting in induction of EMT in mammary epithelial cells upon loss of p53 (Chang et al., 2011). Loss of p53 was positively correlated with expression of ZEB1 and negatively correlated with expression of miR-200c and [E-Cadherin](#) in 106 breast tumor specimens. miRNA microarray analysis of 43 primary tumors (10 colon, 10 bladder, 13 breast and 10 lung cancers) and matched lymph node metastases revealed that miR-200c and other miR-200 family members are downregulated in metastases compared to primary tumors (Baffa et al., 2009). Moreover, miR-200c and other miR-200 family members were shown to be underexpressed in the aggressive claudin-low subtype of breast cancer, which displays an EMT-like gene expression signature (Herschkowitz et al., 2011). In contrast, luminal breast cancers, which have a more epithelial-like phenotype and a better clinical prognosis, express high levels of miR-200c (Bockmeyer et al., 2011). Besides ZEB family, miR-200c can modify metastasis by targeting [HMGB1](#), [ZNF217](#) and a truncated form of [VEGFR-1](#) (Chang et al., 2014a; Bai et al., 2014b; Mezquita et al., 2014). miR-200c was also found to be an inhibitor of tumor progression and therapy resistance by targeting [KRAS](#) and ZNF217 (Bai et al., 2014b; Kopp et al., 2014).

Re-expression of the miR-200 family in aggressive breast cancer cells was shown to inhibit experimental lung metastasis (Ahmad et al., 2011) and decreased expression of it is associated with lymph node metastases in triple negative breast cancer (Berber et al., 2014). In contrast, another study reported that miR-200c is upregulated in breast cancer patients with lymph node metastasis (Wang et al., 2013). It was also shown to promote colonization of breast cancer cells (Dykxhoorn et al., 2009). The level of miR-200c was also found to be high in patients with various cancers including breast cancer that develop poly-metastases and it was reasoned that miR-200c is aiding colonization in the late stages of metastasis by reverting EMT (Lussier et al., 2011). In vitro assays, miR-200c suppresses migration and invasion of breast cancer cells through various mechanisms,

including targeting of [ZEB1/ZEB2](#), [PLCG1](#), [moesin](#) and [fibronectin](#) (Korpai et al., 2008; Uhlmann et al., 2010; Howe et al., 2011; Gerhauser, 2013).

miR-200c also targets stem cell factors such as [BMI1](#), and downregulation of miR-200c was shown to be characteristic of breast cancer stem cells (Shimono et al., 2009) and DNA methylation was found to be the cause of the repression in breast cancer stem cell like populations (Lim et al., 2013). A natural compound, resveratrol, is increasing the activity of [Ago2](#) and as a result inhibiting breast cancer stem cell-like characteristics by increasing the activity of tumor suppressor miRNAs including miR-200c (Hagiwara et al., 2012).

Furthermore, miRNA microarray analysis revealed that miR-200c is downregulated in breast cancer cells with acquired resistance to cisplatin (Pogribny et al., 2010). It was also found to be downregulated in doxorubicin resistant MCF-7 and BT474 breast cancer cells (Chen et al., 2013c; Kopp et al., 2012). It was also associated with trastuzumab resistance, which was found to be reverse by upregulation of miR-200c through the blockage of [TGF- \$\beta\$](#) signaling (Bai et al., 2014b).

miR-200c is also associated with increase in radiosensitivity in breast cancer cells by inhibiting cell proliferation, and by increasing apoptosis and DNA double-strand breaks. [TBK1](#) was found to be a direct target of miR-200c and its downregulation by miR-200c is partially responsible for increased apoptosis (Lin et al., 2013).

Entity CAFs and microenvironment

Oncogenesis

Eleven dysregulated miRNAs including miR-200c were identified in cancer-associated fibroblasts (CAFs) cultured from six resected breast tumor tissues that had not previously received radiotherapy or chemotherapy treatment. MiR-200c was found to be up-regulated in CAFs compared to normal fibroblasts (NFs) (Zhao et al., 2012). miR-200c targets [Ft1/VEGFR1](#) gene which play an important role in enhancement of cell invasion in CAFs isolated from murine lung adenocarcinomas (Roybal et al., 2011).

Entity Colorectal cancer

Prognosis

Kaplan-Meier survival analysis of 24 colorectal cancer patients suggested that high expression of miR-200c was associated with decreased overall survival (Xi et al., 2006). miR-200c levels in plasma and serum can serve as a potential noninvasive biomarker for CRC prognosis/screening and predicting metastasis (Zhang et al., 2013; Toiyama et al., 2014). Fluoropyrimidines treated two separate groups of individuals showed high levels of miR-200c, along with other members of miR-200 family, and found associated with longer overall and disease-free survival (Diaz et al., 2014).

Oncogenesis

Analysis of miR-200c expression in 24 colorectal cancer (CRC) biopsies and matched normal samples by qRT-PCR revealed that miR-200c is overexpressed in CRC tumors compared to normal tissue (Xi et al., 2006). Furthermore, microarray miRNA analysis of 43 primary tumors (10 colon, 10 bladder, 13 breast and 10 lung cancers) and matched lymph node metastases revealed that miR-200c and other miR-200 family members are downregulated in metastases compared to primary tumors (Baffa et al., 2009). miR-200c was also among miRNAs found upregulated in CRC tissue as compared to normal colonic mucosa shown in a microarray analysis followed by RT-PCR (Tsunoda et al., 2011). K-Ras driven expression of miR-200c and other miRNAs in a 3D culture specific manner suggested a role for miR-200c in regulating colorectal tumor development in vivo (Tsunoda et al., 2011; Ota et al., 2012). In an independent study, miR-200c was found to be associated with the development of CRC (Chen et al., 2012). By directly targeting [ZEB1](#), miR-200c inhibited metastasis in CRC cells SW480/620 (Chen et al., 2012). Epigenetically regulated low expression of miR-200c contributes to EMT and metastatic potential of CRC, and transfecting CRC cells lines with miR-200c lead to increased proliferation but reduced invasion and migration (Hur et al., 2013). miR-200c regulates [Sox2](#) expression in a negative feedback loop in CRC and this regulation is associated with stemness, growth and metastatic potential of CRC (Lu et al., 2014). On the contrary, miR-200c has also been shown to work as oncogene in CRC where it takes part in inhibiting apoptosis and its silencing leads towards upregulation of [Pten](#) and p53 tumor suppressor genes (Chen et al., 2014b).

Entity Endometrial cancer

Disease

Endometrial carcinoma; endometrial carcinosarcoma.

Prognosis

miR-200c was shown to be a prognostic marker of overall survival. High levels of miR-200c were associated with lower chance of survival in patients with endometrioid endometrial cancer (Torres et al., 2013).

Oncogenesis

miRNA microarray analysis of four endometrial endometrioid carcinomas and four normal endometrial tissue samples showed that miR-200c and other miR-200 family members were overexpressed in cancerous compared to normal tissue (Lee et al., 2011). These results were supported by other studies which showed that miR-200c expression is significantly upregulated in endometrial tumors compared to normal tissues (Karaayvaz et al., 2012) as well as to complex atypical hyperplasia (CAH) and simple hyperplasia (SH) cases (Lee et al., 2012). Inhibition of miR-200c decreased the growth of endometrial carcinoma cells (Lee et al., 2011). It was shown that it inhibits the expression of [BRD7](#), which was reported as a potential tumor suppressor gene that prevents [B-catenin](#) from entering into nucleus. Inhibition of BRD7 by miR-200c results in increased expression of B-catenin transcriptional target genes, [cyclin D1](#) and [c-myc](#) (Park et al., 2012). Inhibition of miR-200c decreased the growth of endometrial carcinoma cells (Lee et al., 2011). In contrast, an analysis of miR-200c expression levels in five endometrial cancer and normal endometrial cell lines suggested that miR-200c is lower in cell lines derived from aggressive cancer compared to those derived from less aggressive cancer or normal endometrial epithelium (Cochrane et al., 2009). Restoration of miR-200c expression in aggressive endometrial cancer cells reduced their migration and invasion and increased their sensitivity to microtubule-targeting chemotherapeutic agents, at least in part through targeting [TUBB3](#) (Cochrane et al., 2009; Cochrane et al., 2010; Howe et al., 2011). In a panel of 23 endometrial carcinosarcomas, which are composed of mixed populations of epithelial-like and mesenchymal-like cells, miR-200c and other miR-200 family members were found to be downregulated in the mesenchymal components of the tumors compared to the epithelial components (Castilla et al., 2011) and it was found to be methylated during EMT in both in vitro and in vivo models (Díaz-Martín et al., 2014). These results are consistent with the established role of the miR-200 family in suppression of epithelial- mesenchymal transition.

Entity Esophageal cancer

Prognosis

In a panel of 98 esophageal cancer patients treated with preoperative chemotherapy and surgery, expression of miR-200c was associated with shortened overall survival and poor response to chemotherapy, potentially through upregulation of the Akt signaling pathway (Hamano et al., 2011). In another study higher miR-200c expression in serum collected from 64 esophageal cancer patients who have received neoadjuvant chemotherapy has shown to be associated with poor response to chemotherapy and shortened progression free survival (Tanaka et al., 2013).

Oncogenesis

qRT-PCR analysis of miR-200 expression levels in 17 patients with [Barrett's esophagus](#) and 20 patients with esophageal adenocarcinoma indicated that miR-200c is downregulated during cancer progression from normal epithelium through Barrett's

esophagus to esophageal adenocarcinoma (Smith et al., 2011). In contrast, another study on 98 esophageal cancer patients treated with preoperative chemotherapy and surgery found that miR-200c was expressed at higher levels in the tumor than in normal tissue (Hamano et al., 2011).

Entity [Gastric cancer](#)

Prognosis Significantly higher expression level of miR-200c in blood has been observed in gastric cancer patients as compared to controls and also found as good predictor of overall and progression free survival in gastric cancer patients (Valladares-Ayerbes et al., 2012). miR-200c, miR-200b and miR-125 were found to be targeting most of the genes driving mesenchymal subtype of gastric cancer; a subtype which is associated with poor overall survival in gastric cancer. Functional analysis showed that miR-200b suppresses ZEB1, augments E-cadherin and inhibit cell migration and tumor growth in a mouse model (Song et al., 2014).

Oncogenesis Three miRNAs from miR-200 family (miR-200a, -200b, -200c) found downregulated in gastric adenocarcinoma and miR-200a, when upregulated, suppressed EMT and tumor growth by modulating [Wnt/ \$\beta\$ -catenin signaling pathway](#) through targeting ZEB1 and ZEB2 (Cong et al., 2013). Co-delivering miR-200c with docetaxel by nanoparticles significantly enhanced cytotoxicity of docetaxel and suppressed tumor growth in vivo possibly by decreasing TUBB3 levels and by reversing EMT (Liu et al., 2013). miR-200b and miR-200c, when overexpressed in gastric cancer cells, reduced DNA methylation by targeting [DNMT3A](#), [DNMT3B](#) and SP1, and also reduced tumor growth and migration capacity by re-expressing of [p16](#), RASS1A1, and E-cadherin (Tang et al., 2013). Overexpressing miR-200c in gastric cancer tissues and cells (SGC7901 and SGC7901/DDP) led to enhancing cisplatin sensitivity in these cells possibly by targeting [RhoE](#) (Chang et al., 2014b). miR-141, another member of miR-200 family, also found suppressing proliferation, colony formation, migration and invasion capabilities of gastric cancer cells partially by targeting [HDGF](#) (Chen et al., 2014a). A study conducted on miRNA binding site SNPs located in the 3'UTRs of genes involved in gastric cancer susceptibility revealed that ephrin-A1 (EFNA1) gene is significantly associated with risk of gastric cancer as miR-200c binding site SNP (rs12904 G>A) in the 3'UTR of EFNA1 can significantly modulate EFNA1 expression (Li et al., 2014b).

Entity [Germ cell tumors](#)

Disease Germinoma; yolk sac tumors.

Prognosis Diagnosis. Microarray analysis of 25 germ cell tumors and subsequent validation by qRT-PCR in 10 independent samples identified miR-200c overexpression in yolk sac tumors compared to germinoma (Murray et al., 2010).

Entity [Head and neck cancer](#)

Disease [Squamous cell carcinoma](#); spindle cell carcinoma.

Oncogenesis miR-200c was significantly downregulated in a panel of 30 spindle cell carcinomas (which display a mesenchymal-like phenotype) compared to normal mucosa as determined by qRT-PCR (Zidar et al., 2011). In contrast, expression levels of miR-200c in 30 squamous cell carcinomas were comparable to normal tissue. A xenotransplantation study has shown that miR-200c directly targets BMI1 and overexpression of miR-200c or BMI1 knockdown inhibited lung metastasis and prolonged the survival of mice suggesting therapeutic potential miR-200c in head and neck squamous cell carcinoma (Lo et al., 2011). [HGF](#)-driven downregulation of miR-200c leads to enhanced ZEB1/E-cadherin mediated epithelial to mesenchymal transition in head and neck squamous cell carcinoma (Susuki et al., 2011). miR-200c, along with other miRNAs, play important roles e.g., regulation of stemness and epithelial mesenchymal transition in head and neck tumor cells (Tu et al., 2013). Targeting HPV 16 E6-p300 interaction with a CH1-domain inhibitor resulted in enhanced functional reactivation of p53 tumor suppressor as a result of upregulation of miR-200c and [miR-34a](#) expression levels (Xie et al., 2014). In head and neck squamous cell carcinoma, promoter hypermethylation of miR-200c targets (Zeb1/Zeb2) has been reported to somehow mask the effects associated with miR-200 family regulation of EMT and migration (Tamagawa et al., 2014).

Entity [Huntington' disease](#)

Cytogenetics A significant alteration of miR-200 family members, miR-200a, and miR-200c has been observed in the cerebral cortex and the striatum, at the early stage of disease progression in a mouse model of Huntington's disease. Elevated levels of miR-200c results in downregulation of some target genes, which have been suggested to play important roles in synaptic function, axonal trafficking, neurotransmitter release, neurogenesis, and neuronal survival (Jin et al., 2012).

Entity [Leiomyomas](#)

Oncogenesis It was found that [TIMP2](#), [FBLN5](#), and [VEGFA](#) as direct targets of miR-200c in leiomyomas and the expression of miR-200c was significantly lower in leiomyomas compared to matched myometrium (Chuang et al., 2012).

Entity [Liver cancer](#)

Prognosis **Diagnosis.** miR-200c has been suggested as a biomarker to distinguish [hepatocellular carcinoma](#) from liver metastases (Barshack et al., 2010). miR-429, a member of miR-200 family, was shown to be a prognostic marker in a hepatocellular carcinoma (HCC) tissue microarray study as it was upregulated and shown to promote liver tumor initiating cell properties by targeting [Rb binding protein 4](#) (Li et al., 2014a).

Oncogenesis miRNA microarray analysis of 92 primary hepatocellular carcinomas and 9 HCC cell lines identified miR-200c as a microRNA that is upregulated by p53 (Kim et al., 2011). Increased expression of miR-200c results in downregulation of transcriptional repressors ZEB1 and ZEB2, suggesting a role for p53-mediated regulation of miR-200c in suppression of EMT. miR-200c was reported to be underexpressed in benign liver tumors compared to HCC (Ladeiro et al., 2008); miR-200c levels were determined by qRT-PCR in two sets of tumors (first set: 18 benign tumors, 28 hepatocellular carcinomas; second set: 12 benign tumors, 22 hepatocellular carcinomas). miR-200c, along with other miRNAs, was found to be downregulated in both HCC and intrahepatic cholangiocarcinoma (ICC) (Karakatsanis et al., 2013). Targeting liver cancer cells with miR-200b, member of miR-200 family, not alone but simultaneously with DNA methyl transferase inhibitor reduced the metastatic potential of these cells irrespective of E-cadherin levels (Ding et al., 2012). Transcriptome profiling of 23 ICC and combined HCC tumor specimens using microarrays have revealed miR-200c/EMT as common signaling pathway activated in ICC stem cells. Furthermore, [NCAM1](#), known hepatic stem cell marker, was found to be a direct target of miR-200c (Oishi et al., 2012). While analyzing the expression of a member of miR-200 family, miR-429, in 138 pathology diagnosed HCC patients, this miRNA was found upregulated in tumor tissues and

contributing to cell proliferation and inhibiting apoptosis (Huang et al., 2013). Simultaneous silencing of miR-141 and miR-200c has been reported to be responsible for developing HCC with bile duct tumor thrombosis by activation of ZEB-1 mediated EMT in a study conducted on patients having HCC with or without bile duct tumor thrombus (Yeh et al., 2014).

Entity [Lung cancer](#)

Prognosis qRT-PCR analysis of miR-200c expression levels in 70 [non-small cell lung cancer](#) (NSCLC) patients revealed that high expression of miR-200c was associated with reduced overall survival (Liu et al., 2011). Another study investigated serum microRNAs as cancer biomarkers showed that miR200c is associated with NSCLC, suggesting a potential usage for diagnosis (Liu et al., 2012b). Re-expression of miR-200 family miRNAs has been found to target and downregulate the previously identified prognostic biomarkers in metastatic NSCLC suggesting the importance of these miRNAs in regulating metastatic potential of lung cancer (Pacurari et al., 2013).

Oncogenesis Treatment of immortalized human bronchial epithelial cells with tobacco carcinogens was shown to induce an EMT-like phenotype and stem-cell like properties (Tellez et al., 2011). Quantification of miRNA levels by qRT-PCR in combination with bisulfite sequencing and chromatin immunoprecipitation revealed that these changes are accompanied by epigenetic silencing of miR-200c and other EMT-regulating microRNAs, suggesting that loss of miR-200c contributes to transformation of lung epithelial cells. In contrast, miRNA microarray analysis of six NSCLCs and matched adjacent normal tissue revealed that miR-200c is upregulated in NSCLC compared to healthy tissue (Liu et al., 2011). This finding was further validated in 70 lung carcinomas and matched normal tissue by qRT-PCR.

Several studies have reported that miR-200c can repress invasion and metastasis of lung cancer cells. Firstly, low expression of miR-200c and other miR-200 family members was associated with increased metastatic potential in a syngeneic mouse model of lung adenocarcinoma, and re-expression of miR-200 family members in these cell lines prevented EMT and metastasis (Gibbons et al., 2009). Secondly, miR-200c was shown to be downregulated by promoter hypermethylation in invasive NSCLC cell lines, and re-expression of miR-200c reduced the invasive potential of these cell lines (Ceppi et al., 2010). Furthermore, microarray miRNA analysis of 43 primary tumors (10 colon, 10 bladder, 13 breast and 10 lung cancers) and matched lymph node metastases revealed that miR-200c and other miR-200 family members are downregulated in metastases compared to primary tumors (Baffa et al., 2009). Finally, low expression of miR-200c in 69 primary lung tumors was correlated with lymph node metastases (Ceppi et al., 2010).

Mechanistically, the Notch ligand [Jagged2](#) was shown to suppress expression of miR-200 family members, resulting in induction of EMT and increased metastatic potential (Yang et al., 2011). Moreover, miR-200c and fellow miR-200 family member miR-200b target VEGFR, which also contributes to invasion and metastasis (Roybal et al., 2011).

It was demonstrated that miR-200c which is normally downregulated in lung cancer tissue, is upregulated by transfection in H460 cells resulted in higher levels of apoptotic cells in comparison with untransfected ones (Bai et al., 2014a). In addition, miR-200c enhanced the antitumor effect of resveratrol (RESV). Drug sensitivity can be restored in EMT driven erlotinib ([EGFR](#) inhibitor) resistant NSCLC by using a single agent, silibinin, which fully reverses the high miR-21/low miR-200c signature and represses mesenchymal markers [SNAIL](#), ZEB and [N-Cadherin](#) (Cufi et al., 2013). Moreover, VEGF family is an important regulator of angiogenesis and [VEGFR2](#) has been identified as direct target of miR-200c. Ectopic miR-200c expression radio-sensitized the A549 cells by VEGF-VEGFR2 pathway leading to inhibition of its downstream pro-survival signaling and angiogenesis (Shi et al., 2013). Finally, acquired resistance to EGFR inhibitors in lung cancer cells is found to be associated with EMT (characterized by downregulation of miR-200c) and/or stem like properties (increased ALDH1A1 levels, increase of side population and self renewal capability) (Shien et al., 2013).

Entity [Lymphoma](#)

Prognosis High expression levels of miR-200c was found to be associated with decreased overall survival and time from initial diagnosis to the first relapse in [diffuse large B-cell lymphoma](#) (DLBCL) (Berglund et al., 2013).

Oncogenesis miR-200c was found directly targeting polycomb protein BMI1 in radiation induced thymic lymphoma (RITL) model of BALB/c mice and adenovirus mediated overexpression of miR-200c reduced tumorigenesis in vivo suggesting it as a novel therapeutic method to treat RITL (Cui et al., 2014). Genome-wide expression profiling of nine H. pylori-positive and nine H. pylori-negative gastric diffuse large B-cell lymphomas and further confirmation in 30 samples for each has revealed that miR-200c inhibits ZEB1 in H. pylori-positive gastric diffuse large B-cell lymphoma which, in turn, upregulates [BCL6](#) and results in less aggressive behavior of H. pylori-positive gastric diffuse large B-cell lymphomas (Huang et al., 2014).

Entity [Malignant pleural mesothelioma](#)

Prognosis **Diagnosis.** miR-200c has been proposed as a biomarker to distinguish malignant pleural mesothelioma from lung adenocarcinoma and lung metastases of other carcinomas. miRNA microarray expression profiling of 10 lung adenocarcinomas and 15 mesotheliomas revealed that miR-200c is reduced in mesothelioma (Gee et al., 2010). This result was further confirmed by qRT-PCR in a set of 100 mesotheliomas and 32 lung adenocarcinomas. Similarly, microRNA microarray analysis of 7 malignant pleural mesotheliomas and 97 carcinomas of various origins also identified miR-200c as underexpressed in mesotheliomas compared to the carcinoma samples, and differential expression levels of miR-200c and two other microRNAs could successfully be used to distinguish between malignant pleural mesothelioma and other types of cancer (Benjamin et al., 2010).

Entity [Melanoma](#)

Oncogenesis Analysis of miR-200c expression levels in a panel of 10 melanoma cell lines by qRT-PCR showed that miR-200c is overexpressed in many of these cell lines compared to normal melanocytes (Elson-Schwab et al., 2010). On the contrary, overexpression of miR-200c in melanoma cells significantly decreased proliferation, migratory capacity and drug resistance by targeting BMI-1, [ABCG2](#), ABCG5, and [MDR1](#) and enhancing E-cadherin levels. Overexpression of miR-200c also inhibited melanoma xenograft growth and metastasis in vivo (Liu et al., 2012a). Decreased levels of miR-200a, miR-200c, and miR-203 correlated with increasing tumor thickness in a series of 23 frozen primary melanomas. A functional validation study using an anti-miR200 strategy demonstrated that loss of miR-200 expression in melanoma cell lines reduced E-cadherin expression (van Kempen et al., 2012). Overexpressing miR-200c in mouse melanoma B16F10 CD44+CD133+ CSCs led to the reduced cell proliferation, colony formation, cell migration and invasion potential in vitro as well as tumorigenicity in vivo but not in B16F10 cells and B16F10 non-CD44+ CD133+ CSCs (Dou et al., 2013).

Entity [Oral squamous carcinoma](#)

Prognosis	Significant expression alteration of miR-200 family including miR-200c was shown in oral squamous carcinoma patients compared to healthy controls tested from saliva. This demonstrates a potential biomarker property of miR-200c that can be used in clinical application with oral rinse (Wiklund et al., 2011b).
Entity	<u>Ovarian cancer</u>
Prognosis	High expression of miR-200c was found correlated with decreased progression-free and overall survival in a panel of 20 serous ovarian cancer patients (Nam et al., 2008). In contrast, a study investigating microRNA expression profiles in a total of 144 patients with epithelial ovarian cancer found that low expression of miR-200c was associated with increased progression-free and overall survival (Marchini et al., 2011). Similarly, high expression of miR-200c was correlated with response to chemotherapy and decreased risk of disease recurrence in a panel of 57 patients with serous ovarian carcinoma (Leskela et al., 2010). In a study for identification of differentially expressed miRNAs in high-grade serous ovarian carcinoma (HGSC), clear cell ovarian carcinoma (CCC) and ovarian surface epithelium (OSE), high miR-200c-3p expression has been associated with poor progression-free (p = 0.031) and overall (p = 0.026) survival in HGSC patients (Kim et al., 2014). Diagnosis. MiR-200c along with other miR-200 family miRNAs (miR-141, miR-200a, miR-200b) has been found preferentially upregulated in epithelial ovarian cancer suggesting its role as a potential biomarker for this cancer type (Chen et al., 2013d).
Oncogenesis	miR-200c was found to be overexpressed in a panel of 20 serous ovarian carcinomas compared to 8 normal ovarian tissues by miRNA microarray analysis (Nam et al., 2008). Similarly, increased expression of miR-200c compared to normal ovary (n=15) was reported for serous, endometrioid and clear cell ovarian carcinoma in a series of 69 cancer specimens. Expression of miR-200c was correlated with E-Cadherin levels in 36 primary ovarian carcinomas (Park et al., 2008). The regulatory effect of miR-200c on EMT has been shown to be mediated through targeting of ZEB1 and ZEB2, which transcriptionally repress E-Cadherin (Gregory et al., 2008; Korpala et al., 2008; Park et al., 2008). In this same line of comparative study, miRNA microarray and qPCR analysis, it has been shown elevated expression level of miR-200c on ovarian carcinoma effusions (Vaksman et al., 2011). Re-expression of miR-200c in aggressive ovarian cancer cell lines was shown to reduce their migratory capacity; however, this effect appears to be independent of E-Cadherin expression (Cochrane et al., 2010). Furthermore, forced expression of miR-200c has been reported to sensitize ovarian cancer cells to paclitaxel treatment due to downregulation of miR-200c target gene TUBB3 (Cochrane et al., 2009; Cochrane et al., 2010). Reduction in endogenous PTEN levels and upregulation of phospho-Akt levels were reported in miR-200c transfected ovarian cancer stem cells (OCSCs) (Luo et al., 2013). miR-200c was also shown to be downregulated in a subpopulation of the ovarian cancer cell line OVCAR3 expressing the cancer stem cell marker CD133 (Guo et al., 2011). In CD117+ CD44+ OCSCs, miR-200c expression has been reduced. Overexpression of miR-200c in these OCSCs upregulated E-cadherin expression, downregulated ZEB-1 and Vimentin expression in vitro. Also miR-200c upregulation showed inhibitory effect in CD117+ CD44+ OCSCs in xenograft growth and lung metastasis in nude mice (Chen et al., 2013b).
Entity	<u>Pancreatic cancer</u>
Prognosis	In a panel of 99 pancreatic cancer patients, high expression of miR-200c was associated with increased overall survival (Yu et al., 2010).
Oncogenesis	Downregulation of miR-200c and other miR-200 family members has been observed in gemcitabine-resistant pancreatic cancer cell lines (Li et al., 2009; Ali et al., 2010). miR-200c has also been suggested to have a stemness-inhibiting function in pancreatic cancer cells through targeting of stem cell factors such as Bmi1 (Wellner et al., 2009). A double-negative feedback loop between ZEB family transcription factors and the miR-200 family was shown to regulate EMT in different cell systems, including pancreatic cancer cells (Burk et al., 2008). Consistently, high expression of miR-200c was shown to be associated with decreased invasive behavior in a panel of six pancreatic cancer cell lines, and miR-200c expression was correlated with E-Cadherin levels in pancreatic cancer specimens and cell lines (Yu et al., 2010). Overexpression of miR-200c in pancreatic cancer cell lines resulted in upregulation of E-Cadherin expression and reduced invasion but stimulated proliferation. miRNA expression profiling of various stages in a mouse model of multistep tumorigenesis of the pancreas revealed that miR-200c is downregulated in metastases and metastasis-like tumors (Olson et al., 2009). Moreover, miR-200c also targets components of the Notch pathway, which is aberrantly activated in pancreatic cancer (Brabletz et al., 2011). Undifferentiated, aggressive pancreatic adenocarcinomas were shown to have higher expression of ZEB1 and Notch pathway components and lower expression of miR-200c compared to differentiated tumors. In contrast to the studies described above, which suggest a metastasis-suppressing function for miR-200c in pancreatic cancer, a comparison of 16 pancreatic ductal adenocarcinoma cell lines found that miR-200c expression was upregulated in the highly metastatic cell lines (Mees et al., 2010). Interaction of MUC1 and ZEB1 at the promoter of miR-200c/141, results in transcriptional repression of these miRNAs leading to enhanced progression of pancreatic cancer (Mohr et al., 2013). In addition to regulation of proteins that modulate EMT in pancreatic adenocarcinoma, miR-200c has also been found to target cell surface mucins (MUC4 and MUC16), which play essential role in progression and metastasis in pancreatic adenocarcinoma (Radhakrishnan et al., 2013). In another study it was shown how metformin provokes the death of cancer stem cells in human pancreatic cancer cells (Bao et al., 2012). It was further demonstrated that metformin depleted a set of expression of cancer stem cell markers together with repression of miRNAs including miR-200c.
Entity	<u>Prolactinoma cancer</u>
Oncogenesis	miR-200c was shown to be upregulated in a rat prolactinoma cell line, MMQ. A marine drug SZ-685C induces apoptosis of these cells via downregulation of miR-200c. Moreover, overexpression of miR-200c was found to be attenuating the apoptotic effect of SZ-685C (Chen et al., 2013a).
Entity	<u>Prostate cancer</u>
Prognosis	Plasma levels of miR-200c has been identified as a potential biomarker to differentiate localized prostate cancer from metastatic castration resistant prostate cancer (Watahiki et al., 2013).
Oncogenesis	miRNA sequencing demonstrated that miR-200c was upregulated in primary prostate carcinoma tissue (Szczyrba et al., 2011). In contrast, prostate cancer cells with EMT phenotype were found to have stem-cell like properties and express low levels of miR-200 family members (Kong et al., 2010). In approximately 50% of prostate cancer patients, chromosomal translocations that juxtapose the androgen-sensitive transmembrane protease, serine 2 (TMPRSS2) gene promoter to the oncogenic ETS-family transcription factor ERG result in excessive ERG overexpression which in turn directly represses miR-200c and promotes EMT by upregulating ZEB1 (Kim et al., 2013). Overexpression of miR-200c reversed EMT and stem-cell like properties, in part due to

targeting of [Notch-1](#). miR-200c was also shown to target the Notch ligand [Jagged1](#), resulting in decreased proliferation of metastatic prostate cancer cells (Vallejo et al., 2011).

Entity	Renal cancer
Disease	Clear cell carcinoma (CCC); chromophobe renal cell carcinoma (ChCC).
Prognosis	Diagnosis. miR-200c has been found to be specifically expressed in ChCC and has been suggested as one of the microRNAs that can be used to distinguish between RCC subtypes (Fridman et al., 2010). In addition, miR-200c is one of the five miRNAs used as a biomarker subset allowing to characterize clear-cell renal cell carcinoma (ccRCC), papillary RCC (pRCC) types 1 and 2 and normal tissue with high accuracy (Wach et al., 2013).
Oncogenesis	miR-200c was found to be significantly downregulated in CCC compared to normal kidney in a panel of 16 CCCs, 4 ChCCs and 6 normal kidneys both by microarray analysis and by qRT-PCR (Nakada et al., 2008). Furthermore, miR-200c expression was inversely correlated with expression of its target gene ZEB1 in these specimens. The downregulation of miR-200c in CCC was also confirmed by a second study comparing a total of 25 CCC and matched adjacent normal tissue (Liu et al., 2010). miR-200c has also been shown to be one of the most downregulated miRNAs in a comparative study with 70 matched pairs of clear cell renal cell carcinoma and normal kidney tissues (White et al., 2011). miR-200c negatively affects metastasis of RCC cells by upregulating E-cadherin upon ZEB1 in addition to its effective role on AKT protein. Hence, AKT-miR-200c-E-cadherin pathway may have importance in EMT within RCC. In a series of functional studies of mir-200c, it has been shown that induction of miR-200c expression by Ochratoxin A (OTA) in porcine renal proximal tubular cells attenuates Nrf2 and HO-1 expression and elevates ROS and profibrotic TGF- β expression (Stachurska et al., 2013). Furthermore, it has been demonstrated that the renal cortical content of miR-200c was increased with aging. Increased miR-200c contents were associated with reduced expression of its target, ZEB2 (Sataranatarajan et al., 2012). Finally, multidrug resistance linked proteins appears to be prominently influenced by a set of five miRNAs including mir-200c used to discriminate renal tumor from normal tissue (Wach et al., 2013).
Entity	Thyroid carcinoma
Prognosis	Diagnosis. Deregulated miR-200c, along with other miRNAs, has been reported as a marker for metastatic medullary thyroid carcinoma (Santarpia et al., 2013).
Oncogenesis	The expression of miR-200 family members, including miR-200c, was found to be downregulated in undifferentiated, aggressive anaplastic thyroid carcinoma compared to both normal tissue and well-differentiated papillary and follicular thyroid carcinomas (Braun et al., 2010). Overexpression of the miR-200 family induced mesenchymal-to-epithelial transition and reduced invasion of ATC cells. Overexpression of mir-200c in metastatic medullary thyroid carcinoma improves E-cadherin levels by directly targeting ZEB1 and ZEB2 or by enhanced expression of TGF- β (Santarpia et al., 2013).
Entity	Wilms tumor
Oncogenesis	miRNA expression was analyzed in tissue samples including alveolar rhabdomyosarcoma (RMA) and malignant rhabdoid tumor (MRT) as well as in the rhabdomyosarcoma (RMS) cell lines (Rh30 and RD). It has been shown that miR-200c expression inhibits migration, and miR-200c was shown to be expressed at a lower level in RMA than in MRT (Armeanu-Ebinger et al., 2012).
Entity	Microbiota
Cytogenetics	The intestinal levels of miR-200c, together with 5 other miRNAs, varied upon Listeria infection and for 5 of these miRNAs including miR-200c, this alteration was found to be dependent on the presence of intestinal microbiota of mice (Archambaud et al., 2013).
Entity	Obesity
Cytogenetics	microRNA expression analysis showed mouse miR-200c (mmu-miR-200c) downregulation in the presence of high fat diet in C57BL/6 mice (Chartoumpakis et al., 2012). Leptin deficient ob/ob mice manifest up-regulated miR-200a, miR-200b and miR-429 levels and leptin treatment decreases the amount of these miRNAs. Besides, through overexpression and downregulation studies it was shown that miR-200a might be a target for obesity since its inverse expression relationship with leptin and insulin signaling (Crépin et al., 2014).
Entity	Stem cells, differentiation and reprogramming
Cytogenetics	Direct transfection of three mature miRNAs (miR-200c, -302s and -369s) with increased expression levels in embryonic stem cells and induced pluripotent stem cells can reprogram mouse and human cells to pluripotency. Transfection of miRNAs reduced the risk of mutations and tumorigenesis compared to induced pluripotent stem cells (iPSCs) by introduction of four transcription factors Oct3/4, Sox2, c-Myc and Klf4 (Miyoshi et al., 2011; Miyazaki et al., 2012). miR-200 family also regulates two of the Yamanaka factors Oct4/Sox2 in a specific manner and induces somatic cell reprogramming with the involvement of the miR-200/ZEB2 pathway (Wang et al., 2013). miR-200c, along with miR-150 , has been reported to play an important role in human embryonic stem cell differentiation towards endothelial lineage and chick embryonic blood vessel formation by targeting ZEB1 (Luo et al., 2013).

Bibliography

Prognostic Values of microRNAs in Colorectal Cancer.

Xi Y, Formentini A, Chien M, Weir DB, Russo JJ, Ju J, Kornmann M, Ju J.
Biomark Insights. 2006;2:113-121.
PMID [18079988](#)

A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells.

Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, Spaderna S, Brabletz T.
EMBO Rep. 2008 Jun;9(6):582-9. Epub 2008 May 16.
PMID [18483486](#)

The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1.

Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ.
 Nat Cell Biol. 2008 May;10(5):593-601. Epub 2008 Mar 30.
 PMID [18376396](#)

The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2.

Korpai M, Lee ES, Hu G, Kang Y.
 J Biol Chem. 2008 May 30;283(22):14910-4. Epub 2008 Apr 14.
 PMID [18411277](#)

MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations.

Ladeiro Y, Couchy G, Balabaud C, Bioulac-Sage P, Pelletier L, Rebouissou S, Zucman-Rossi J.
 Hepatology. 2008 Jun;47(6):1955-63.
 PMID [18433021](#)

Genome-wide microRNA expression profiling in renal cell carcinoma: significant down-regulation of miR-141 and miR-200c.

Nakada C, Matsuura K, Tsukamoto Y, Tanigawa M, Yoshimoto T, Narimatsu T, Nguyen LT, Hijiya N, Uchida T, Sato F, Mimata H, Seto M, Moriyama M.
 J Pathol. 2008 Dec;216(4):418-27.
 PMID [18925646](#)

MicroRNA expression profiles in serous ovarian carcinoma.

Nam EJ, Yoon H, Kim SW, Kim H, Kim YT, Kim JH, Kim JW, Kim S.
 Clin Cancer Res. 2008 May 1;14(9):2690-5.
 PMID [18451233](#)

The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2.

Park SM, Gaur AB, Lengyel E, Peter ME.
 Genes Dev. 2008 Apr 1;22(7):894-907.
 PMID [18381893](#)

miR-200 expression regulates epithelial-to-mesenchymal transition in bladder cancer cells and reverses resistance to epidermal growth factor receptor therapy.

Adam L, Zhong M, Choi W, Qi W, Nicoloso M, Arora A, Calin G, Wang H, Siefker-Radtke A, McConkey D, Bar-Eli M, Dinney C.
 Clin Cancer Res. 2009 Aug 15;15(16):5060-72. Epub 2009 Aug 11.
 PMID [19671845](#)

MicroRNA expression profiling of human metastatic cancers identifies cancer gene targets.

Baffa R, Fassan M, Volinia S, O'Hara B, Liu CG, Palazzo JP, Gardiman M, Rugge M, Gomella LG, Croce CM, Rosenberg A.
 J Pathol. 2009 Oct;219(2):214-21.
 PMID [19593777](#)

MicroRNA-200c mitigates invasiveness and restores sensitivity to microtubule-targeting chemotherapeutic agents.

Cochrane DR, Spoelstra NS, Howe EN, Nordeen SK, Richer JK.
 Mol Cancer Ther. 2009 May;8(5):1055-66. Epub 2009 May 12.
 PMID [19435871](#)

miR-200 enhances mouse breast cancer cell colonization to form distant metastases.

Dykxhoorn DM, Wu Y, Xie H, Yu F, Lal A, Petrocca F, Martinvalet D, Song E, Lim B, Lieberman J.
 PLoS One. 2009 Sep 29;4(9):e7181.
 PMID [19787069](#)

Contextual extracellular cues promote tumor cell EMT and metastasis by regulating miR-200 family expression.

Gibbons DL, Lin W, Creighton CJ, Rizvi ZH, Gregory PA, Goodall GJ, Thilaganathan N, Du L, Zhang Y, Pertsemidis A, Kurie JM.
 Genes Dev. 2009 Sep 15;23(18):2140-51.
 PMID [19759262](#)

Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells.

Li Y, VandenBoom TG 2nd, Kong D, Wang Z, Ali S, Philip PA, Sarkar FH.
 Cancer Res. 2009 Aug 15;69(16):6704-12. Epub 2009 Aug 4.
 PMID [19654291](#)

MicroRNA dynamics in the stages of tumorigenesis correlate with hallmark capabilities of cancer.

Olson P, Lu J, Zhang H, Shai A, Chun MG, Wang Y, Libutti SK, Nakakura EK, Golub TR, Hanahan D.
 Genes Dev. 2009 Sep 15;23(18):2152-65.
 PMID [19759263](#)

Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells.

Shimono Y, Zabala M, Cho RW, Lobo N, Dalerba P, Qian D, Diehn M, Liu H, Panula SP, Chiao E, Dirbas FM, Somlo G, Pera RA, Lao K, Clarke MF.
 Cell. 2009 Aug 7;138(3):592-603.
 PMID [19665978](#)

The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs.

Wellner U, Schubert J, Burk UC, Schmalhofer O, Zhu F, Sonntag A, Waldvogel B, Vannier C, Darling D, zur Hausen A, Brunton VG, Morton J, Sansom O, Schuler J, Stemmler MP, Herzberger C, Hopt U, Keck T, Brabletz S, Brabletz T.

Nat Cell Biol. 2009 Dec;11(12):1487-95. Epub 2009 Nov 22.
PMID [19935649](#)

Gemcitabine sensitivity can be induced in pancreatic cancer cells through modulation of miR-200 and miR-21 expression by curcumin or its analogue CDF.

Ali S, Ahmad A, Banerjee S, Padhye S, Dominiak K, Schaffert JM, Wang Z, Philip PA, Sarkar FH.
Cancer Res. 2010 May 1;70(9):3606-17. Epub 2010 Apr 13.
PMID [20388782](#)

Differential diagnosis of hepatocellular carcinoma from metastatic tumors in the liver using microRNA expression.

Barshack I, Meiri E, Rosenwald S, Lebanony D, Bronfeld M, Aviel-Ronen S, Rosenblatt K, Polak-Charcon S, Leizerman I, Ezagouri M, Zepeniuk M, Shabes N, Cohen L, Tabak S, Cohen D, Bentwich Z, Rosenfeld N.
Int J Biochem Cell Biol. 2010 Aug;42(8):1355-62. Epub 2009 Mar 6.
PMID [20619223](#)

A diagnostic assay based on microRNA expression accurately identifies malignant pleural mesothelioma.

Benjamin H, Lebanony D, Rosenwald S, Cohen L, Gibori H, Barabash N, Ashkenazi K, Goren E, Meiri E, Morgenstern S, Perelman M, Barshack I, Goren Y, Edmonston TB, Chajut A, Aharonov R, Bentwich Z, Rosenfeld N, Cohen D.
J Mol Diagn. 2010 Nov;12(6):771-9. Epub 2010 Sep 23.
PMID [20864637](#)

Downregulation of microRNAs directs the EMT and invasive potential of anaplastic thyroid carcinomas.

Braun J, Hoang-Vu C, Dralle H, Huttelmaier S.
Oncogene. 2010 Jul 22;29(29):4237-44. Epub 2010 May 24.
PMID [20498632](#)

Loss of miR-200c expression induces an aggressive, invasive, and chemoresistant phenotype in non-small cell lung cancer.

Ceppi P, Mudduluru G, Kumarswamy R, Rapa I, Scagliotti GV, Papotti M, Allgayer H.
Mol Cancer Res. 2010 Sep;8(9):1207-16. Epub 2010 Aug 9.
PMID [20696752](#)

Loss of miR-200c: A Marker of Aggressiveness and Chemoresistance in Female Reproductive Cancers.

Cochrane DR, Howe EN, Spoelstra NS, Richer JK.
J Oncol. 2010;2010:821717. Epub 2009 Dec 15.
PMID [20049172](#)

MicroRNA-200 family members differentially regulate morphological plasticity and mode of melanoma cell invasion.

Elson-Schwab I, Lorentzen A, Marshall CJ.
PLoS One. 2010 Oct 4;5(10). pii: e13176.
PMID [20957176](#)

Accurate molecular classification of renal tumors using microRNA expression.

Fridman E, Dotan Z, Barshack I, David MB, Dov A, Tabak S, Zion O, Benjamin S, Benjamin H, Kuker H, Avivi C, Rosenblatt K, Polak-Charcon S, Ramon J, Rosenfeld N, Spector Y.
J Mol Diagn. 2010 Sep;12(5):687-96. Epub 2010 Jul 1.
PMID [20595629](#)

Downregulated microRNAs in the differential diagnosis of malignant pleural mesothelioma.

Gee GV, Koestler DC, Christensen BC, Sugarbaker DJ, Ugolini D, Ivaldi GP, Resnick MB, Houseman EA, Kelsey KT, Marsit CJ.
Int J Cancer. 2010 Dec 15;127(12):2859-69.
PMID [21351265](#)

Epithelial to mesenchymal transition is mechanistically linked with stem cell signatures in prostate cancer cells.

Kong D, Banerjee S, Ahmad A, Li Y, Wang Z, Sethi S, Sarkar FH.
PLoS One. 2010 Aug 27;5(8):e12445.
PMID [20805998](#)

The miR-200 family controls beta-tubulin III expression and is associated with paclitaxel-based treatment response and progression-free survival in ovarian cancer patients.

Leskela S, Leandro-Garcia LJ, Mendiola M, Barriuso J, Inglada-Perez L, Munoz I, Martinez-Delgado B, Redondo A, de Santiago J, Robledo M, Hardisson D, Rodriguez-Antona C.
Endocr Relat Cancer. 2010 Dec 21;18(1):85-95. Print 2011.
PMID [21051560](#)

Identifying mRNA targets of microRNA dysregulated in cancer: with application to clear cell Renal Cell Carcinoma.

Liu H, Brannon AR, Reddy AR, Alexe G, Seiler MW, Arreola A, Oza JH, Yao M, Juan D, Liou LS, Ganesan S, Levine AJ, Rathmell WK, Bhanot GV.
BMC Syst Biol. 2010 Apr 27;4:51.
PMID [20420713](#)

EP300--a miRNA-regulated metastasis suppressor gene in ductal adenocarcinomas of the pancreas.

Mees ST, Mardin WA, Wendel C, Baeumer N, Willscher E, Senninger N, Schleicher C, Colombo-Benkmann M, Haier J.
Int J Cancer. 2010 Jan 1;126(1):114-24.
PMID [19569050](#)

The two most common histological subtypes of malignant germ cell tumour are distinguished by global microRNA profiles, associated with differential transcription factor expression.

Murray MJ, Saini HK, van Dongen S, Palmer RD, Muralidhar B, Pett MR, Piipari M, Thornton CM, Nicholson JC, Enright AJ, Coleman N.
Mol Cancer. 2010 Nov 8;9:290.

PMID [21059207](#)

Alterations of microRNAs and their targets are associated with acquired resistance of MCF-7 breast cancer cells to cisplatin.

Pogribny IP, Filkowski JN, Tryndyak VP, Golubov A, Shpileva SI, Kovalchuk O.
Int J Cancer. 2010 Oct 15;127(8):1785-94.

PMID [20099276](#)

miR-200bc/429 cluster targets PLCgamma1 and differentially regulates proliferation and EGF-driven invasion than miR-200a/141 in breast cancer.

Uhlmann S, Zhang JD, Schwager A, Mannsperger H, Riazalhosseini Y, Burmester S, Ward A, Korf U, Wiemann S, Sahin O.
Oncogene. 2010 Jul 29;29(30):4297-306. Epub 2010 May 31.

PMID [20514023](#)

MicroRNA, hsa-miR-200c, is an independent prognostic factor in pancreatic cancer and its upregulation inhibits pancreatic cancer invasion but increases cell proliferation.

Yu J, Ohuchida K, Mizumoto K, Sato N, Kayashima T, Fujita H, Nakata K, Tanaka M.
Mol Cancer. 2010 Jun 28;9:169.

PMID [20579395](#)

Phosphoglucose isomerase/autocrine motility factor mediates epithelial-mesenchymal transition regulated by miR-200 in breast cancer cells.

Ahmad A, Aboukameel A, Kong D, Wang Z, Sethi S, Chen W, Sarkar FH, Raz A.
Cancer Res. 2011 May 1;71(9):3400-9. Epub 2011 Mar 9.

PMID [21389093](#)

MicroRNA profiles of healthy basal and luminal mammary epithelial cells are distinct and reflected in different breast cancer subtypes.

Bockmeyer CL, Christgen M, Muller M, Fischer S, Ahrens P, Langer F, Kreipe H, Lehmann U.
Breast Cancer Res Treat. 2011 Mar 17. [Epub ahead of print]

PMID [21409395](#)

The ZEB1/miR-200 feedback loop controls Notch signalling in cancer cells.

Brabletz S, Bajdak K, Meidhof S, Burk U, Niedermann G, Firat E, Wellner U, Dimmler A, Faller G, Schubert J, Brabletz T.
EMBO J. 2011 Feb 16;30(4):770-82. Epub 2011 Jan 11.

PMID [21224848](#)

Micro-RNA signature of the epithelial-mesenchymal transition in endometrial carcinosarcoma.

Castilla MA, Moreno-Bueno G, Romero-Perez L, Van De Vijver K, Biscuola M, Lopez-Garcia MA, Prat J, Matias-Guiu X, Cano A, Oliva E, Palacios J.
J Pathol. 2011 Jan;223(1):72-80. Epub 2010 Oct 29.

PMID [21125666](#)

p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs.

Chang CJ, Chao CH, Xia W, Yang JY, Xiong Y, Li CW, Yu WH, Rehman SK, Hsu JL, Lee HH, Liu M, Chen CT, Yu D, Hung MC.
Nat Cell Biol. 2011 Mar;13(3):317-23. Epub 2011 Feb 20.

PMID [21336307](#)

Description of the CD133+ subpopulation of the human ovarian cancer cell line OVCAR3.

Guo R, Wu Q, Liu F, Wang Y.
Oncol Rep. 2011 Jan;25(1):141-6.

PMID [21109969](#)

Overexpression of miR-200c induces chemoresistance in esophageal cancers mediated through activation of the Akt signaling pathway.

Hamano R, Miyata H, Yamasaki M, Kurokawa Y, Hara J, Moon JH, Nakajima K, Takiguchi S, Fujiwara Y, Mori M, Doki Y.
Clin Cancer Res. 2011 May 1;17(9):3029-38. Epub 2011 Jan 19.

PMID [21248297](#)

MicroRNA expression signatures of bladder cancer revealed by deep sequencing.

Han Y, Chen J, Zhao X, Liang C, Wang Y, Sun L, Jiang Z, Zhang Z, Yang R, Chen J, Li Z, Tang A, Li X, Ye J, Guan Z, Gui Y, Cai Z.
PLoS One. 2011 Mar 28;6(3):e18286.

PMID [21464941](#)

Comparative oncogenomics identifies breast tumors enriched in functional tumor-initiating cells.

Herschkowitz JI, Zhao W, Zhang M, Usary J, Murrow G, Edwards D, Knezevic J, Greene SB, Darr D, Troester MA, Hilsenbeck SG, Medina D, Perou CM, Rosen JM.
Proc Natl Acad Sci U S A. 2011 Jun 1. [Epub ahead of print]

PMID [21633010](#)

Targets of miR-200c mediate suppression of cell motility and anoikis resistance.

Howe EN, Cochrane DR, Richer JK.
Breast Cancer Res. 2011 Apr 18;13(2):R45. [Epub ahead of print]

PMID [21501518](#)

p53 regulates epithelial-mesenchymal transition through microRNAs targeting ZEB1 and ZEB2.

Kim T, Veronese A, Pichiotti F, Lee TJ, Jeon YJ, Volinia S, Pineau P, Marchio A, Palatini J, Suh SS, Alder H, Liu CG, Dejean A, Croce CM.
J Exp Med. 2011 May 9;208(5):875-83. Epub 2011 Apr 25.

PMID [21518799](#)

The expression of the miRNA-200 family in endometrial endometrioid carcinoma.

Lee JW, Park YA, Choi JJ, Lee YY, Kim CJ, Choi C, Kim TJ, Lee NW, Kim BG, Bae DS.
Gynecol Oncol. 2011 Jan;120(1):56-62. Epub 2010 Oct 28.

PMID [21035172](#)

High expression of serum miR-21 and tumor miR-200c associated with poor prognosis in patients with lung cancer.

Liu XG, Zhu WY, Huang YY, Ma LN, Zhou SQ, Wang YK, Zeng F, Zhou JH, Zhang YK.

Med Oncol. 2011 Apr 24. [Epub ahead of print]

PMID [21516486](#)

MicroRNA-200c attenuates tumour growth and metastasis of presumptive head and neck squamous cell carcinoma stem cells.

Lo WL, Yu CC, Chiou GY, Chen YW, Huang PI, Chien CS, Tseng LM, Chu PY, Lu KH, Chang KW, Kao SY, Chiou SH.

J Pathol. 2011 Mar;223(4):482-95. doi: 10.1002/path.2826. Epub 2011 Jan 5.

PMID [21294122](#)

MicroRNA expression characterizes oligometastasis(es).

Lussier YA, Xing HR, Salama JK, Khodarev NN, Huang Y, Zhang Q, Khan SA, Yang X, Hasselle MD, Darga TE, Malik R, Fan H, Perakis S, Filippo M, Corbin K, Lee Y, Posner MC, Chmura SJ, Hellman S, Weichselbaum RR.

PLoS One. 2011;6(12):e28650. doi: 10.1371/journal.pone.0028650. Epub 2011 Dec 13.

PMID [22174856](#)

[Differential expression of colon cancer microRNA in microarray study].

Ma Q, Yang L, Wang C, Yu YY, Zhou B, Zhou ZG.

Sichuan Da Xue Xue Bao Yi Xue Ban. 2011 May;42(3):344-8.

PMID [21826996](#)

Association between miR-200c and the survival of patients with stage I epithelial ovarian cancer: a retrospective study of two independent tumour tissue collections.

Marchini S, Cavaliere D, Fruscio R, Calura E, Garavaglia D, Nerini IF, Mangioni C, Cattoretti G, Clivio L, Beltrame L, Katsaros D, Scarampi L, Menato G, Perego P, Chiorino G, Buda A, Romualdi C, D'Incalci M.

Lancet Oncol. 2011 Mar;12(3):273-85. Epub 2011 Feb 21.

PMID [21345725](#)

Reprogramming of mouse and human cells to pluripotency using mature microRNAs.

Miyoshi N, Ishii H, Nagano H, Haraguchi N, Dewi DL, Kano Y, Nishikawa S, Tanemura M, Mimori K, Tanaka F, Saito T, Nishimura J, Takemasa I, Mizushima T, Ikeda M, Yamamoto H, Sekimoto M, Doki Y, Mori M.

Cell Stem Cell. 2011 Jun 3;8(6):633-8. doi: 10.1016/j.stem.2011.05.001.

PMID [21620789](#)

miR-200 Inhibits lung adenocarcinoma cell invasion and metastasis by targeting Flt1/VEGFR1.

Roybal JD, Zang Y, Ahn YH, Yang Y, Gibbons DL, Baird BN, Alvarez C, Thilaganathan N, Liu DD, Saintigny P, Heymach JV, Creighton CJ, Kurie JM.

Mol Cancer Res. 2011 Jan;9(1):25-35. Epub 2010 Nov 29.

PMID [21115742](#)

miR-200 family expression is downregulated upon neoplastic progression of Barrett's esophagus.

Smith CM, Watson DI, Leong MP, Mayne GC, Michael MZ, Wijnhoven BP, Hussey DJ.

World J Gastroenterol. 2011 Feb 28;17(8):1036-44.

PMID [21448356](#)

Regulation of microRNA expression by hepatocyte growth factor in human head and neck squamous cell carcinoma.

Susuki D, Kimura S, Naganuma S, Tsuchiyama K, Tanaka T, Kitamura N, Fujieda S, Itoh H.

Cancer Sci. 2011 Dec;102(12):2164-71. doi: 10.1111/j.1349-7006.2011.02096.x. Epub 2011 Oct 12.

PMID [21899661](#)

Downregulation of Sec23A protein by miRNA-375 in prostate carcinoma.

Szczyrba J, Nolte E, Wach S, Kremmer E, Stohr R, Hartmann A, Wieland W, Wullich B, Grasser FA.

Mol Cancer Res. 2011 Jun;9(6):791-800. doi: 10.1158/1541-7786.MCR-10-0573. Epub 2011 May 18.

PMID [21593139](#)

EMT and stem cell-like properties associated with miR-205 and miR-200 epigenetic silencing are early manifestations during carcinogen-induced transformation of human lung epithelial cells.

Tellez CS, Juri DE, Do K, Bernauer AM, Thomas CL, Damiani LA, Tessema M, Leng S, Belinsky SA.

Cancer Res. 2011 Apr 15;71(8):3087-97. Epub 2011 Mar 1.

PMID [21363915](#)

Oncogenic KRAS regulates miR-200c and miR-221/222 in a 3D-specific manner in colorectal cancer cells.

Tsunoda T, Takashima Y, Yoshida Y, Doi K, Tanaka Y, Fujimoto T, Machida T, Ota T, Koyanagi M, Kuroki M, Sasazuki T, Shirasawa S.

Anticancer Res. 2011 Jul;31(7):2453-9.

PMID [21873159](#)

miRNA profiling along tumour progression in ovarian carcinoma.

Vaksman O, Stavnes HT, Kaern J, Trope CG, Davidson B, Reich R.

J Cell Mol Med. 2011 Jul;15(7):1593-602. doi: 10.1111/j.1582-4934.2010.01148.x.

PMID [20716115](#)

Targeting Notch signalling by the conserved miR-8/200 microRNA family in development and cancer cells.

Vallejo DM, Caparros E, Dominguez M.

EMBO J. 2011 Feb 16;30(4):756-69. Epub 2011 Jan 11.

PMID [21224847](#)

miRNA profiling for clear cell renal cell carcinoma: biomarker discovery and identification of potential controls and consequences of miRNA

dysregulation.

White NM, Bao TT, Grigull J, Youssef YM, Girgis A, Diamandis M, Fatoohi E, Metias M, Honey RJ, Stewart R, Pace KT, Bjarnason GA, Youssef GM. J Urol. 2011 Sep;186(3):1077-83. doi: 10.1016/j.juro.2011.04.110. Epub 2011 Jul 23.
PMID [21784468](#)

Coordinated epigenetic repression of the miR-200 family and miR-205 in invasive bladder cancer.

Wiklund ED, Bramsen JB, Hulf T, Dyrskjot L, Ramanathan R, Hansen TB, Villadsen SB, Gao S, Ostensfeld MS, Borre M, Peter ME, Orntoft TF, Kjems J, Clark SJ. Int J Cancer. 2011a Mar 15;128(6):1327-34.
PMID [20473948](#)

MicroRNA alterations and associated aberrant DNA methylation patterns across multiple sample types in oral squamous cell carcinoma.

Wiklund ED, Gao S, Hulf T, Sibbritt T, Nair S, Costea DE, Villadsen SB, Bakholdt V, Bramsen JB, Sørensen JA, Krogdahl A, Clark SJ, Kjems J. PLoS One. 2011b;6(11):e27840. doi: 10.1371/journal.pone.0027840. Epub 2011 Nov 22.
PMID [22132151](#)

A miR-200b/200c/429-binding site polymorphism in the 3' untranslated region of the AP-2? gene is associated with cisplatin resistance.

Wu Y, Xiao Y, Ding X, Zhuo Y, Ren P, Zhou C, Zhou J. PLoS One. 2011;6(12):e29043. doi: 10.1371/journal.pone.0029043. Epub 2011 Dec 14.
PMID [22194984](#)

The Notch ligand Jagged2 promotes lung adenocarcinoma metastasis through a miR-200-dependent pathway in mice

Yang Y, Ahn YH, Gibbons DL, Zang Y, Lin W, Thilaganathan N, Alvarez CA, Moreira DC, Creighton CJ, Gregory PA, Goodall GJ, Kurie JM. J Clin Invest. 2011 Apr 1;121(4):1373-85. Epub 2011 Mar 14.
PMID [21403400](#)

Down-regulation of microRNAs of the miR-200 family and miR-205, and an altered expression of classic and desmosomal cadherins in spindle cell carcinoma of the head and neck--hallmark of epithelial-mesenchymal transition.

Zidar N, Bostjancic E, Gale N, Kojc N, Poljak M, Glavac D, Cardesa A. Hum Pathol. 2011 Apr;42(4):482-8. Epub 2011 Jan 15.
PMID [21237487](#)

Differential expression of miRNAs in rhabdomyosarcoma and malignant rhabdoid tumor.

Armeanu-Ebinger S, Herrmann D, Bonin M, Leuschner I, Warmann SW, Fuchs J, Seitz G. Exp Cell Res. 2012 Dec 10;318(20):2567-77. doi: 10.1016/j.yexcr.2012.07.015. Epub 2012 Sep 18.
PMID [23000453](#)

Metformin inhibits cell proliferation, migration and invasion by attenuating CSC function mediated by deregulating miRNAs in pancreatic cancer cells.

Bao B, Wang Z, Ali S, Ahmad A, Azmi AS, Sarkar SH, Banerjee S, Kong D, Li Y, Thakur S, Sarkar FH. Cancer Prev Res (Phila). 2012 Mar;5(3):355-64. doi: 10.1158/1940-6207.CAPR-11-0299. Epub 2011 Nov 15.
PMID [22086681](#)

Differential expression of microRNAs in adipose tissue after long-term high-fat diet-induced obesity in mice.

Chartoumpekis DV, Zaravinos A, Ziros PG, Iskrenova RP, Psyrogiannis AI, Kyriazopoulou VE, Habeos IG. PLoS One. 2012;7(4):e34872. doi: 10.1371/journal.pone.0034872. Epub 2012 Apr 4.
PMID [22496873](#)

miR-200c inhibits invasion and migration in human colon cancer cells SW480/620 by targeting ZEB1.

Chen ML, Liang LS, Wang XK. Clin Exp Metastasis. 2012 Jun;29(5):457-69. doi: 10.1007/s10585-012-9463-7. Epub 2012 Mar 10.
PMID [22407310](#)

miR-200c is aberrantly expressed in leiomyomas in an ethnic-dependent manner and targets ZEBs, VEGFA, TIMP2, and FBLN5.

Chuang TD, Panda H, Luo X, Chegini N. Endocr Relat Cancer. 2012 Jul 22;19(4):541-56. doi: 10.1530/ERC-12-0007. Print 2012 Aug.
PMID [22685266](#)

miR-200b restoration and DNA methyltransferase inhibitor block lung metastasis of mesenchymal-phenotype hepatocellular carcinoma.

Ding W, Dang H, You H, Steinway S, Takahashi Y, Wang HG, Liao J, Stiles B, Albert R, Rountree CB. Oncogenesis. 2012 Jun 11;1:e15. doi: 10.1038/oncsis.2012.15.
PMID [23552699](#)

Stilbene derivatives promote Ago2-dependent tumour-suppressive microRNA activity.

Hagiwara K, Kosaka N, Yoshioka Y, Takahashi RU, Takeshita F, Ochiya T. Sci Rep. 2012;2:314. doi: 10.1038/srep00314. Epub 2012 Mar 15.
PMID [22423322](#)

Interrogation of brain miRNA and mRNA expression profiles reveals a molecular regulatory network that is perturbed by mutant huntingtin.

Jin J, Cheng Y, Zhang Y, Wood W, Peng Q, Hutchison E, Mattson MP, Becker KG, Duan W. J Neurochem. 2012 Nov;123(4):477-90. doi: 10.1111/j.1471-4159.2012.07925.x. Epub 2012 Sep 28.
PMID [22906125](#)

Prognostic significance of miR-205 in endometrial cancer.

Karaayvaz M, Zhang C, Liang S, Shroyer KR, Ju J. PLoS One. 2012;7(4):e35158. doi: 10.1371/journal.pone.0035158. Epub 2012 Apr 13.
PMID [22514717](#)

miR-200c sensitizes breast cancer cells to doxorubicin treatment by decreasing TrkB and Bmi1 expression.

Kopp F, Oak PS, Wagner E, Roidl A.
 PLoS One. 2012;7(11):e50469. doi: 10.1371/journal.pone.0050469. Epub 2012 Nov 29.
 PMID [23209748](#)

Expression of miRNAs and PTEN in endometrial specimens ranging from histologically normal to hyperplasia and endometrial adenocarcinoma.

Lee H, Choi HJ, Kang CS, Lee HJ, Lee WS, Park CS.
 Mod Pathol. 2012 Nov;25(11):1508-15. doi: 10.1038/modpathol.2012.111. Epub 2012 Jul 6.
 PMID [22766795](#)

miR-200c inhibits melanoma progression and drug resistance through down-regulation of BMI-1.

Liu S, Tetzlaff MT, Cui R, Xu X.
 Am J Pathol. 2012a Nov;181(5):1823-35. doi: 10.1016/j.ajpath.2012.07.009. Epub 2012 Sep 13.
 PMID [22982443](#)

High expression of serum miR-21 and tumor miR-200c associated with poor prognosis in patients with lung cancer.

Liu XG, Zhu WY, Huang YY, Ma LN, Zhou SQ, Wang YK, Zeng F, Zhou JH, Zhang YK.
 Med Oncol. 2012b Jun;29(2):618-26. doi: 10.1007/s12032-011-9923-y. Epub 2011 Apr 24.
 PMID [21516486](#)

Circulating miRNAs as surrogate markers for circulating tumor cells and prognostic markers in metastatic breast cancer.

Madhavan D, Zucknick M, Wallwiener M, Cuk K, Modugno C, Scharpf M, Schott S, Heil J, Turchinovich A, Yang R, Benner A, Riethdorf S, Trumpp A, Sohn C, Pantel K, Schneeweiss A, Burwinkel B.
 Clin Cancer Res. 2012 Nov 1;18(21):5972-82. doi: 10.1158/1078-0432.CCR-12-1407. Epub 2012 Sep 4.
 PMID [22952344](#)

Emerging methods for preparing iPS cells.

Miyazaki S, Yamamoto H, Miyoshi N, Takahashi H, Suzuki Y, Haraguchi N, Ishii H, Doki Y, Mori M.
 Jpn J Clin Oncol. 2012 Sep;42(9):773-9. doi: 10.1093/jjco/hys108. Epub 2012 Jul 23. (REVIEW)
 PMID [22826352](#)

Transcriptomic profiling reveals hepatic stem-like gene signatures and interplay of miR-200c and epithelial-mesenchymal transition in intrahepatic cholangiocarcinoma.

Oishi N, Kumar MR, Roessler S, Ji J, Forgues M, Budhu A, Zhao X, Andersen JB, Ye QH, Jia HL, Qin LX, Yamashita T, Woo HG, Kim YJ, Kaneko S, Tang ZY, Thorgerirsson SS, Wang XW.
 Hepatology. 2012 Nov;56(5):1792-803. doi: 10.1002/hep.25890. Epub 2012 Aug 22.
 PMID [22707408](#)

KRAS up-regulates the expression of miR-181a, miR-200c and miR-210 in a three-dimensional-specific manner in DLD-1 colorectal cancer cells.

Ota T, Doi K, Fujimoto T, Tanaka Y, Ogawa M, Matsuzaki H, Kuroki M, Miyamoto S, Shirasawa S, Tsunoda T.
 Anticancer Res. 2012 Jun;32(6):2271-5.
 PMID [22641662](#)

The interactions between MicroRNA-200c and BRD7 in endometrial carcinoma.

Park YA, Lee JW, Choi JJ, Jeon HK, Cho Y, Choi C, Kim TJ, Lee NW, Kim BG, Bae DS.
 Gynecol Oncol. 2012 Jan;124(1):125-33. doi: 10.1016/j.ygyno.2011.09.026. Epub 2011 Oct 19.
 PMID [22015043](#)

Molecular events in matrix protein metabolism in the aging kidney.

Sataranatarajan K, Feliars D, Mariappan MM, Lee HJ, Lee MJ, Day RT, Yalamanchili HB, Choudhury GG, Barnes JL, Van Remmen H, Richardson A, Kasinath BS.
 Aging Cell. 2012 Dec;11(6):1065-73. doi: 10.1111/ace.12008. Epub 2012 Oct 19.
 PMID [23020145](#)

Circulating miR-200c as a diagnostic and prognostic biomarker for gastric cancer.

Valladares-Ayerbes M, Reboredo M, Medina-Villaamil V, Iglesias-Diaz P, Lorenzo-Patino MJ, Haz M, Santamarina I, Blanco M, Fernandez-Tajes J, Quindos M, Carral A, Figueroa A, Anton-Aparicio LM, Calvo L.
 J Transl Med. 2012 Sep 6;10:186. doi: 10.1186/1479-5876-10-186.
 PMID [22954417](#)

Loss of microRNA-200a and c, and microRNA-203 expression at the invasive front of primary cutaneous melanoma is associated with increased thickness and disease progression.

van Kempen LC, van den Hurk K, Lazar V, Michiels S, Winnepenninckx V, Stas M, Spatz A, van den Oord JJ.
 Virchows Arch. 2012 Oct;461(4):441-8. doi: 10.1007/s00428-012-1309-9. Epub 2012 Sep 6.
 PMID [22956368](#)

Expression of microRNAs in the urine of patients with bladder cancer.

Wang G, Chan ES, Kwan BC, Li PK, Yip SK, Szeto CC, Ng CF.
 Clin Genitourin Cancer. 2012 Jun;10(2):106-13. doi: 10.1016/j.clgc.2012.01.001. Epub 2012 Mar 3.
 PMID [22386240](#)

Infiltration related miRNAs in bladder urothelial carcinoma.

Xie P, Xu F, Cheng W, Gao J, Zhang Z, Ge J, Wei Z, Xu X, Liu Y.
 J Huazhong Univ Sci Technolog Med Sci. 2012 Aug;32(4):576-80. doi: 10.1007/s11596-012-0099-2. Epub 2012 Aug 11.
 PMID [22886973](#)

MiRNA expression analysis of cancer-associated fibroblasts and normal fibroblasts in breast cancer.

Zhao L, Sun Y, Hou Y, Peng Q, Wang L, Luo H, Tang X, Zeng Z, Liu M.
Int J Biochem Cell Biol. 2012 Nov;44(11):2051-9. doi: 10.1016/j.biocel.2012.08.005. Epub 2012 Aug 13.
 PMID [22964023](#)

The intestinal microbiota interferes with the microRNA response upon oral *Listeria* infection.

Archambaud C, Sismeiro O, Toedling J, Soubigou G, Becavin C, Lechat P, Lebreton A, Ciaudo C, Cossart P.
MBio. 2013 Dec 10;4(6):e00707-13. doi: 10.1128/mBio.00707-13.
 PMID [24327339](#)

High expression of microRNA-200c predicts poor clinical outcome in diffuse large B-cell lymphoma.

Berglund M, Hedstrom G, Amini RM, Enblad G, Thunberg U.
Oncol Rep. 2013 Feb;29(2):720-4. doi: 10.3892/or.2012.2173. Epub 2012 Dec 10.
 PMID [23232598](#)

A novel marine drug, SZ-685C, induces apoptosis of MMQ pituitary tumor cells by downregulating miR-200c.

Chen CH, Xiao WW, Jiang XB, Wang JW, Mao ZG, Lei N, Fan X, Song BB, Liao CX, Wang HJ, She ZG, Zhu YH.
Curr Med Chem. 2013a;20(16):2145-54.
 PMID [23317100](#)

MicroRNA-200c overexpression inhibits tumorigenicity and metastasis of CD117+CD44+ ovarian cancer stem cells by regulating epithelial-mesenchymal transition.

Chen D, Zhang Y, Wang J, Chen J, Yang C, Cai K, Wang X, Shi F, Dou J.
J Ovarian Res. 2013b Jul 10;6(1):50. doi: 10.1186/1757-2215-6-50.
 PMID [23842108](#)

miRNA-200c increases the sensitivity of breast cancer cells to doxorubicin through the suppression of E-cadherin-mediated PTEN/Akt signaling.

Chen Y, Sun Y, Chen L, Xu X, Zhang X, Wang B, Min L, Liu W.
Mol Med Rep. 2013c May;7(5):1579-84. doi: 10.3892/mmr.2013.1403. Epub 2013 Mar 28.
 PMID [23546450](#)

Candidate microRNA biomarkers in human epithelial ovarian cancer: systematic review profiling studies and experimental validation.

Chen Y, Zhang L, Hao Q.
Cancer Cell Int. 2013d Aug 27;13(1):86. doi: 10.1186/1475-2867-13-86.
 PMID [23978303](#)

Downregulated microRNA-200a promotes EMT and tumor growth through the wnt/b-catenin pathway by targeting the E-cadherin repressors ZEB1/ZEB2 in gastric adenocarcinoma.

Cong N, Du P, Zhang A, Shen F, Su J, Pu P, Wang T, Zjang J, Kang C, Zhang Q.
Oncol Rep. 2013 Apr;29(4):1579-87. doi: 10.3892/or.2013.2267. Epub 2013 Jan 31.
 PMID [23381389](#)

Silibinin suppresses EMT-driven erlotinib resistance by reversing the high miR-21/low miR-200c signature in vivo.

Cufi S, Bonavia R, Vazquez-Martin A, Oliveras-Ferraro C, Corominas-Faja B, Cuyas E, Martin-Castillo B, Barrajon-Catalan E, Visa J, Segura-Carretero A, Joven J, Bosch-Barrera J, Micol V, Menendez JA.
Sci Rep. 2013;3:2459. doi: 10.1038/srep02459.
 PMID [23963283](#)

Overexpression of microRNA-200c in CD44+CD133+ CSCs inhibits the cellular migratory and invasion as well as tumorigenicity in mice.

Dou J, He XF, Cao WH, Zhao FS, Wang XY, Liu YR, Wang J.
Cell Mol Biol (Noisy-le-grand). 2013 Oct 13;Suppl 59:OL1861-8.
 PMID [24120113](#)

Epigenetic impact of dietary isothiocyanates in cancer chemoprevention.

Gerhauser C.
Curr Opin Clin Nutr Metab Care. 2013 Jul;16(4):405-10. doi: 10.1097/MCO.0b013e328362014e. (REVIEW)
 PMID [23657153](#)

MicroRNA-429 Modulates Hepatocellular Carcinoma Prognosis and Tumorigenesis.

Huang XY, Yao JG, Huang HD, Wang C, Ma Y, Xia Q, Long XD.
Gastroenterol Res Pract. 2013;2013:804128. doi: 10.1155/2013/804128. Epub 2013 Sep 24.
 PMID [24204382](#)

MicroRNA-200c modulates epithelial-to-mesenchymal transition (EMT) in human colorectal cancer metastasis.

Hur K, Toiyama Y, Takahashi M, Balaguer F, Nagasaka T, Koike J, Hemmi H, Koi M, Boland CR, Goel A.
Gut. 2013 Sep;62(9):1315-26. doi: 10.1136/gutjnl-2011-301846. Epub 2012 Jun 26.
 PMID [22735571](#)

Expression of microRNAs, miR-21, miR-31, miR-122, miR-145, miR-146a, miR-200c, miR-221, miR-222, and miR-223 in patients with hepatocellular carcinoma or intrahepatic cholangiocarcinoma and its prognostic significance.

Karakatsanis A, Papaconstantinou I, Gazouli M, Lyberopoulou A, Polymeneas G, Voros D.
Mol Carcinog. 2013 Apr;52(4):297-303. doi: 10.1002/mc.21864. Epub 2011 Dec 27.
 PMID [22213236](#)

TMPRSS2-ERG gene fusions induce prostate tumorigenesis by modulating microRNA miR-200c.

Kim J, Wu L, Zhao JC, Jin HJ, Yu J.

Oncogene. 2013 Nov 4. doi: 10.1038/onc.2013.461. [Epub ahead of print]
PMID [24186205](#)

Epigenetic modulation of the miR-200 family is associated with transition to a breast cancer stem-cell-like state.

Lim YY, Wright JA, Attema JL, Gregory PA, Bert AG, Smith E, Thomas D, Lopez AF, Drew PA, Khew-Goodall Y, Goodall GJ. J Cell Sci. 2013 May 15;126(Pt 10):2256-66. doi: 10.1242/jcs.122275. Epub 2013 Mar 22.
PMID [23525011](#)

miR-200c enhances radiosensitivity of human breast cancer cells.

Lin J, Liu C, Gao F, Mitchel RE, Zhao L, Yang Y, Lei J, Cai J. J Cell Biochem. 2013 Mar;114(3):606-15. doi: 10.1002/jcb.24398.
PMID [22991189](#)

Targeted delivery of miR-200c/DOC to inhibit cancer stem cells and cancer cells by the gelatinases-stimuli nanoparticles.

Liu Q, Li RT, Qian HQ, Wei J, Xie L, Shen J, Yang M, Qian XP, Yu LX, Jiang XQ, Liu BR. Biomaterials. 2013 Sep;34(29):7191-203. doi: 10.1016/j.biomaterials.2013.06.004. Epub 2013 Jun 24.
PMID [23806972](#)

Enrichment of ovarian cancer stem-like cells is associated with epithelial to mesenchymal transition through an miRNA-activated AKT pathway.

Luo X, Dong Z, Chen Y, Yang L, Lai D. Cell Prolif. 2013 Aug;46(4):436-46. doi: 10.1111/cpr.12038.
PMID [23869765](#)

MUC1 regulates expression of multiple microRNAs involved in pancreatic tumor progression, including the miR-200c/141 cluster.

Mohr AM, Bailey JM, Lewallen ME, Liu X, Radhakrishnan P, Yu F, Tappich W, Hollingsworth MA. PLoS One. 2013 Oct 15;8(10):e73306. doi: 10.1371/journal.pone.0073306. eCollection 2013.
PMID [24143167](#)

The microRNA-200 family targets multiple non-small cell lung cancer prognostic markers in H1299 cells and BEAS-2B cells.

Pacurari M, Addison JB, Bondalapati N, Wan YW, Luo D, Qian Y, Castranova V, Ivanov AV, Guo NL. Int J Oncol. 2013 Aug;43(2):548-60. doi: 10.3892/ijo.2013.1963. Epub 2013 May 27.
PMID [23708087](#)

MicroRNA-200c modulates the expression of MUC4 and MUC16 by directly targeting their coding sequences in human pancreatic cancer.

Radhakrishnan P, Mohr AM, Grandgenett PM, Steele MM, Batra SK, Hollingsworth MA. PLoS One. 2013 Oct 25;8(10):e73356. doi: 10.1371/journal.pone.0073356. eCollection 2013.
PMID [24204560](#)

A miRNA signature associated with human metastatic medullary thyroid carcinoma.

Santarpia L, Calin GA, Adam L, Ye L, Fusco A, Giunti S, Thaller C, Paladini L, Zhang X, Jimenez C, Trimarchi F, El-Naggar AK, Gagel RF. Endocr Relat Cancer. 2013 Oct 14;20(6):809-23. doi: 10.1530/ERC-13-0357. Print 2013 Dec.
PMID [24127332](#)

MiR-200c increases the radiosensitivity of non-small-cell lung cancer cell line A549 by targeting VEGF-VEGFR2 pathway.

Shi L, Zhang S, Wu H, Zhang L, Dai X, Hu J, Xue J, Liu T, Liang Y, Wu G. PLoS One. 2013 Oct 30;8(10):e78344. doi: 10.1371/journal.pone.0078344. eCollection 2013.
PMID [24205206](#)

Acquired resistance to EGFR inhibitors is associated with a manifestation of stem cell-like properties in cancer cells.

Shien K, Toyooka S, Yamamoto H, Soh J, Jida M, Thu KL, Hashida S, Maki Y, Ichihara E, Asano H, Tsukuda K, Takigawa N, Kiura K, Gazdar AF, Lam WL, Miyoshi S. Cancer Res. 2013 May 15;73(10):3051-61. doi: 10.1158/0008-5472.CAN-12-4136. Epub 2013 Mar 29.
PMID [23542356](#)

Cross-talk between microRNAs, nuclear factor E2-related factor 2, and heme oxygenase-1 in ochratoxin A-induced toxic effects in renal proximal tubular epithelial cells.

Stachurska A, Ciesla M, Kozakowska M, Wolfram S, Boesch-Saadatmandi C, Rimbach G, Jozkowicz A, Dulak J, Loboda A. Mol Nutr Food Res. 2013 Mar;57(3):504-15. doi: 10.1002/mnfr.201200456. Epub 2012 Dec 28.
PMID [23281030](#)

Circulating miR-200c levels significantly predict response to chemotherapy and prognosis of patients undergoing neoadjuvant chemotherapy for esophageal cancer.

Tanaka K, Miyata H, Yamasaki M, Sugimura K, Takahashi T, Kurokawa Y, Nakajima K, Takiguchi S, Mori M, Doki Y. Ann Surg Oncol. 2013 Dec;20 Suppl 3:S607-15. doi: 10.1245/s10434-013-3093-4. Epub 2013 Jul 10.
PMID [23838916](#)

miR-200b and miR-200c as prognostic factors and mediators of gastric cancer cell progression.

Tang H, Deng M, Tang Y, Xie X, Guo J, Kong Y, Ye F, Su Q, Xie X. Clin Cancer Res. 2013 Oct 15;19(20):5602-12. doi: 10.1158/1078-0432.CCR-13-1326. Epub 2013 Aug 30.
PMID [23995857](#)

Diagnostic and prognostic significance of miRNA signatures in tissues and plasma of endometrioid endometrial carcinoma patients.

Torres A, Torres K, Pesci A, Ceccaroni M, Paszkowski T, Cassandrini P, Zamboni G, Maciejewski R. Int J Cancer. 2013 Apr 1;132(7):1633-45. doi: 10.1002/ijc.27840. Epub 2012 Oct 17.
PMID [22987275](#)

MicroRNA aberrances in head and neck cancer: pathogenetic and clinical significance.

Tu HF, Lin SC, Chang KW.

Curr Opin Otolaryngol Head Neck Surg. 2013 Apr;21(2):104-11. doi: 10.1097/MOO.0b013e32835e1d6e. (REVIEW)

PMID [23340306](#)

MicroRNA profiles classify papillary renal cell carcinoma subtypes.

Wach S, Nolte E, Theil A, Stohr C, T Rau T, Hartmann A, Ekici A, Keck B, Taubert H, Wullich B.

Br J Cancer. 2013 Aug 6;109(3):714-22. doi: 10.1038/bjc.2013.313. Epub 2013 Jun 25.

PMID [23799849](#)

Overexpressions of MicroRNA-9 and MicroRNA-200c in Human Breast Cancers Are Associated with Lymph Node Metastasis.

Wang J, Zhao H, Tang D, Wu J, Yao G, Zhang Q.

Cancer Biother Radiopharm. 2013 Apr 25. [Epub ahead of print]

PMID [23617747](#)

Plasma miRNAs as Biomarkers to Identify Patients with Castration-Resistant Metastatic Prostate Cancer.

Watahiki A, Macfarlane RJ, Gleave ME, Crea F, Wang Y, Helgason CD, Chi KN.

Int J Mol Sci. 2013 Apr 10;14(4):7757-70. doi: 10.3390/ijms14047757.

PMID [23574937](#)

Plasma miR-200c and miR-18a as potential biomarkers for the detection of colorectal carcinoma.

Zhang GJ, Zhou T, Liu ZL, Tian HP, Xia SS.

Mol Clin Oncol. 2013 Mar;1(2):379-384. Epub 2013 Jan 7.

PMID [24649179](#)

Synergistic antitumor activity of resveratrol and miR-200c in human lung cancer.

Bai T, Dong DS, Pei L.

Oncol Rep. 2014a May;31(5):2293-7. doi: 10.3892/or.2014.3090. Epub 2014 Mar 14.

PMID [24647918](#)

MiR-200c suppresses TGF- β signaling and counteracts trastuzumab resistance and metastasis by targeting ZNF217 and ZEB1 in breast cancer.

Bai WD, Ye XM, Zhang MY, Zhu HY, Xi WJ, Huang X, Zhao J, Gu B, Zheng GX, Yang AG, Jia LT.

Int J Cancer. 2014b Sep 15;135(6):1356-68. doi: 10.1002/ijc.28782. Epub 2014 Mar 3.

PMID [24615544](#)

miR-205 and miR-200c: Predictive Micro RNAs for Lymph Node Metastasis in Triple Negative Breast Cancer.

Berber U, Yilmaz I, Narli G, Haholu A, Kucukodaci Z, Demirel D.

J Breast Cancer. 2014 Jun;17(2):143-8. doi: 10.4048/jbc.2014.17.2.143. Epub 2014 Jun 27.

PMID [25013435](#)

miR-200c inhibits metastasis of breast cancer cells by targeting HMGB1.

Chang BP, Wang DS, Xing JW, Yang SH, Chu Q, Yu SY.

J Huazhong Univ Sci Technolog Med Sci. 2014a Apr;34(2):201-6. doi: 10.1007/s11596-014-1259-3. Epub 2014 Apr 8.

PMID [24710933](#)

MicroRNA-200c regulates the sensitivity of chemotherapy of gastric cancer SGC7901/DDP cells by directly targeting RhoE.

Chang L, Guo F, Wang Y, Lv Y, Huo B, Wang L, Liu W.

Pathol Oncol Res. 2014b Jan;20(1):93-8. doi: 10.1007/s12253-013-9664-7. Epub 2013 Jul 3.

PMID [23821457](#)

miR-141 suppresses proliferation and motility of gastric cancer cells by targeting HDGF.

Chen B, Huang T, Jiang J, Lv L, Li H, Xia S.

Mol Cell Biochem. 2014a Mar;388(1-2):211-8. doi: 10.1007/s11010-013-1912-3. Epub 2013 Nov 26.

PMID [24276755](#)

The roles of miR-200c in colon cancer and associated molecular mechanisms.

Chen J, Wang W, Zhang Y, Hu T, Chen Y.

Tumour Biol. 2014b Jul;35(7):6475-83. doi: 10.1007/s13277-014-1860-x. Epub 2014 Mar 30.

PMID [24682933](#)

The over-expression of miR-200a in the hypothalamus of ob/ob mice is linked to leptin and insulin signaling impairment.

Crepin D, Benomar Y, Riffault L, Amine H, Gertler A, Taouis M.

Mol Cell Endocrinol. 2014 Mar 25;384(1-2):1-11. doi: 10.1016/j.mce.2013.12.016. Epub 2014 Jan 4.

PMID [24394757](#)

Down regulation of miR200c promotes radiation-induced thymic lymphoma by targeting BMI1.

Cui J, Cheng Y, Zhang P, Sun M, Gao F, Liu C, Cai J.

J Cell Biochem. 2014 Jun;115(6):1033-42. doi: 10.1002/jcb.24754.

PMID [24375660](#)

A core microRNA signature associated with inducers of the epithelial-to-mesenchymal transition.

Diaz-Martin J, Diaz-Lopez A, Moreno-Bueno G, Castilla MA, Rosa-Rosa JM, Cano A, Palacios J.

J Pathol. 2014 Feb;232(3):319-29. doi: 10.1002/path.4289.

PMID [24122292](#)

Role of miR-200 family members in survival of colorectal cancer patients treated with fluoropyrimidines.

Diaz T, Tejero R, Moreno I, Ferrer G, Cordeiro A, Artells R, Navarro A, Hernandez R, Tapia G, Monzo M.

J Surg Oncol. 2014 Jun;109(7):676-83. doi: 10.1002/jso.23572. Epub 2014 Feb 10.

PMID [24510588](#)

Inhibition of ZEB1 by miR-200 characterizes Helicobacter pylori-positive gastric diffuse large B-cell lymphoma with a less aggressive behavior.

Huang WT, Kuo SH, Cheng AL, Lin CW.

Mod Pathol. 2014 Aug;27(8):1116-25. doi: 10.1038/modpathol.2013.229. Epub 2014 Jan 3.

PMID [24390222](#)**Differential microRNA expression signatures and cell type-specific association with Taxol resistance in ovarian cancer cells.**

Kim YW, Kim EY, Jeon D, Liu JL, Kim HS, Choi JW, Ahn WS.

Drug Des Devel Ther. 2014 Feb 24;8:293-314. doi: 10.2147/DDDT.S51969. eCollection 2014.

PMID [24591819](#)**The proto-oncogene KRAS is targeted by miR-200c.**

Kopp F, Wagner E, Roidl A.

Oncotarget. 2014 Jan 15;5(1):185-95.

PMID [24368337](#)**Expression of miRNAs and ZEB1 and ZEB2 correlates with histopathological grade in papillary urothelial tumors of the urinary bladder.**

Lee H, Jun SY, Lee YS, Lee HJ, Lee WS, Park CS.

Virchows Arch. 2014 Feb;464(2):213-20. doi: 10.1007/s00428-013-1518-x. Epub 2013 Dec 4.

PMID [24306957](#)**Epigenetic modification of MiR-429 promotes liver tumour-initiating cell properties by targeting Rb binding protein 4.**

Li L, Tang J, Zhang B, Yang W, Liugao M, Wang R, Tan Y, Fan J, Chang Y, Fu J, Jiang F, Chen C, Yang Y, Gu J, Wu D, Guo L, Cao D, Li H, Cao G, Wu M, Zhang MQ, Chen L, Wang H.

Gut. 2014a Feb 26. doi: 10.1136/gutjnl-2013-305715. [Epub ahead of print]

PMID [24572141](#)**G-A variant in miR-200c binding site of EFNA1 alters susceptibility to gastric cancer.**

Li Y, Nie Y, Cao J, Tu S, Lin Y, Du Y, Li Y.

Mol Carcinog. 2014b Mar;53(3):219-29. doi: 10.1002/mc.21966. Epub 2012 Oct 12.

PMID [23065816](#)**Regulation of colorectal carcinoma stemness, growth, and metastasis by an miR-200c-Sox2-negative feedback loop mechanism.**

Lu YX, Yuan L, Xue XL, Zhou M, Liu Y, Zhang C, Li JP, Zheng L, Hong M, Li XN.

Clin Cancer Res. 2014 May 15;20(10):2631-42. doi: 10.1158/1078-0432.CCR-13-2348. Epub 2014 Mar 21.

PMID [24658157](#)**Unlocking Doors without Keys: Activation of Src by Truncated C-terminal Intracellular Receptor Tyrosine Kinases Lacking Tyrosine Kinase Activity.**

Mezquita B, Mezquita P, Pau M, Mezquita J, Mezquita C.

Cells. 2014 Feb 14;3(1):92-111. doi: 10.3390/cells3010092.

PMID [24709904](#)**Integrated microRNA network analyses identify a poor-prognosis subtype of gastric cancer characterized by the miR-200 family.**

Song F, Yang D, Liu B, Guo Y, Zheng H, Li L, Wang T, Yu J, Zhao Y, Niu R, Liang H, Winkler H, Zhang W, Hao X, Chen K.

Clin Cancer Res. 2014 Feb 15;20(4):878-89. doi: 10.1158/1078-0432.CCR-13-1844. Epub 2013 Dec 18.

PMID [24352645](#)**Role of miR-200c/miR-141 in the regulation of epithelial-mesenchymal transition and migration in head and neck squamous cell carcinoma.**

Tamagawa S, Beder LB, Hotomi M, Gunduz M, Yata K, Grenman R, Yamanaka N.

Int J Mol Med. 2014 Apr;33(4):879-86. doi: 10.3892/ijmm.2014.1625. Epub 2014 Jan 14.

PMID [24424572](#)**Serum miR-200c is a novel prognostic and metastasis-predictive biomarker in patients with colorectal cancer.**

Toiyama Y, Hur K, Tanaka K, Inoue Y, Kusunoki M, Boland CR, Goel A.

Ann Surg. 2014 Apr;259(4):735-43. doi: 10.1097/SLA.0b013e3182a6909d.

PMID [23982750](#)**Targeting HPV16 E6-p300 interaction reactivates p53 and inhibits the tumorigenicity of HPV-positive head and neck squamous cell carcinoma.**

Xie X, Piao L, Bullock BN, Smith A, Su T, Zhang M, Teknos TN, Arora PS, Pan Q.

Oncogene. 2014 Feb 20;33(8):1037-46. doi: 10.1038/onc.2013.25. Epub 2013 Mar 11.

PMID [23474763](#)**Expression profile of microRNA-200 family in hepatocellular carcinoma with bile duct tumor thrombus.**

Yeh TS, Wang F, Chen TC, Yeh CN, Yu MC, Jan YY, Chen MF.

Ann Surg. 2014 Feb;259(2):346-54. doi: 10.1097/SLA.0000000000000223.

PMID [24135722](#)**Citation***This paper should be referenced as such :*

Mutlu M, Saatçi Ö, Raza U, Eyüpoglu E, Yurdusev E, Sahin Ö

MIR200C (microRNA 200c)

Atlas Genet Cytogenet Oncol Haematol. 2015;19(4):270-285.

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On line version : <http://AtlasGeneticsOncology.org/Genes/MIR200CID51054ch12p13.html>

History of this paper:

Jurmeister, S ; Uhlmann, S ; Sahin, Ö. MIR200C (microRNA 200c). Atlas Genet Cytogenet Oncol Haematol. 2012;16(2):92-99.

<http://documents.irevues.inist.fr/bitstream/handle/2042/46935/08-2011-MIR200CID51054ch12p13.pdf>

External links

	Nomenclature
HGNC (Hugo)	MIR200C 31580
	Cards
Atlas	MIR200CID51054ch12p13
Entrez Gene (NCBI)	MIR200C 406985 microRNA 200c
Aliases	MIRN200C; mir-200c
GeneCards (Weizmann)	MIR200C
Ensembl hg19 (Hinxton)	[Gene_View] chr12:7072862-7072929 [Contig_View] MIR200C [Vega]
Ensembl hg38 (Hinxton)	[Gene_View] chr12:7072862-7072929 [Contig_View] MIR200C [Vega]
TCGA cBioPortal	MIR200C
AceView (NCBI)	MIR200C
Genatlas (Paris)	MIR200C
WikiGenes	406985
SOURCE (Princeton)	MIR200C
	Genomic and cartography
GoldenPath hg19 (UCSC)	MIR200C - chr12:7072862-7072929 + 12p13.31 [Description] (hg19-Feb_2009)
GoldenPath hg38 (UCSC)	MIR200C - 12p13.31 [Description] (hg38-Dec_2013)
Ensembl	MIR200C - 12p13.31 [CytoView hg19] MIR200C - 12p13.31 [CytoView hg38]
Mapping of homologs : NCBI	MIR200C [Mapview hg19] MIR200C [Mapview hg38]
OMIM	612092
	Gene and transcription
Genbank (Entrez)	LM608608
RefSeq transcript (Entrez)	
RefSeq genomic (Entrez)	NC_000012 NC_018923 NT_009759 NW_004929382
Consensus coding sequences : CCDS (NCBI)	MIR200C
Gene Expression	MIR200C [NCBI-GEO] MIR200C [EBI - ARRAY_EXPRESS] MIR200C [SEEK] MIR200C [MEM]
Gene Expression Viewer (FireBrowse)	MIR200C [Firebrowse - Broad]
SOURCE (Princeton)	Expression in : [Datasets] [Normal Tissue Atlas] [carcinoma Classification] [NCI60]
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Domain families : Pfam (Sanger)	
Domain families : Pfam (NCBI)	
DMDM Disease mutations	406985
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