

**miRNA-mRNA INTERACTION NETWORK
REGULATING CHEMOTHERAPY RESISTANCE IN TRIPLE
NEGATIVE BREAST CANCER**

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

By

Ridho Assidicky

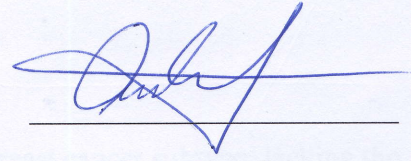
June 2019

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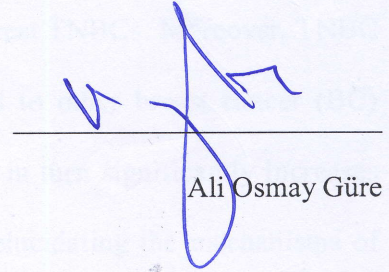
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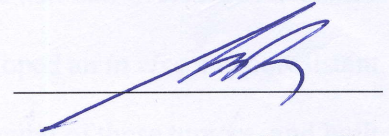


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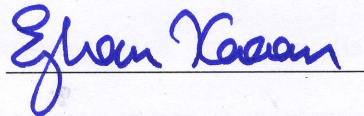


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ABSTRACT

miRNA-mRNA INTERACTION NETWORK REGULATING CHEMOTHERAPY RESISTANCE IN TRIPLE NEGATIVE BREAST CANCER

Ridho Assidicky

M.S. in Molecular Biology and Genetics

Advisor: Özgür Şahin

June, 2019

Triple negative breast cancer (TNBC) is the most aggressive breast cancer subtype, lacking the expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2). Compared to other subtypes, which can be treated with targeted therapies, chemotherapy is the major regimen used to treat TNBCs. Moreover, TNBC patients have better response rate to chemotherapy compared to other breast cancer (BC) subtypes. However, patients develop resistance rapidly, which in turn significantly increases the mortality rate. Therefore, there is urgent unmet need for elucidating the mechanisms of chemotherapy resistance in TNBC and identifying novel targets that can overcome resistance or potentiate the efficacy of chemotherapy. In this line, we developed an *in vivo* chemoresistant TNBC xenograft model, performed whole transcriptome sequencing of these tumors, and built a miRNA-mRNA interaction network regulating TNBC chemoresistance. We identified an ECM glycoprotein (“EG”) as the central chemoresistance driver gene, and a candidate potential tumor suppressor miRNA (“TSM”) sensitizing cells to EG-induced chemoresistance. Mechanistically, TSM was downregulated by hypoxia in chemoresistant tumor microenvironment that, in turn, led to upregulation of an integrin family protein (“IFP”), which encodes a subunit of receptor recognizing EG. We further showed that TSM directly binds the 3’-UTR of IFP, represses its expression, and inhibits FAK/Src signaling, PI3K signaling and

MAPK signaling pathways, which constitute the major pathways in cell survival. Importantly, overexpression of TSM or inhibition of the IFP overcame EG-driven chemotherapy resistance *in vitro* and potentiated the efficacy of chemotherapy *in vivo*. Overall, we built the first miRNA-mRNA interaction network of TNBC chemoresistance and identified a hypoxia-regulated novel tumor suppressor miRNA, TSM, or its target IFP as potential targets overcoming chemoresistance or potentiating the efficacy of chemotherapy in TNBCs

Keyword: TNBC, chemotherapy, chemoresistance, ECM glycoprotein (EG), tumor suppressor miRNA (TSM), integrin family protein (IFP), hypoxia, integrin signaling, chemosensitization.



ÖZET

ÜÇLÜ NEGATİF MEME KANSERİNDE KEMOTERAPİ DİRENCİNİ DÜZENLEYEN miRNA-mRNA ETKİLEŞİM AĞI

Ridho Assidicky

Moleküler Biyoloji ve Genetik, Yüksek Lisans

Tez danışmanı: Özgür Şahin

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Triple (üçlü) negatif meme kanseri (TNMK) en agresif meme kanseri alt türü olup östrojen reseptörü (ER), progesteron reseptörü (PR) ve insan epidermal büyüme faktörü reseptörü 2'yi (HER2) ifade etmemektedir. TNMK üzerinde hedefli terapi ajanlarının etkinliğinin kısıtlı olması, konvansiyonel kemoterapiyi yegane tedavi opsiyonu kılmaktadır. Bununla birlikte, TNMK diğer meme kanseri alt türlerine kıyasla başlangıçta kemoterapiye daha yüksek oranda yanıt vermektedir. Ancak hastalar kısa sürede direnç geliştirmekte ve bu, ölüm oranlarının belirgin düzeyde artışına sebebiyet vermektedir. Bu nedenle TNMK'de ilaç direncinin altında yatan mekanizmaların aydınlatılması ve direnci kırabilecek ya da kemoterapinin etkisini güçlendirebilecek yeni hedeflerin belirlenmesi hususunda ivedilikle karşılanmayı bekleyen bir ihtiyaç bulunmaktadır. Bu doğrultuda, bir *in vivo* kemoterapi dirençli TNMK ksenograft modeli geliştirdik ve tüm transkriptom ve miRNA-mRNA etkileşim ağı analizlerini yaptık. Bir ekstrasellüler matriks glikoproteinini ("EG") kemoterapi direncine sebebiyet veren merkezi gen olarak ve EG-güdümlü kemoterapi direncini kırma potansiyeline sahip bir aday tümör baskılayıcı miRNA ("TSM") belirledik. Mekanizmal açıdan, TSM ifadesi kemoterapi dirençli tümör mikroçevresinde hipoksi tarafından azaltılmakta ve bu, EG reseptörü alt birimi olan bir integrin ailesi proteinin ("IFP") artışına yol açmaktadır. Devamında, TSM'nin doğrudan IFP'nin 3'-UTR'sine bağlandığını, IFP ifadesini baskıladığını ve başlıca hücre sağkalım

yolaklarını teşkil eden FAK/Src, PI3K ve MAPK sinyal yolaklarını engellediğini gösterdik. Daha önemlisi, TSM'nin aşırı ifade edilmesi ya da IFP'nin engellenmesi, EG-güdümlü *in vitro* kemoterapi direncini kırmış ve *in vivo* kemoterapi etkinliğini güçlendirmiştir.

Sonuç olarak, TNMK kemoterapi direncinin ilk miRNA-mRNA etkileşim ağını oluşturduk ve hipoksi tarafından düzenlenen yeni bir tümör baskılayıcı miRNA, TSM'yi ya da hedefi IFP'yi kemoterapi direncinin üstesinden gelme ya da kemoterapinin etkinliğini güçlendirmede potansiyel hedefler olarak belirledik.

Anahtar kelimeler: TNMK, kemoterapi, kemoterapi direnci, EG, tümör baskılayıcı miRNA (TSM), integrin ailesi proteini (IFP), hipoksi, integrin yolağı, kemoterapi direncinin kırılması.

To my parents

And

To my significant other



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Abbreviations

AGO	Argonaute
AKT	serine/threonine-specific protein kinase, also known as protein kinase B
ERK	Extracellular signal-regulated kinase
APS	Ammonium persulfate
AR	Androgen receptor
ECM	Extracellular matrix
ARRB1	Arrestin Beta 1
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2 protein
B-CLL	B-Cell chronic lymphocytic leukemia
BRAF	B-Raf Proto-Oncogene, Serine/Threonine Kinase
BRCA1	Breast cancer type 1 susceptibility protein
BSA	Bovine serum albumin
CCND1	Cyclin D1
cDNA	Complementary DNA
CST	Cell Signaling Technologies
DAVID	The database for annotation, visualization and integrated discovery
DGCR8	DiGeorge syndrome chromosomal region 8
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsRNA	Double stranded RNA
ECL	Enhanced chemiluminescence
EGFR	Epidermal growth factor receptor
ELK1	Proto-oncogene, ETS-like gene 1
EMT	Epithelial–mesenchymal transition
MMP	Matrix metalloproteinase

ER	Estrogen receptor
ERBB2	Proto-oncogene erb-b2 receptor tyrosine kinase 2, commonly referred as HER2
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FN/FN1	Fibronectin
GEO	Gene expression omnibus
GFP	Green fluorescence protein
GS score	Gene Signature score
GSEA	Gene Set Enrichment Analysis
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factor
HIF1A	Hypoxia-inducible factor alpha subunit
HR	Hazard ratio
RFS	Relapse free survival
IPA	Ingenuity Pathway Analysis
ITGA5	Integrin subunit alpha 5
ITGB1	Integrin subunit beta 1
IVIS	<i>In vivo</i> imaging system
kDa	kilo Dalton
KEGG	Kyoto encyclopedia of genes and genomes
KRAS	Proto-oncogene, Kirsten Rat Sarcoma
LB	Lysogeny broth
MAPK	Mitogen-activated protein kinase
MFP	Mammary fat pad
MFS	Metastasis free survival
miRNA	microRNA
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin, serine/threonine-protein kinase

OS	Overall survival
P/S	Penicillin/Streptomycin
PARP	Poly (ADP-ribose) polymerase protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pCR	Pathological complete response
PDX	Patient derived xenograft
PI3K	Phosphoinositide 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PR	Progesterone receptor
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative Real Time polymerase chain reaction
RB1	Retinoblastoma protein 1
RCB	Residual cancer burden
RFP	Red fluorescence protein
RFS	Relapse-free survival
RGD	Arg-Gly-Asp motif
RIPA	Radioimmunoprecipitation assay
RISC	RNA-induced silencing complex
RNA	Ribonucleotide acid
RNAi	RNA interference
RPPA	Reverse phase protein array
rRNA	Ribosomal RNA
SDS-PAGE	Sodium dodecyl sulfate-gel electrophoresis
shRNA	Small hairpin RNA
siRNA	Small interfering RNA

SIRT1	Sirtuin 1
Src	Src proto-oncogene, non-receptor tyrosine kinase
TAE	Tris-acetate EDTA
TBST	Tris buffer saline Tween20
TCGA	The Cancer Genome Atlas
TEMED	Thermo Scientific Pierce Tetramethylethylenediamine
TGF- β	Transforming growth factor beta
TNBC	Triple negative breast cancer
TP53	Tumor protein 53
UTR	Untranslated region
v/v	Volume/volume
w/v	Weight/volume

Chapter 1

Introduction

1.1. Breast cancer

Breast cancer is the most common cancer and the leading cause of cancer-associated deaths among women worldwide [1]. In 2017, The American Cancer Society reported that more than 250.000 women are expected to be diagnosed with invasive breast carcinoma in the US, and around 40.000 BC patients will die eventually due to the disease (American Cancer Society, 2017). Even though breast cancer is mostly seen in women of age 40-45 or more, there is a clear trend that the incidence age is decreasing in the recent years [2].

Breast cancer is a heterogeneous disease encompassing very different biological entities, and therefore requires distinct clinical treatments. Classification based on the classical immunohistochemistry markers distinguishes breast cancer into 3 subtypes: human epidermal growth factor receptor (HER2+/ERBB2+)-overexpressed, hormone receptor positive (Estrogen Receptor (ER)+/Progesterone Receptor (PR)+), and triple negative breast cancer (TNBC) [3]. According to the gene expression profiling, breast cancer can be classified into five subtypes: Luminal A, Luminal B, HER2-enriched, Basal-like and Normal-like [4] [5]. Luminal A and Luminal B breast cancer possess similarity to hormone receptor positive subtype where they both express estrogen receptor alpha (ER α). Tumors with this characteristic often respond well to endocrine therapies, such as tamoxifen and aromatase inhibitors [6]. HER2-enriched subtype (ERBB2+), as the name suggests, has elevated levels of HER2 protein receptor that is mostly due to gene amplification [7]. HER2-targeted therapies, such as trastuzumab or lapatinib, are common treatment options for this subtype [8]. Lastly, Basal-like subtype mostly resembles TNBC in terms of hormone and HER2 receptor expressions, leaving chemotherapy as the only

treatment option for the patients [5]. Approximately, 80% of breast cancer cases fall into one of these five breast cancer molecular subtypes. Therefore, classification of patients by subtype is not only important for the treatment selection, but also useful in predicting the prognostic outcome of breast cancer patients (**Figure 1.1**) [9].

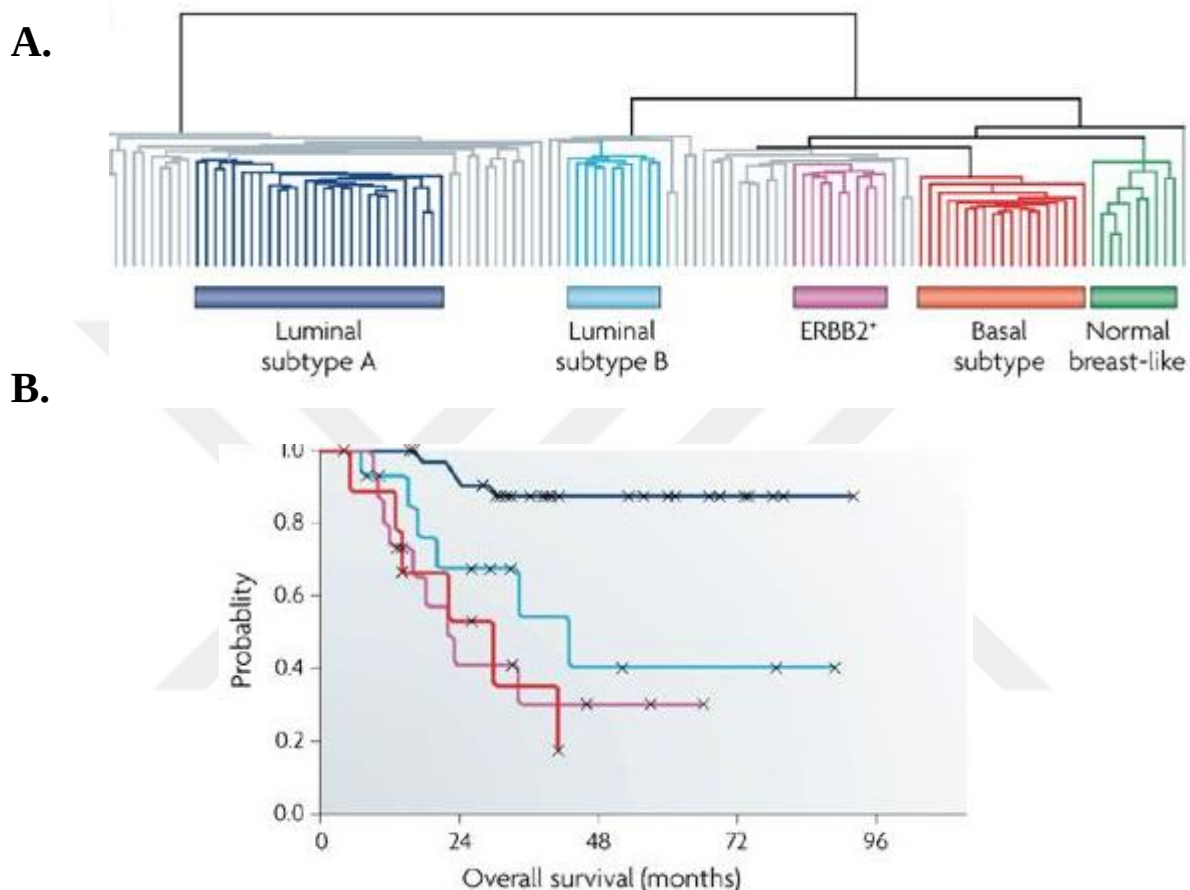


Figure 1. 1. Classification of breast cancer subtypes based on gene expression profiling and survival outcome of these subtype. (A) A dendrogram classifies 115 breast tumors into 5 subtypes as Luminal A, Luminal B, ERBB⁺ / HER2-enriched, Basal and Normal-like subtypes. Samples in grey do not correspond to any of the subtype. **(B)** Survival analysis of different breast cancer subtypes which is represented as probability of overall survival. Luminal A subtype shows better prognosis followed by Luminal B and HER2-enriched whereas Basal subtype has the worst overall survival compared to others [9]. *See copyright permission in Appendix Section.*

1.1.1. Triple negative breast cancer (TNBC)

TNBC tends to be high grade/stage and is the most aggressive subtype of breast cancer, accounting for about 15% of the all breast cancer cases. Gene expression-based subtyping demonstrated that most of the TNBCs are classified as basal subtype with the highest risk of developing distant recurrence and subsequent death within 5 years of diagnosis compared to other subtypes [10, 11]. Despite their common characteristics with respect to the absence of ER, PR, and HER2 expression, TNBCs display heterogeneity with respect to gene expression profiles [12]. Analysis performed by The Cancer Genome Atlas (TCGA) Research Network that combines different platforms including reverse-phase protein arrays (RPPA), DNA copy numbers, and exome sequencing revealed that genetic alterations in TNBCs are mostly occurred in the genes involved in phosphatidylinositol 3-kinase (PI3K) signaling and DNA damage repair mechanisms [13]. Dysregulation of PI3K pathway involves loss-of-function mutations in prominent negative regulator of PI3K pathway; *PTEN*, or gain-of-function mutations in PI3K catalytic subunit; *PIK3CA*, as well as in PI3K downstream signaling; mTOR signaling associated genes [10]. TNBCs with highly activated PI3K pathway due to abovementioned mutations respond well to PI3K/mTOR inhibitors, such as NVP-BEZ235 in pre-clinical settings [14]. Meanwhile, aberrant regulation of DNA damage repair mechanism is frequently related with the loss-of-function mutations in *RB1*, *TP53*, and *BRCA1* genes and therefore, this type of TNBC responds well to DNA-damaging agents, such as cisplatin [14, 15]. Further efforts in subtyping TNBC also identified other biomarkers participating in cell cycle, growth factor signaling, immune cell signaling, cell motility and even hormone-regulated pathways [14, 15]. Taken together, understanding the basis of heterogeneity of TNBCs in terms of gene expression profiles can yield better patient stratification for the most favorable treatment.

1.2. Chemotherapy

The use of chemical substances to cure cancer in the middle of nineteenth century started the era of chemotherapy that complements previously other major therapies, such as surgery and radiotherapy. Chemotherapy fulfilled the need of systemic cancer treatment that could not be achieved with local therapies [16]. Interestingly, some anti-cancer agents were discovered through screening of natural compounds without clear mechanisms of action [17, 18]. Other chemotherapeutic agents, like cisplatin and Adriamycin (also known as doxorubicin), which were originally known as anti-bacterial substances, were later found to have anti-cancer effects [19, 20]. Nowadays, conventional chemotherapies still constitute an important part of a preferable cancer therapy even though the era of “targeted therapy” had been set out along with our better understanding on molecular and genetics basis of cancer.

In case of TNBC, chemotherapy remains as mainstay treatment regardless of its high toxicity and side effects. Fortunately, TNBC has been shown to respond better to chemotherapy compared to other subtypes of breast cancer [21]. However, the success of chemotherapy is limited by many factors. Its lack of specificity inflicts side effects to patients, such as nausea, bleeding and loss of hair since proliferative normal cells are also affected during the treatment [22]. Moreover, chemotherapy often further increases cellular heterogeneity in cancer cell population, some of which may develop resistance during the course of treatment [23]. Ultimately, conventional chemotherapy often shows poor prognosis in TNBC due to rapid relapse and aggressiveness [23, 24]. The use of chemotherapy as neo-adjuvant treatment increased pathological complete response (pCR) rate of TNBC patients and could prolong their survival [25], yet residual disease after neo-adjuvant treatment leads to worse prognosis in TNBC patients, especially in the first 3 years after treatment [11, 26].

1.2.1. Development of resistance against chemotherapy

One of the many challenges for chemotherapy regimens in clinics is the development of resistance, which in most cases is inevitable. Initially, many TNBCs respond well to chemotherapy. However, they can acquire resistance over time through several distinct mechanisms, including genetic and epigenetic alterations [27]. It has been shown that chemotherapy resistance contributes up to 90% of failure in treatment rate of metastatic patients. [28]. The high rate of relapse is linked to small cohorts of cancer cells which gain aberrant pro-survival signals, resulting in more aggressive phenotype after systemic chemotherapy treatment [29]. It is still being debated whether the development of resistance is due to genetic mutations throughout medication or due to pre-existing genetic variation within primary cancer cell population or even due to both [30, 31]. The rationale of chemotherapy resistance is an evolutionary process to partially accomplish hallmarks of cancer: evading apoptosis and limitless replicative potential [32, 33].

1.3. Tumor microenvironment and chemotherapy resistance

Tumor cells do not exist as isolated entities in a given organ. Instead, they are engaged with other cellular components comprising non-transformed adjacent cells and complex stromal environments [34]. Notably, stromal tissues consist of many elements including tumor vasculature, fibroblasts, and extracellular matrix (ECM) which, in fact, often represents to the majority of tumor mass. Further, various molecules in ECM, which are secreted by normal neighbor cells as well as fibroblasts, have been shown to be crucial in promoting tumorigenesis [29, 35]. Different cytokines, chemokines, and matrix re-modeling enzymes are also enriched

in tumor microenvironment, implying advanced and dynamic interaction between tumor cells, normal tissues, and even immune cells [36].

The ECM consists of assembled structural proteins and other macromolecules that provides three dimensional structure serving as scaffold to tumor cells [37]. Importantly, tumor microenvironment-associated ECM is distinct from ECM of healthy tissues with respect to its composition. The changes in ECM of tumor includes high density of collagen that contributes to the stiffness of malignant tissue. Besides promoting aggressiveness of tumor, this stiffened ECM acts as physical barrier for which chemotherapy agents must pass through, and therefore leads to reduction of therapy efficacy [38, 39]. Interestingly, malignant tumors often acquire the ability to produce their own ECM with different proteomic signatures that might be worth exploited for biomarker of late stage cancer [40]. Some of these well-known differentially expressed ECM proteins comparing low and high grade breast cancer constitute fibronectin (*FN1*) and collagen type I alpha 2 (*COL1A2*) [41].

Vascularization is also an important process in tumor microenvironment that is needed for sustainable tumor growth. However, in a progressing and advanced tumor, growth of tumor often outstrips vascular supply which then creates less oxygenated area; a condition called as hypoxia [42]. Hypoxic tumor environment frequently enforces cancer to switch their metabolic pathways that might contribute to cancer aggressiveness [43]. For instance, hypoxia-inducible factor (HIF) that constitutes the major protein executor in hypoxia response was found to promote tumorigenesis [42]. HIF induces cancer cell proliferation and even invasion, partially through some HIF-regulated ECM proteins such as vimentin, fibronectin, matrix metalloproteinase (MMP), and cytokine receptor CXCR4 [44]. Importantly, hypoxia has been associated with the reduced efficacy of many cancer treatments including chemotherapy,

radiotherapy, and immunotherapy [45]. A number of anti-cancer agents including some anthracyclines, antimetabolites and alkylating agents have been reported to work less effectively in hypoxic environment [46].

The interaction of cancer cells with their microenvironment can dictate the activity of intracellular signaling. For example, type I collagen (Col-I), a prominent ECM component, triggers dormant breast cancer cells to enter proliferative stage through activation of β 1-integrin, Src and extracellular signal-regulated kinase (ERK) [47]. Similarly, interaction of laminin, another component of ECM, with integrins also induces EGFR activation and promotes NF- κ B mediated survival along with anti-apoptotic response [48, 49]. More importantly, ECM ligation activates several cell signaling processes that play pivotal role in cell survival and anti-apoptotic mechanisms, including PI3K, MAPK, and FAK-Src pathways [50, 51]. Taken together, ECM contributes to the development of chemotherapy resistance through alteration of tumors' biophysical properties and modulation of intracellular signaling pathways that favors self-survival and apoptotic escape.

1.4. MicroRNAs and their biogenesis

MicroRNAs (miRNAs) are short (18-25 nucleotides) non-coding RNAs that function in regulating the expression of many genes mainly through complementary base pairing to the 3'-untranslated region (3'-UTR) of target mRNAs. According to miRBase (www.mirbase.org), more than 2600 miRNAs are encoded by human genome, and 60% of human genes are predicted to be regulated by miRNAs [52, 53], suggesting the importance of miRNAs in molecular and cellular organizations.

A large number of miRNA genes are located in the regions quite distant from other annotated genes, indicating that they represent single transcriptional unit (intergenic miRNA gene) [54]. Around 40% of these intergenic miRNAs are expressed within same primary transcripts, resulting in polycistronic miRNAs [55]. A well-studied example of miRNAs co-expressed in polycistronic precursor is miR-17/92 cluster that produces six different mature miRNAs (miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a-1) [56]. There is a hypothesis that miRNAs generated from common polycistronic transcript are homologous and regulate overlapping targets. Some other miRNAs derived from same transcript may have different targets, yet still co-regulate the same biological pathways [57, 58]. A few miRNA genes are located in another gene (intragenic miRNA genes), and have the same transcriptional regulatory sites and orientation as its host gene [54]. The host gene might be either coding or non-coding gene where miRNA genes may reside within intron (intronic miRNA gene) or within exon (exonic miRNA gene) of their host genes.

The biogenesis of miRNAs starts with transcription of miRNA gene by RNA polymerase II into large primary transcript called pri-miRNA, which is equipped with 5' cap and 3' poly-A tail structures [59]. Pri-miRNA is a long double stranded RNA (dsRNA), that is later cleaved by Drosha, an RNase III-type enzyme, into shorter (~60 nucleotides) hairpin dsRNA called pre-miRNA [60]. Drosha that forms a protein complex with other proteins, such as Pasha (DGCR8), delivers pre-miRNA into cytoplasm with the help of nuclear transport exportin 5 [61]. Another type of RNase III in cytoplasm called Dicer recognizes pre-miRNAs and excises them into ~22 nucleotides of mature miRNA-miRNA* duplex. This dsRNA structure of mature miRNA is subsequently converted into single stranded mature miRNA after being loaded into Argonaute (AGO) protein by expelling single miRNA* strand [62, 63]. The interaction of miRNA-AGO

creates functional RNA-induced silencing complex (RISC) where it regulates gene expression via direct binding to mRNA targets (**Figure 1.2**) [64].

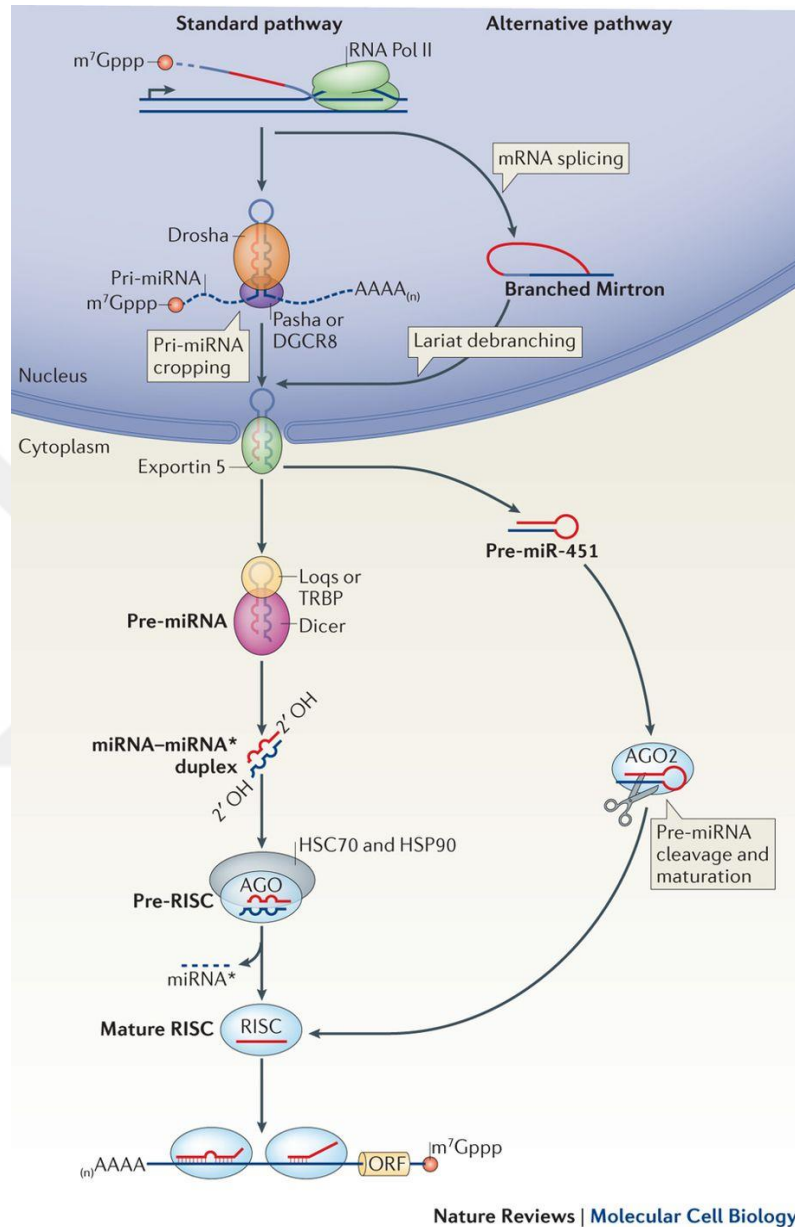


Figure 1. 2. miRNA biogenesis pathway. The scheme shows miRNA synthesis process starting from its transcription in the nucleus until being translocated and becoming functional in the cytoplasm [64]. *See copyright permission in Appendix Section.*

The binding of miRNAs to their target mRNAs initiates destabilization of mRNA by inducing mRNA cleavage and later, impeding translation [65]. Particularly in animals, the interaction of miRNA to 3'-UTR of mRNA target occurs in the sequences near 5' terminus of miRNA called "seed" sequence. This 6-8 nucleotide-long sequence is mostly conserved among different species and determines the range of miRNA targets. The principle of miRNA seed sequence leads to the understanding on how single miRNA is able to regulate various expressions of mRNA targets, and how single mRNA might be controlled by multiple miRNAs [66-68]. Moreover, the existence of seed sequence is useful to classify miRNAs in a family which at the same time, being exploited by many algorithms to predict hundreds of miRNA targets [67, 69].

1.5. Dysregulation of miRNA in cancer and miRNA as potential therapy

Considering the significant function of miRNAs in regulating gene expression, it is not surprising that many miRNAs are dysregulated in different types of cancer. The first report on miRNA dysregulation in cancer came from a study by Carlo Croce's group in 2002. They identified that the deletions of miR-15 and miR-16 genes at 13q14 chromosome region were associated with the development of B-cell chronic lymphocytic leukemia (B-CLL) [70]. Later, miR-15 and miR-16 were shown to act as tumor suppressors by inhibiting the expression of an anti-apoptotic protein, Bcl-2 [71]. Over the following years, studies on tumorigenesis-linked miRNAs have been exponentially increasing thanks to the advanced miRNA profiling and sequencing technologies. miRNAs have been a major focus of cancer research due to their potential to be biomarkers for diagnosis, prognosis, and even as an alternative treatment option for many cancers.

Dysregulation of miRNAs in cancer occurs through different mechanisms including abnormalities in transcriptional control, chromosomal and epigenetic changes and failure in the miRNA biogenesis machinery [72]. Transcriptional control abnormalities could arise from the dysregulation of crucial transcription factors themselves, such as p53 or general transcription machinery. For instance, p53 can bind to the promoter of miR-34 family and induce their expression [73]. Interestingly, miR-34 can also induce p53 expression by targeting negative regulator of p53, SIRT1, providing a positive feedback loop [74]. Next, chromosomal and epigenetic changes on the genome including amplification, deletion and translocation of miRNA genes as well as aberrant methylation of miRNA promoters could be some other factors dysregulating miRNA expression. Dysregulation of miR-15 and miR-16 in B-CLL as described above is an example of how deletion of miRNA gene alters healthy cells becoming cancerous. Conversely, oncogenic miR-17-92 cluster gene is often amplified in human lung cancer that subsequently promotes cell proliferation [75]. Meanwhile, the defects in the miRNA biogenesis machinery would also affect in the dysregulation of large fraction of miRNAs that might lead to the development of certain tumor. For example, mutations in DGCR8 and Drosha that are important for miRNA maturation has been identified in Wilms tumor and led to the decreased expression of tumor suppressor miRNAs, e.g. Let-7a and miR-200 family [76].

Many studies have explored miRNAs to be harnessed in clinical treatment for cancers due to their promising effect and minimum toxicity. The miRNA therapeutic strategy utilizes either miRNA-resembling oligonucleotides (miRNA mimics) or miRNA-targeting molecules (antimiRs) [77]. miR-16 mimics, for example, has been reported to enter Phase I clinical trials for malignant pleural mesothelioma and advanced non-small cell lung cancer [78, 79]. Another example is antimiR-10b, which was confirmed to decrease tumor growth in an orthotopic glioblastoma model and reduce metastasis in orthotopic breast cancer model [80, 81]. Perhaps,

the most advanced miRNA therapeutic strategy is pinned to miR-34 mimics that are delivered using nanoparticle carrier [82]. The liposome-encapsulated miR-34 mimics (MRX34) had been shown to significantly reduce non-small-cell lung cancer (NSCLC) tumor growth without generating immune response upon systemic delivery [83]. Therefore, lipid-based nanoparticle is often applied as delivery system for therapeutic miRNAs or other oligonucleotides due to its flexible modification and reduced potential in eliciting immune-rejection [82]. Importantly, the Food and Drug Administration (FDA) has recently approved liposome-based siRNA therapeutics, and considering the similar physicochemical properties and the requirement for RISC for their intracellular function for siRNAs and miRNAs [84], it may not be surprising to see the first miRNA-based therapeutics in clinics soon. Taken together, miRNA therapeutics with an efficient delivery method could be promising alternative treatments for combating cancer.

1.6. miRNA-mRNA interaction networks in cancer

Cancer is a complex disease that occurs due to aberrations in molecular interactions comprising pathway signaling, regulation of gene and cell metabolism [85, 86]. In tumorigenesis, for instance, healthy tissues undergo multiple steps of mutations in both coding and non-coding genome that influences different biological pathways before they display malignant phenotype [87, 88]. The cross-talks and feedback loops that often happen in cancer signaling also increase the complexity of this disease [85]. Importantly, the development of high-throughput sequencing technologies in the last two decades enables us to obtain a comprehensive -omics data from tissue samples [89]. Furthermore, the advancements in algorithm and mathematical modeling bridge the use of large data obtained from sequencing and to perform simulation on their dynamic molecular interactions [85]. As a result, the integrative analysis of biological

networks allows us to embrace the complexity of cancer and changes our view to cancer not only as a single gene- or protein-based disease, but also as a system biology-based disease [90]. This will eventually lead us to gather more knowledge in cancer biology and also better predictions for therapeutic strategies.

miRNA-mRNA interactions are essential in human transcriptome control where their dysregulation may result in cancer progression [91, 92]. However, as a single miRNA is predicted to target multiple mRNAs and conversely, single mRNA is predicted to be targeted by many miRNAs [66-68], it is necessary to apply system biology approaches to visualize this complexity and infer useful, testable hypotheses from the identified miRNA-mRNA interaction networks [93]. Such an approach has been utilized in many predictive studies. For example, analysis of miRNA-mRNA network has been useful to map oncogenic cellular pathways following the modulation of human papillomaviruses 16 (HPV16) oncoprotein in human foreskin keratinocytes [94]. Similar network analysis also has been applied to study the effect of metformin, a common drug for type II diabetes patients that also had been examined under Phase III clinical trial for breast cancer. Using miRNA-mRNA interaction network model, metformin was later known to regulate cell survival, cell cycle, and angiogenesis pathways in breast cancer [95].

In line with this, miRNA-mRNA interaction network analysis could be the state-of-art approach to comprehensively elucidate chemoresistance in TNBC whose cancer heterogeneity is high [12]. The result of this *in silico* analysis has the potential to provide preliminary data and testable hypotheses using *in vitro* and *in vivo* experimental models [96]. Finally, miRNA-mRNA network is useful not only to comprehend the biological process, but also to determine the driver genes of chemoresistance and direct the investigation for therapeutic strategy [93].

1.7. The rationale and the aims of the study

Chemotherapy resistance is a serious clinical problem in cancer, especially for those with limited therapy options e.g. TNBC patients. The development of resistance is molecularly orchestrated through the aberrations in both coding (mRNA) and non-coding genome (miRNAs, circular RNAs, long non-coding RNAs). Therefore, in this thesis, we aimed to elucidate the mechanism/s of chemotherapy resistance in TNBC by using *in silico*, *in vitro*, and *in vivo* approaches, and target major alterations to overcome chemoresistance in TNBC. Our study starts with establishing *in vivo* models representing chemo-sensitive and -resistant TNBC tumors, then to apply tumor-derived transcriptomics data to build a miRNA-mRNA network, and to validate the molecular mechanisms/interactions on the network through cutting-edge *in vitro* and *in vivo* experiments.

Our aims in this thesis are as following:

- To build the first miRNA-mRNA interaction network of chemoresistance in TNBC
- To discover novel molecular mechanisms of chemotherapy resistance in TNBCs involving miRNAs, proteins and their interactions.
- To validate the role of identified miRNAs and their target proteins using *in vitro* and *in vivo* approaches to overcome chemotherapy resistance in TNBCs.

Chapter 2

Materials

2.1. Buffers

1x Anode Buffer I	300 mM Tris, 20% (v/v) methanol
1x Anode Buffer II	25 mM Tris, 20% (v/v) methanol
1x Cathode Buffer	40 mM 6-aminocaproic acid, 20% (v/v) methanol
1x PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na ₂ HPO ₄ , 2 mM KH ₂ PO ₄ (pH: 7.4)
1x SDS-PAGE Running Buffer	25 mM Tris, 14.41 g/l glycine, 1% (v/v) SDS
1x TAE	40 mM Tris, 20 mM acetic acid, 1 mM EDTA
1x TBST	20 mM Tris, 8 g/l NaCl 0.2% (v/v) Tween20
RIPA lysis buffer	150 mM NaCl, 1% (v/v) NP-40, 0.5% (v/v) Sodium DOC, 50 mM Tris-HCl (pH:8.0), 50 mM NAF, 1 mM NaVO ₄ , 4% (v/v) Protease inhibitor, 4% (v/v) Phosphatase inhibitor

2.2. Chemicals and reagents

4x Protein Loading Dye	250 mM Tris HCl (pH:6.8), 10% (w/v) SDS, 0.1% (w/v) Bromophenol blue, 50% Glycerol (v/v), 25% (v/v) β -mercaptoethanol
6-aminocaproic acid	Sigma Aldrich, St Louis, MO, USA
6x DNA loading Dye	Thermo Fisher Scientific, Waltham, MA, USA
Acetic acid	Sigma Aldrich, St Louis, MO, USA
Acrylamide/bis-acrylamide	AppliChem, Darmstadt, Germany

Agar	Sigma Aldrich, St Louis, MO, USA
Agarose	Promega, Madison, WI, USA
Ammonium peroxisulfate	Carlo Erba, Cornaredu, Italy
Bovine Serum Albumin	Santa Cruz, Dallas, TX, USA
Protease inhibitor cocktail	Roche Applied Science, Mannheim, Germany
D-Luciferin Potassium salt	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 100bp DNA Ladder	New England Biolabs, Ipswich, MA, USA
Gene Ruler 1kb DNA Ladder	New England Biolabs, Ipswich, MA, USA
Isoflurane	Piramal Critical Care, Bethlehem, PA, USA
Isopropanol	Sigma Aldrich, St Louis, MO, 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
Ponceau 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
Yeast Extract	Sigma Aldrich, St Louis, MO, USA

2.3. Enzymes and enzyme buffers

10X Cut Smart Buffer	New England Biolabs, Ipswich, MA, USA
10X T4 DNA ligase Buffer	Thermo Fisher Scientific, Waltham, MA, USA
Not1 Restriction enzyme	New England Biolabs, Ipswich, MA, USA
Phusion Polymerase	New England Biolabs, Ipswich, MA, USA
T4 DNA Ligase	Thermo Fisher Scientific, Waltham, MA, USA
T4 Kinase	Thermo Fisher Scientific, Waltham, MA, USA
TaqMan ABgene universal mix	Thermo Fisher Scientific, Waltham, MA, USA
XhoI Restriction enzyme	New England Biolabs, Ipswich, MA, USA

2.4. Media and supplements

DMEM (Phenol Red, Low Glucose)	Lonza, Basel, Switzerland
Fetal bovine serum (FBS)	Biowest, Nuaille, France
Fibronectin bovine plasma	Sigma Aldrich, St Louis, MO, USA

LB Agar	1.5% (w/v) agar, 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl
LB Broth	10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl
Matrigel	BD, Franklin Lakes, NJ, USA
Non-essential amino acids	Lonza, Basel, Switzerland
OptiMEM	Invitrogen, Carlsbad, CA, USA
Penicillin/streptomycin	Lonza, Basel, Switzerland

2.5. Kits

BCA Protein Assay Kit	Pierce, Rockford, IL, USA
Cell Titer-Glo cell viability assay kit	Promega, Madison, WI, USA
MycoAlert detection kit	Lonza, Basel, Switzerland
Trans-lentiviral packaging kit	Dharmacon, Lafayette, Colorado, USA
Dual luciferase reporter kit	Promega, Madison, WI, USA
First strand cDNA synthesis kit	Thermo Fisher Scientific, Waltham, MA, USA
Gel and PCR clean up kit	MN, Duren, Germany
Plasmid isolation kit	MN, Duren, Germany
Plasmid Maxi kit	Qiagen, Hilden, Germany
TaqMan miRNA Assays	Applied Biosystems, Foster City, CA, USA

2.6. Equipment

AxioVision 4.3 microscopy	Carl Zeiss, Munich, Germany
Amersham Imager 600	GE Healthcare, Chicago, Illinois, USA
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	Bellco, Vineland, NJ, USA
Hypoxia Chamber	StemCell Technologies, Vancouver, Canada
LightCycler 96	Roche Applied Science, Mannheim, Germany
Mini-PROTEAN Gel casting module	Bio-Rad, Hercules, CA, USA
Mini-PROTEAN Tetra Cell	Bio-Rad, 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
Thermocycler	Thermo Fisher Scientific, Waltham, MA, USA
Power supplies for electrophoresis	Bio-Rad, Hercules, CA, USA
Semidry western blot transfer unit	Bio-Rad, Hercules, CA, USA
Synergy HT Microplate Reader	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

2.7. 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
Filtered pipette tips (10 µl, 20 µl, 200 µl, 1000 µl)	Greiner bio-one, Frickenhausen, Germany
Cell scrapers	Greiner bio-one, Frickenhausen, Germany
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 (5 ml, 10 ml, 25 ml)	Corning Incorporated, Corning, NY, USA
PVDF Membrane	Biorad, Hercules, CA, USA
Reaction tubes (500 µl, 1.5 ml, 2 ml)	Axygen, Corning, NY, USA
Storage bottles (250 ml, 500 ml, 1 L)	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

Chapter 3

Methods

3.1. *In vitro* experiment

3.1.1. Culturing human breast cancer cell lines

Human breast cancer cell line MDA-MB-231 was obtained from ATCC (Manassas, VA, USA) whereas MDA-MB-231.Luc2.GFP (shortly 231.Luc2.GFP), a modified and labeled version of MDA-MB-231, was a kind gift from Dr. Dihua Yu (MD Anderson Cancer Center, Houston, TX, USA). Both cells were cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 50 U/ml penicillin/streptomycin, 1% non-essential amino acids and 10% fetal bovine serum (FBS). Mycoplasma contamination test was performed regularly using MycoAlert mycoplasma detection kit (Lonza, Basel, Switzerland).

3.1.2. Cell seeding on fibronectin-coated well

The concentration of fibronectin (Sigma Aldrich, St Louis, MO, USA) was adjusted to 20 µg/mL in PBS and as much as 50 µL or 500 µL were added to 96 or 6-well plates, respectively. Plates were incubated in 4° C overnight and then washed once with PBS. Then, 7000 cells/well of 96-well plate or 200.000 cells/well of 6-well plate were seeded on top of fibronectin-coated well within 80 µL or 1500 µL of 2% bovine serum albumin (BSA) containing DMEM for 96-well or 6-well plate, respectively.

3.1.3. Transient transfection using miRNA mimics, siRNAs and reporter constructs

Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA) and OptiMEM were used for transient transfection that is conducted around 12 hours after cell seeding on fibronectin-coated well. For

one well of 96-well plate, miRNA mimics and siRNAs were transfected at concentration of 40 nM and 20 nM, respectively, which were diluted in 20 μ L OptiMEM to make mix A. Mix B was prepared by adding 0.2 μ L of Lipofectamine to 20 μ L OptiMEM. Each mixture was vortexed for 10 seconds and incubated at room temperature for 5 minutes, then both vials were mixed in 1:1 (v/v) ratio and vortexed again for 20 seconds followed by incubation for 20 minutes at room temperature. 40 μ L of the final mixture was added on top of the seeded cells. The plates were incubated at 37° C, 5% CO₂ for further treatments or experiments.

For one well of 6-well plate, mix A was prepared using 40 nM miRNAs or 20 nM siRNAs that were diluted in 250 μ L OptiMEM. At the same time, 2 μ L lipofectamine was added to 250 μ L OptiMEM to make mix B. Both vials were vortexed, mixed and incubated similar to that of 96-well format. Finally, 500 μ L of transfection mixture were added on top of cells in 6-well plate.

Reporter construct carrying 3' UTR of human ITGA5 was co-transfected with miRNA mimics on top of cells grown in 96-well plate. For one well, 50 ng of reporter construct and 20 nM of miRNA mimics were dissolved in OptiMEM and mixed with lipofectamine similar to of the protocol for single miRNA mimics transfection in 96-well plate format.

3.1.4. Construction of 3'-UTR plasmid

The 3'-UTR sequence of ITGA5 harboring binding sites for miR-326 was amplified from genomic DNA of MDA-MB-231 cells using primers shown in **Table 3.1**.

Table 3. 1. Primer sequences for 3'-UTR cloning

Gene Symbol	Gene ID	Direction	Primer
ITGA5	3678	Forward	5'-ccgctcgagcccaatttcagactcccattcctg-3'
		Reverse	5'-atttgcggccgcgttctggtcagtgggggcac-3'

psiCHECKTM-2 plasmid was used for reporter vector, and this plasmid together with 3'-UTR amplicons were double digested with *XhoI* and *NotI* restriction enzymes using protocol described in **Table 3.2**. Double digestion reaction was conducted at 37° C for 1.5 hours followed by inactivating enzyme at 65° C for 10 minutes.

Table 3. 2. Double digestion protocol

Name	Concentration/Volume
DNA	2 µg (X µL)
<i>XhoI</i>	1.25 µL
<i>NotI</i>	1.25 µL
Cut Smart Buffer	10 µL
Nuclease-free H ₂ O	(37.5-X) µL

Ligation of digested vector and insert was performed using protocol shown in **Table 3.3** at 22°C for 1 hour. Vector and insert concentration were calculated as follows:

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

Table 3. 3. Ligation reaction Protocol

Name	Concentration/Volume
<i>T4 DNA Ligase</i>	1 μ L
<i>T4 DNA Ligase Buffer (10X)</i>	2 μ L
psiCHECKTM-2 (double digested vector)	50 ng
Insert DNA (double digested 3'-UTR amplicon)	50 ng
Nuclease-free H₂O	up to 20 μ L

Following ligation, 10 μ L ligation product was transformed into *DH5 α* bacteria strain through heat shock treatment at 42° C for 45 seconds. 1 mL of LB media was added to transformed bacteria and incubated at 37° C on shaker for 45 minutes. The transformed bacteria were then selectively cultured by spreading onto LB agar plates containing 500 μ g/mL Ampicillin and incubated at 37° C overnight. Next day, viable colonies from each transformation were picked and amplified in 5 mL LB media at 37° C shaker overnight. To clarify the construct sequence, restriction digestion reaction was carried out using the same protocol described in **Table 3.2**. The bacteria were grown to amplify the vectors in 100-200 mL LB culture. Maxi-prep isolation kit was used to isolate vectors which were subsequently sent for sequencing using psiCHECKTM-2 universal sequencing primers. psiCHECKTM-2_Rluc_Forward: 5'-cgtccagatgaaatgggtaag-3'; psiCHECKTM-2_Reverse: 5'-cgaggtccgaagactcattt-3'.

3.1.5. Dual luciferase reporter assay

MDA-MB-231 cells were seeded into 96-well plate with the concentration of 5000 cells/ well and transfected with reporter construct as well as miRNA mimics as described in **Section 3.1.3**. 60 μ L of 1X passive lysis buffer was added to lyse the cells and followed by incubation on

shaker at room temperature for 15 minutes to enhance the cell lysis. The lysate from each well was then transferred to 96-well opaque white plates. 40 μ L of reagent I was added and measured with Synergy HT microplate reader (Biotek, Winooski, VT, USA) to measure *firefly luciferase* activity. After addition of reagent II, *renilla luciferase* activity was measured. The data was normalized by taking the ratio of *renilla luciferase* activity over *firefly luciferase* activity.

3.1.6. Doxorubicin treatment and cell viability assays

Doxorubicin treatment was carried out 12 hours after miRNA mimics or siRNA transfection according to the designated doses. In case of experiments without transfection, drug was given 24 hours after cell seeding. Doxorubicin was added on top of cells in 40 μ L and 200 μ L media into 96-well plate and 6-well plate, respectively. Cell viability assay was performed with Cell Titer-Glo cell viability assay kit (Promega, Madison, WI, USA) at 48 hours post-drug treatment. Before starting the experiments using Cell Titer-Glo, both reagent and cells containing 96-well plate were incubated for 30 minutes at room temperature. Then, 30 μ L of Cell Titer-Glo reagent was added to each well of 96-well plate and incubated for 10 minutes on shaker at room temperature. The lysate from each well was transferred to corresponding well of 96-well opaque white plate, and luminescence signal was measured via Synergy HT microplate reader (Biotek, Winooski, VT, USA).

3.1.7. Hypoxia assay

MDA-MB-231 cells were seeded into 6-well plate and incubated at 37° C in normoxic condition overnight. Next day, plates were transferred into Hypoxia Incubator Chamber (StemCell Technologies, Vancouver, Canada) with 1% oxygen, 5% CO₂, and 94% nitrogen at 37° C. At certain time points, cells were harvested by trypsinization and proceeded for RNA isolation.

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

3.1.8.1. RNA isolation

Cells were seeded in 6-well plates followed by transfection and/or drug treatment as described in **Section 3.1.3** and **3.1.6**. Then, medium was removed, cells were washed with 1X PBS and collected through trypsinization into 1.5 mL reaction tube. TRIsure (Bioline, Luckenwalde, Germany) reagent was used to perform RNA isolation according to the manufacturer's protocol. RNA isolation from tumors was performed by putting tumor fragments into 1.5 mL reaction tubes and homogenization in TRIsure. After homogenization, the same protocol was used for tumors as well.

3.1.8.2. Reverse Transcription for mRNA

cDNA was generated from 2 µg of total RNA for each sample using RevertAid RT Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions as summarized in **Table 3.4**. The reaction was carried out in thermocycler using the program provided in **Table 3.5**. Then, cDNA samples were diluted to 1:20 and proceeded with qRT-PCR experiments.

Table 3. 4. Protocol for reverse transcription reaction

Reagent	Concentration/Volume
RNA	2 µg (X µL)
Oligo-dT	1 µL
RevertAid reaction buffer	4 µL
Ribolock Ribonuclease Inhibitor	1 µL

dNTPs (10mM)	2 μ L
RevertAid H Minus M-MuL V RT	1 μ L
Nuclease-free H₂O	up to 20 μ L

Table 3. 5. Thermocycler program for reverse transcription reaction

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

3.1.8.3. qRT-PCR for mRNA quantification

qRT-PCR was performed in 96-well plates. Master mix was prepared as described in **Table 3.6**

using the primer pair for each gene of interest that listed in **Table 3.7**.

Table 3. 6. Master mix composition used for qRT-PCR reaction

Reagent	Volume
SYBR Green	5 μ L
Forward Primer (20 μM)	0.25 μ L
Reverse Primer (20 μM)	0.25 μ L
Nuclease-free H₂O	2.5 μ L

Table 3. 7. Sequences of forward and reverse primers used for qRT-PCR reaction

Gene Symbol	Gene ID	Forward Primer	Reverse Primer
<i>ACTB</i>	60	5'ccaaccgcgagaagatga-3'	5'ccagaggcgtacagggatag-3'
<i>HIF1A</i>	3091	5'ccacaggacagtacaggatg-3'	5'-tcaagtcgtgctgaataataacc-3'
<i>HPRT</i>	3251	5'-tgaccttgattattttgcataacc-3'	5'-cgagcaagacgttcagtcct-3'
<i>ITGA5</i>	3678	5'-gtcgggggcttcaacttagac-3'	5'-cctggctggctggtattagc-3'

First, 8 μ L of the master mix was added to each well, and then 2 μ L cDNA (20 ng) (see **Section 3.1.8.2**) was added on top of the master mix. The plate was sealed, centrifuged at 1000 rpm for 2 minutes, and placed into LightCycler 96 qRT-PCR Thermocycler (Roche Applied Science, Mannheim, Germany). The program details of thermocycler are provided in **Table 3.8**.

Table 3. 8. Thermocycler program used for qRT-PCR

Pre-incubation							
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C)	Acquisition (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)	Acquisition (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)	Acquisition (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)	Acquisition (per°C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
40	None	00:00:30	2.2	5	0	0	0

3.1.8.4. Reverse transcription for miRNAs

cDNA synthesis of miRNAs was done using Taqman miRNA Assays (Foster City, CA, USA). Master mix and miRNA reverse transcription reaction were prepared according to the manufacturer's protocol as described in **Table 3.9** and **Table 3.10**, respectively.

Table 3. 9. Protocol for Taqman miRNA reverse transcription reaction

Reagent	Volume
RNA	5 µL (10 ng)
dNTPs (100 nM)	0.15 µL
MultiScribe™ Reverse Transcriptase (50 U/µL)	0.25 µL
10X RT Buffer	1.5 µL

RNase Inhibitor (20 U/μL)	0.19 μ L
5X Taqman RT primer	3 μ L
Nuclease-free H₂O	4.91 μ L
Total	15 μ L

Table 3. 10. Thermocycler program for Taqman miRNA reverse transcription

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

3.1.8.5. qRT-PCR for miRNA quantification

miRNA quantification was done using qRT-PCR with Taqman miRNA assays (Foster City, CA, USA) in 96-well plate. For each well, reaction mix was prepared as described in **Table 3.11** and distributed to each well, followed by the addition of 1.33 μ L cDNA prepared in **Section 3.1.8.4**. The plate was sealed and centrifuged at 1000 rpm for 2 minutes. Then, it was placed into LightCycler 96 qRT-PCR Thermocycler (Roche Applied Science, Mannheim, Germany) and run using program described in **Table 3.12**.

Table 3. 11. Taqman miRNA qRT-PCR master mix

Reagent	Volume
Taqman 2X Universal PCR Master Mix	5 μ L

20X Taqman microRNA assay mix	0.5 μ L
Nuclease-free H₂O	3.17 μ L
Total	8.67 μ L

Table 3. 12. Program for Taqman miRNA qRT-PCR amplification

Parameters	Value			
Run Mode	9600 emulation (Default)			
Sample volume	10 μ L			
Thermal cycling parameters	Step	Type	PCR	
		Hold	40 cycles	
			Denature	Anneal
	Time	10 minutes	15 seconds	60 seconds
	Temp ($^{\circ}$ 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 $^{\circ}$ C)			

3.1.9. Protein Biochemistry

3.1.9.1. Protein Isolation

Cells were seeded in 6-well plates followed by transfection and/or drug treatment as described in **Section 3.1.3** and **3.1.6**. Then, medium was collected in 1.5 mL Eppendorf and centrifuged at 5000 rpm for 5 minutes to obtain the apoptotic bodies. Cells were then washed with 1X PBS and collected through either scrapping method (for blotting phospho-proteins) or trypsinization

(for blotting non phospho-proteins). 30-50 μ L RIPA lysis buffer that was freshly prepared as indicated in **Table 3.13** was added onto the cells. Later, the suspension was vortexed every 5 minutes for a total of 30 minutes to ensure complete cell lysis. Then, the suspension was centrifuged at 14,000 rpm for 30 minutes at 4° C. Supernatant was transferred into new 1.5 mL eppendorf and preserved at -80° C for further experiments.

Table 3. 13. RIPA lysis buffer preparation

Components	Stock Conc.	Final Conc.	For 1mL
NaCl	2M	150 mM	75 μ L
NP-40	10%	1%	100 μ L
Sodium DOC	10%	0.5%	50 μ L
SDS	10%	0.1%	10 μ L
Tris HCl (pH 8.0)	2M	50 mM	25 μ L
NAF	1M	50 mM	50 μ L
NaVO₄	100 mM	1 mM	10 μ L
Protease Inhibitor	25X	4%	40 μ L
Phosphatase Inhibitor	10X	4%	40 μ L
ddH₂O			600 μ L

3.1.9.2. Protein Quantification

To quantify isolated proteins (see **Section 3.1.9.1**), BCA protein assay kit (Pierce, Rockford, IL, USA) was used according to manufacturer's instructions. Briefly, nine BSA standard solutions were prepared in the concentration range of 0-2 μ g/ μ L. The standards and protein samples were loaded into different wells on 96-well plates with a ratio of 5:1 between the

standards and the samples. 200 μL of solution A and B mixture (ratio 50:1) was added into each well and incubated for 30 minutes at 37° C. Absorbance was measured at 562 nm wavelength using SynergyHT microplate reader (Biotek, Winooski, VT, USA). A standard curve was plotted according to the absorbance readings of BSA standards, and the calibration was used to calculate the protein concentrations.

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

Protein samples were prepared at the same concentration (16 $\mu\text{g}/\mu\text{L}$) and mixed with 4X protein loading dye that was prepared according to **Table 3.14**. Samples were then vortexed briefly, incubated at 95° C for 3-5 minutes and shortly centrifuged. Preparation of polyacrylamide gels was done according to **Table 3.15**.

Table 3. 14. Protocol for 4X loading dye preparation

Components	Stock Conc.	Final Conc.	For 10 mL
Glycerol	100%	40%	4 mL
Tris HCl (pH6.8)	1M	240 mM	2.4 mL
SDS		8%	0.8 g
Bromophenol blue		0.04%	4 mg
Beta-mercaptoethanol	100%	5%	0.5 mL
ddH₂O			Up to 10 mL

Table 3. 15. Protocol for stacking and resolving gels preparation

Reagent	Stacking gel	Resolving gel		
	5%	8%	10%	12%
ddH₂O	1.36 mL	2.3 mL	1.9 mL	1.6 mL
30% acrylamide mix	340 µL	1.3 mL	1.7 mL	2 mL
1M Tris	(pH=6.8)	(pH=8.8)	(pH=8.8)	(pH=8.8)
	260 µL	1.3 mL	1.3 mL	1.3 mL
10% SDS	20 µL	50 µL	50 µL	50 µL
10%APS	20 µL	50 µL	50 µL	50 µL
TEMED	2 µL	5 µL	5 µL	5 µL
Total Volume	2 mL	5 mL	5 mL	5 mL

First, the resolving gel solution was poured into Mini-Protean Gel casting module (Biorad, Hercules, CA, USA) followed by isopropanol addition on top of the gel. After the resolving gel was polymerized, isopropanol was removed, and the stacking gel was added on top of the resolving gel. Then 10- or 15-well comb was inserted on top of the casting system. After it was polymerized, too, the comb was removed and protein samples were loaded into each well. Electrophoresis was set at 100V initially for 15 minutes and 110-120V for the next 90-120 minutes.

3.1.9.4. Western Blotting

For semi-dry transfer, four 3-mm thick Whatmann papers (GE Healthcare, Little Chalfont, UK) with a size of 7 cm x 9 cm were moistened in anode buffer I, two in anode buffer II and six of them in cathode buffer. PVDF membrane was first dipped in 100% methanol for 3 minutes and then in anode buffer II for 1 minute. Whatmann papers, PVDF membrane and gel (see **Section**

3.1.9.3) were stacked from anode to cathode in Semi-dry Turbo Blot machine. Transfer was run at 25V for 30 minutes. Following the transfer, the PVDF membrane was dipped in Ponceau S stain for 3 minutes and washed with ddH₂O in order to visualize the protein bands. Membranes were then cut at certain sites according to the molecular weight of protein of interest and blocked with 5% (w/v) milk:1X TBS-T or 5% (w/v) BSA:1X TBS-T for 1 hour at room temperature on a rocker platform. After blocking solution was removed, membranes were incubated in primary antibody against the proteins of interest listed in **Table 3.16** either for 1-hour at room temperature or overnight at 4° C.

Table 3. 16. List of primary antibodies used for Western blot.

Name	Manufacturer	Catalog Number	Dilution
Cleaved PARP	CST	5625S	1:1000
ITGA5	Sigma Aldrich	HPA002642	1:1000
phospho-AKT (S473)	CST	9018P	1:1000
phospho-AKT (T308)	CST	4056S	1:1000
phospho-ERK (T202/204)	CST	4376S	1:1000
phospho-FAK (Y397)	Abcam	39967	1:1000
phospho-Src (Y416)	Abcam	2685	1:1000
Total AKT	CST	9272S	1:1000
Total ERK	CST	4695S	1:1000
Total FAK	CST	3285S	1:1000
Total Src	CST	2108S	1:1000
β-actin	MP Biomedicals	691001	1:10000

Once primary antibody incubation is over, the membrane was washed three times with 1X TBS-T at high shaking speed for 10 minutes each. The membrane was then transferred into secondary antibody solution listed in **Table 3.17** and incubated either for 1 hour at room temperature or overnight at 4° C. After that, the membrane was again washed three times with 1X TBS-T at high shaking speed for 10 minutes each. The membrane was then developed by applying Amersham ECL reagent (Amersham Pharmacia Biotech, Amersham, UK) or Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA) on the membrane and then visualized in an Amersham imager (GE Healthcare, Chicago, Illinois, USA) for different exposure times.

Table 3. 17. List of secondary antibodies for Western blot.

Name	Manufacturer	Catalog Number	Dilution
Anti-mouse	CST	7076S	1:10000
Anti-rabbit	CST	7074S	1:10000

3.1.10. Stable transfection using lentiviral vectors

shMIMIC inducible mCMV-turboRFP human lentiviral hsa-miR-326, TRIPZ inducible lentiviral non-silencing shRNA Control, and TRIPZ inducible Lentiviral human ITGA5 shRNA (shortly shITGA5) with clone ID V2THS_133433 were purchased from Dharmacon (Lafayette, CO, USA). All vectors contain red fluorescence protein (RFP) and puromycin resistance genes for detection and selection purposes, respectively. Transduction using miR-326 viral particles into 231.Luc2.GFP cells was performed in 6 well-plates. 20 hours post-transduction, the medium containing virus was changed with fresh medium, allowing cells to grow further. Selection was done by incubating cells in the medium containing 2 µg/mL puromycin for 72 hours. To generate viral particles used for shRNA vectors, 6 µg of vectors along with 4.3 µL of

trans-lentiviral packaging mix were co-transfected into HEK293FT cells in 6-well plate using CaCl_2 reagent as suggested in the manufacturer's protocol (Dharmacon). After 48 hours incubation, the viral particles were collected and transduced into 231.Luc2.GFP cells. Further selection was done by treating cells with the medium containing 2 $\mu\text{g/mL}$ puromycin for 2-3 weeks.

3.2. *In vivo* experiments

3.2.1. Primary tumor xenografts

All animal experiments were carried out under approval of Animal Ethics Committee of Bilkent University (Protocol numbers: 2013/45, 2018/17, 2018/24). Initially, 6-8 weeks old female athymic nu/nu mice were subcutaneously injected with 2×10^6 231.Luc2.GFP cells prepared in 100 μL PBS:matrigel (1:1) solution into the left and right mammary fat pads (MFPs). After tumors became palpable, their growth was measured regularly twice a week using a caliper, and tumor volume was calculated as $(\text{length} \times \text{width}^2)/2$. All mice were sacrificed when the tumors of control group reached to 1500 mm^3 .

3.2.2. *In vivo* doxorubicin treatment and induction of stable vector

In order to develop doxorubicin resistance *in vivo* or test the combinatorial effect of doxorubicin together with miR-326 or shITGA5 expression on tumor growth, mice were treated with doxorubicin regularly. Doxorubicin was delivered once a week *via* intravenous (iv.) injection at a concentration of 5 mg/kg and 2.5 mg/kg of mice body weight for doxorubicin resistance development (in the context of another project in the lab) and combination treatment, respectively. To induce expression from stable vector, 100 $\mu\text{g/mL}$ doxycycline was added into mice drinking water along with 1% sucrose, which compensates for the bitter taste of the water.

3.2.3. *In vivo* imaging system (IVIS) analysis of primary tumors

Before mice were sacrificed, primary tumors were taken for bioluminescence imaging. Mice were anesthetized with isoflurane, and 150 mg/kg D-luciferin (Sigma Aldrich, St Louis, MO, USA) was injected into intraperitoneal (ip.) cavity. Mice were then placed into Lumina III *In Vivo* Imaging System (Perkin Elmer, Waltham, MA, USA), and bioluminescence-based live imaging was conducted. Photon flux measurements were analyzed by Living Image Software.

3.3. Bioinformatics and statistical analyses

3.3.1. Whole transcriptome sequencing and data analysis

After total RNA was isolated from tumors comprising doxorubicin sensitive, doxorubicin resistance and vehicle groups, RNA-Seq and small RNA-Seq expression profiling were performed for each of tumor groups as 4 biological replicates using Illumina Hiseq 2500 platform at McGill University and Genome Quebec Innovation Centre. The sequencing was conducted through paired-end 100bp with around 60 million reads per each pair after rRNA-depletion for each replicate. Data analysis for RNA-Seq was done by aligning raw sequence data with UCSC *Homo sapiens* reference genome (hg19, GRCh37) using Tophat v2.1.0 according to default parameters. Mapped reads were counted using HTSeq with UCSC-hg19 reference genome annotation. Then, differentially expressed genes between doxorubicin sensitive and resistant groups were determined through *Cufdiff* analysis and transformed into R statistical computing environment using Bioconductor package, *edgeR*. Meanwhile, data analysis for miRNA-Seq was done by aligning raw sequence data with UCSC *Homo sapiens* reference genome (hg19, GRCh37) using *Bowtie v1.1.2* according to default parameters. Identification of the differentially expressed miRNA was performed using three different

differential expression algorithms (edgeR, DESeq and NOISEQ). Consensus miRNAs from these three programs were taken for further analyses.

3.3.2. miRNA-mRNA interaction network construction

The threshold of differentially expressed miRNAs and mRNAs between sensitive and resistant xenografts was set as $\log_2FC \leq -0.59$ and $\log_2FC \geq +0.59$, $p\text{-value} < 0.05$, yielding 1280 mRNAs and 68 miRNAs. List of these mRNAs and miRNAs were then uploaded to web-based software application for omics analysis, namely Ingenuity Pathway Analysis / IPA (QIAGEN Inc., <https://qiagenbioinformatics.com/>). In IPA, the miRNAs were initially paired with their predicted mRNA targets using “microRNA target filter” analysis. This resulted in 616 mRNAs being regulated by 42 miRNAs in 1718 interactions. As miRNA-mRNA interactions should be inversely correlated in terms of their expressions, inverse pairing analysis was performed using “expression pairing” filter, reduced the numbers into 463 mRNAs and 41 miRNAs which were incorporated in 870 interactions. List of 463 mRNAs were again uploaded to the IPA core analysis to check the associated pathways. Genes from identified pathways were recollected and constructed in network used IPA “connect” function. In addition, “grow within own data” function provided in IPA was also used to find the closely related genes from the initial uploaded genes. Then, upstream regulators were added to the network followed by miRNA integration. The network was finalized by removing positively correlated miRNA-mRNA interactions. Open source platform-based program, namely Cytoscape (<https://cytoscape.org/>), was also utilized to re-visualize the network obtained from IPA.

3.3.3. Generating gene signatures, GSEA and survival analysis

Doxorubicin resistance miRNA-mRNA gene signature (DoxoR miRNA-mRNA GS Score) was generated comprising of differentially expressed mRNAs between sensitive and resistant

tumors. z-score of DoxoR miRNA-mRNA GS Score were calculated for patients in GSE58812 [97] by subtracting the z-scores of the downregulated mRNAs from the sum of z-scores of the upregulated mRNAs which constitute the predicted targets of differentially expressed miRNAs as described previously [98]. Gene sets for *Gene Set Enrichment Analysis (GSEA)* was retrieved from the GSEA website: <http://software.broadinstitute.org/gsea/index.jsp>. RNA-seq data from doxorubicin resistant and sensitive tumors was used as expression dataset in performing GSEA.

Survival analysis was done using GSE58812 and from online survival analysis tool, KM-plotter [99]. Briefly, patients were separated from the median of corresponding gene expression and defined as having low or high expression of that certain gene. Then patient survival analyses were performed based on candidate gene expression through Kaplan-Meier method. The results were represented either as Kaplan-Meier patient survival curve or as forest plot. In patient survival curves, survival rate of patient based on candidate gene is drawn in function with time, whereas in forest plot, the patient hazard ratio is plotted for each gene

3.3.4. Network activity flow

Activity flow analysis was used to measure and simulate the effects of regulatory interactions between miRNAs, upstream regulators, and proteins in the given network based on random walks on graphs. Briefly, initial activity level of upstream regulators and upregulated proteins and miRNAs were designed as 1.0 while the rest were valued as 0.0. Induction edges and inhibition edges exerted positive and negative effect, respectively, over the interacted nodes (proteins, miRNAs and upstream regulators). The simulation was run in *Java* program through many iterations until activity flow score for each node was getting converged. Later, each node was ranked according to its activity flow score that also reflected the centrality of that node in the given network.

3.3.5. miRNA target prediction and KEGG pathway enrichment analysis

miRWalk2.0 (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/>) [100] was used as target prediction algorithm to predict miRNA gene targets. This online available tool is a comprehensive program for miRNA target prediction that can combine other 11 miRNA target prediction databases including Targetscan (<http://www.targetscan.org>), PITA (<https://genie.weizmann.ac.il/>) and miRDB (<http://mirdb.org/>) in the analysis. In order to get more robust data, a stringent threshold was applied by taking common genes identified from at least 8 of those 12 databases. KEGG pathway enrichment analysis was performed using online tool, DAVID v6.8 (<https://david.ncifcrf.gov/>) [101] to determine which biological pathways are most enriched from the given gene list.

3.3.6. GEO dataset and statistical analyses

Patient datasets were retrieved from the NCBI GEO database (GSE19536 [102], GSE19783 [102], and GSE58812 [97]). Log-rank (Mantel-Cox) test was used to calculate the significance of the differences in survival analysis between two groups. Pearson correlation co-efficient was used for correlation analysis. 2-tailed Student's t-test was performed to compare two experimental groups. Box-plots show median number, and the 25th to 75th quartiles. Upper and lower whiskers denote the minimum and maximum values in the corresponding group. Graphs and curves were drawn using GraphPad software (GraphPad software Inc., La Jolla, CA, USA). Significance threshold was described as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

Chapter 4

Results

4.1. Development and characterization of doxorubicin resistant TNBC tumor model

To elucidate the molecular mechanisms underlying doxorubicin resistance in TNBC, we had developed *in vivo* models of doxorubicin response representing doxorubicin sensitivity and acquired doxorubicin resistance using MDA-MB-231 cell line in the context of other project in the lab. Briefly, MDA-MB-231 cells were injected into the MFPS of female nude mice, and when the tumor volume reached to around 100 mm³, mice were started to be treated with doxorubicin (5 mg/kg) for over 3-4 months. Vehicle treated mice had an average tumor volume of 1500 mm³ in 3 to 4 weeks and were sacrificed. Doxorubicin was effective and caused a regression and stabilization on tumor volume. Some of these tumors were then removed from mice and denoted as “doxorubicin sensitive”. Whereas the rest of the tumor-harboring mice were maintained under doxorubicin treatment. We observed that these initially sensitive tumors increased their volume upon extended doxorubicin treatment, and were collected and denoted as “doxorubicin resistant” samples. To decipher the molecular mechanisms behind the doxorubicin resistance, whole transcriptome sequencing for mRNAs and miRNAs was performed using the RNAs isolated from both doxorubicin sensitive and doxorubicin resistant tumor samples. **Figure 4.1** shows the schematic illustration of generation of resistant and sensitive mice models.

In vivo model for developing doxorubicin resistance

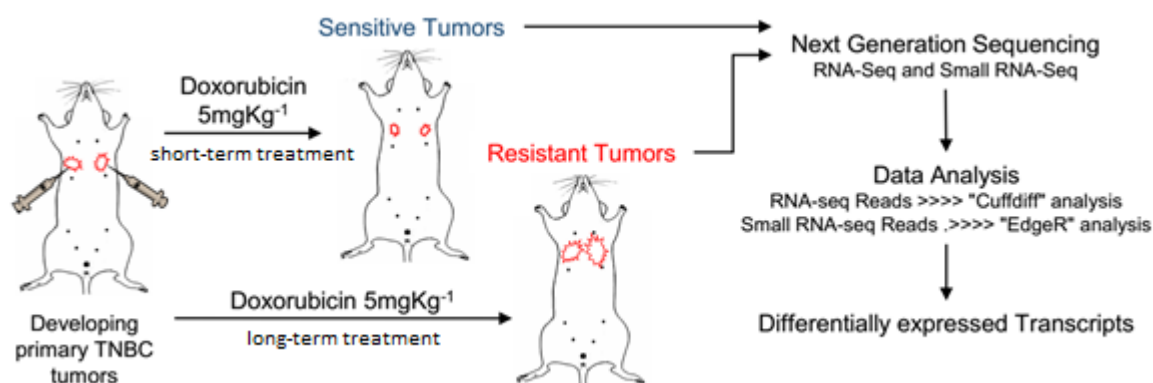


Figure 4. 1. Development of doxorubicin resistance in female nude mice using MDA-MB-231 TNBC cells. Schematic representation of mice models and the analysis flow used for the sensitive and resistant tumors isolated from corresponding models.

Raw data obtained from whole transcriptome analysis was processed to uncover differentially expressed mRNA and small RNAs (**Figure 4.1**, for technical details, see Methods section). As summarized in the funnel diagram below (**Figure 4.2A**), 1280 mRNAs and 68 miRNAs were found to be differentially expressed between doxorubicin resistant and sensitive samples with cutoff $-0.59 \geq \log_2FC \geq +0.59$, $p < 0.05$. Then, miRNA-mRNA interaction analysis was performed using Ingenuity Pathway Analysis (IPA) tool. miRNA-mRNA target filter analysis and inverse pairing analysis filtered these differentially expressed transcriptome into 463 mRNAs and 41 miRNAs to subsequently generate Doxorubicin resistance miRNA-mRNA gene signature (DoxoR miRNA-mRNA GS). TNBC patient survival analysis on GSE58812 dataset showed that patients with low DoxoR miRNA-mRNA GS score had better overall survival (OS) as compared to those having high DoxoR miRNA-mRNA GS score (**Figure 4.2B**), corroborating our *in vivo* chemotherapy resistance model.

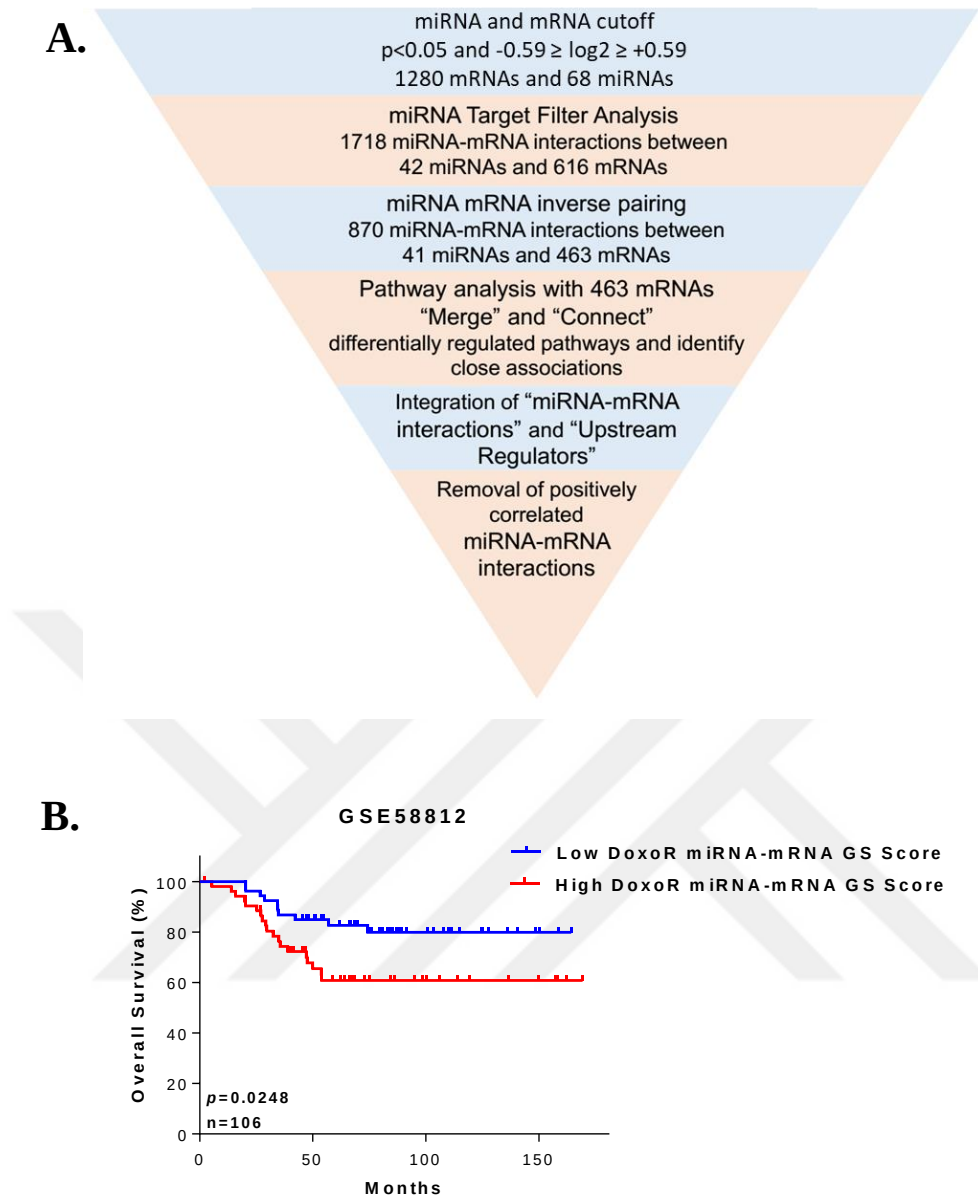


Figure 4. 2. Applied filters to generate miRNA-mRNA interaction network of TNBC chemoresistance and validation of DoxoR miRNA-mRNA GS score in TNBC patients. (A) Workflow describing the step-by-step selection process of candidate miRNAs and mRNAs from sequencing data to develop doxorubicin resistance miRNA-mRNA interaction network. **(B)** Kaplan Meier survival curve showing the overall survival percentage of chemotherapy-treated TNBC patients separated based on high or low DoxoR miRNA-mRNA GS Scores. The data was retrieved from GSE58812.

In order to construct doxorubicin resistance network mapping interactions between mRNA, miRNAs and upstream regulators, our differentially expressed transcriptomes were further processed in IPA through pathway analysis-based selection, network integration of miRNA-mRNA and upstream regulators, and removal of miRNA-mRNA positive interaction (see Methods for details) (**Figure 4.3**).

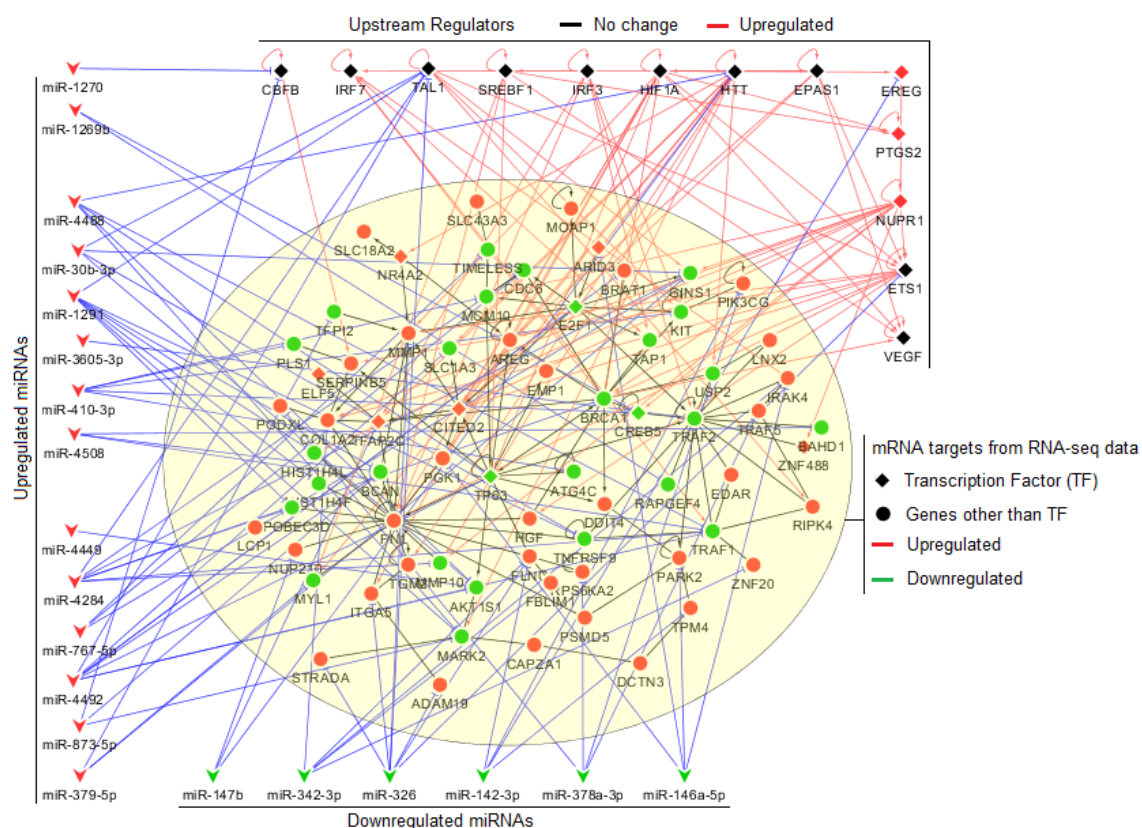


Figure 4. 3. Doxorubicin resistance associated miRNA-mRNA interaction network. The interactions between mRNA, miRNA, and upstream regulators were generated with literature-based IPA analysis. Red and green nodes represent upregulated and down regulated mRNAs or miRNAs, respectively, while black nodes represent upstream regulators (including transcription factors). Red and blue edges represent induction and inhibition interactions, respectively.

As shown above (**Figure 4.3**) we obtained 69 mRNAs, 20 miRNAs and 13 upstream regulators to be included in the doxorubicin resistance associated network. Even though these selected

candidates might be involved in the development of doxorubicin resistance, we aimed to simplify the network and pinpoint the key players. Besides, it would be laborious to test each of the nodes in the network as well as to determine the driver gene(s) for doxorubicin resistance. To narrow down the number of gene candidates and to validate the relevance of network components in TNBC patients, we performed survival analyses of chemotherapy-treated TNBC patients based on the expression level of each of these doxorubicin resistance related genes. The analyses were represented as hazard ratio (HR) that is the ratio of hazard rate between patients with upregulated gene expression over patients with downregulated gene expression. Upregulated mRNAs and upstream regulators were expected to have high HR score, whereas downregulated genes were expected to have low HR score for chemotherapy-treated TNBC patient survival. Therefore, we selected the genes that showed significant HR in at least one of these following survival analyses: relapse-free survival (KM-Plotter), overall survival (GSE58812) or metastasis-free survival (GSE58812) in two different datasets (**Figure 4.4**). Genes that are not significant in any of these analyses are not included in the plot but shown in appendix part.

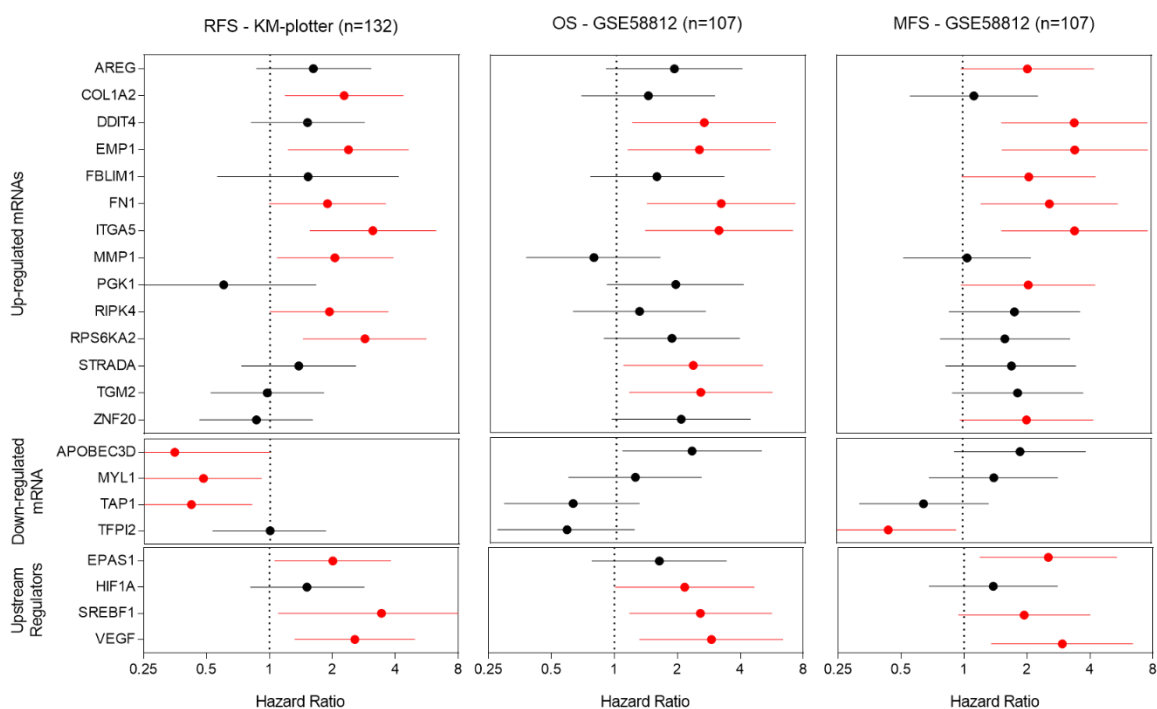
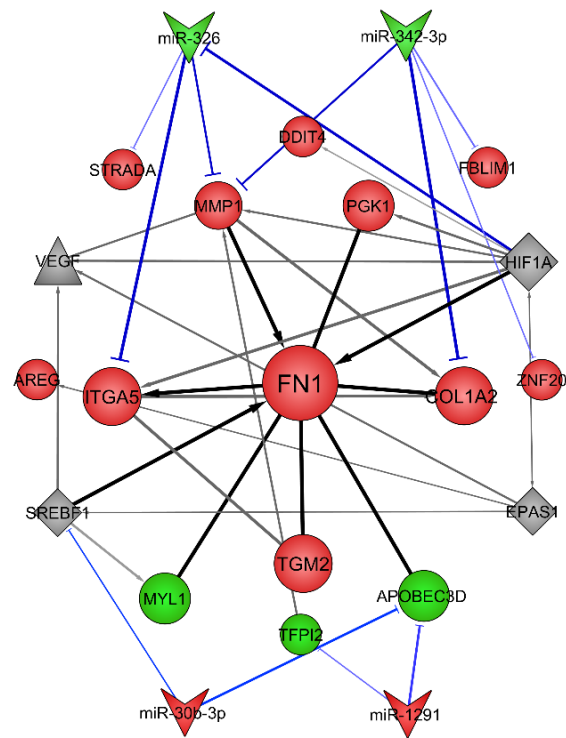


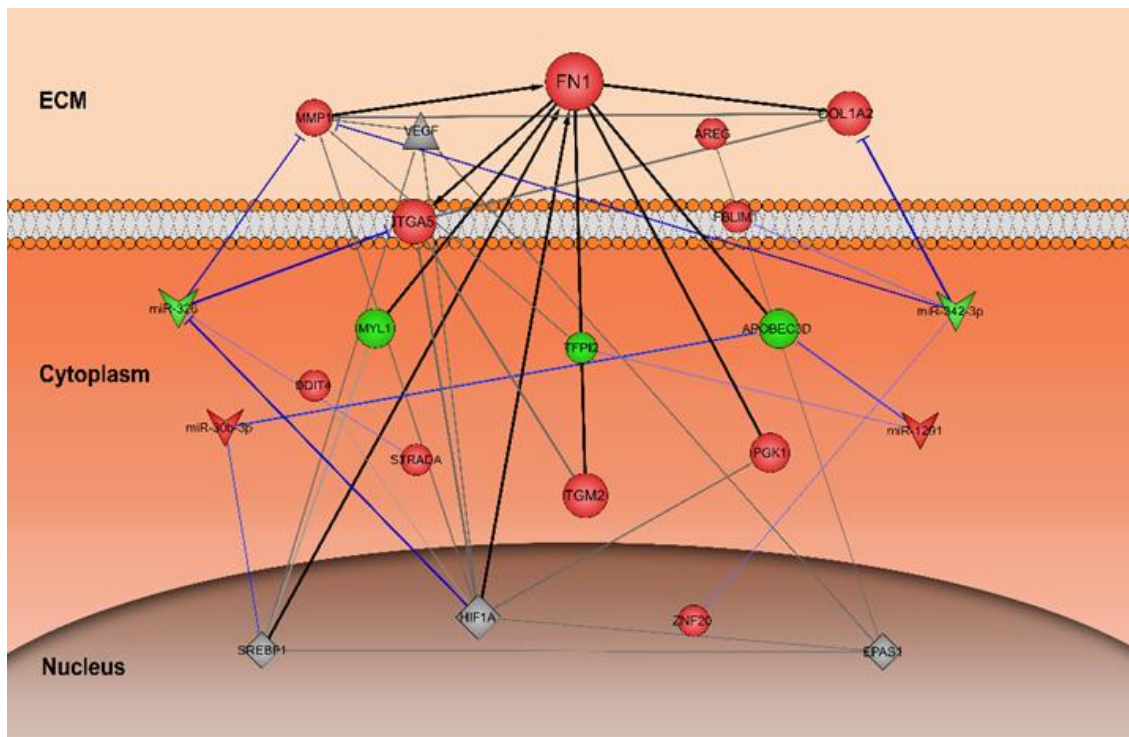
Figure 4. 4. Forest plot showing survival analyses of chemotherapy treated TNBC patients. Patient survival data were obtained from KM-Plotter (n=132) and GSE58812 (n=107) datasets, and analyses were performed according to mRNA expression of genes involved in doxorubicin resistance. Circles indicate the hazard ratio and the horizontal bars show the 95% confidence interval. Red plot indicates significant ($p < 0.05$) HR score.

After selection process using survival analysis was completed, the genes were filtered out into differentially expressed 18 mRNAs and 4 upstream regulators. Doxorubicin resistance network was re-constructed by integrating 14 mRNA (we observed that 4 mRNAs lacked any interactions, thus are unconnected and had to be eliminated) and 4 upstream regulators with their associated miRNAs using IPA platform. Then, we manually constructed and modified the network in Cytoscape (<https://cytoscape.org/>) [103] to get a better network visualization (**Figure 4.5A and B**). Lastly, depending on the interaction type, each gene (node) contributes to the expression (i.e. activity) level of other genes and conversely, activity level of certain gene comes from accumulation of other gene regulations in the given network. To measure the activity level of each gene in our miRNA-mRNA interaction network, we performed “random-walk” simulation using activity flow analysis (see Method section for details). The results clearly show that FN1 had the highest activity flow score, which suggests its centrality in the doxorubicin resistance network (**Figure 4.5C**).

A.



B.



C.

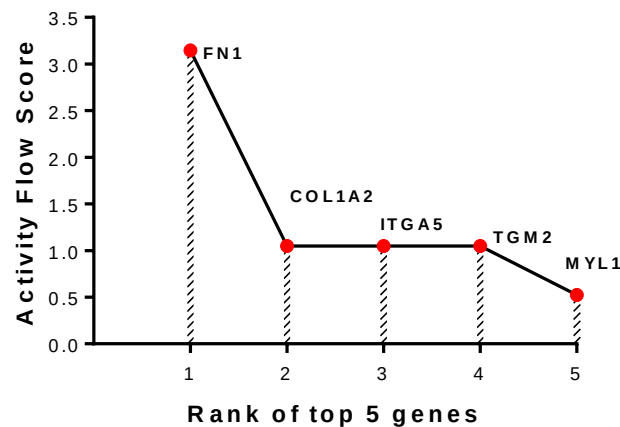
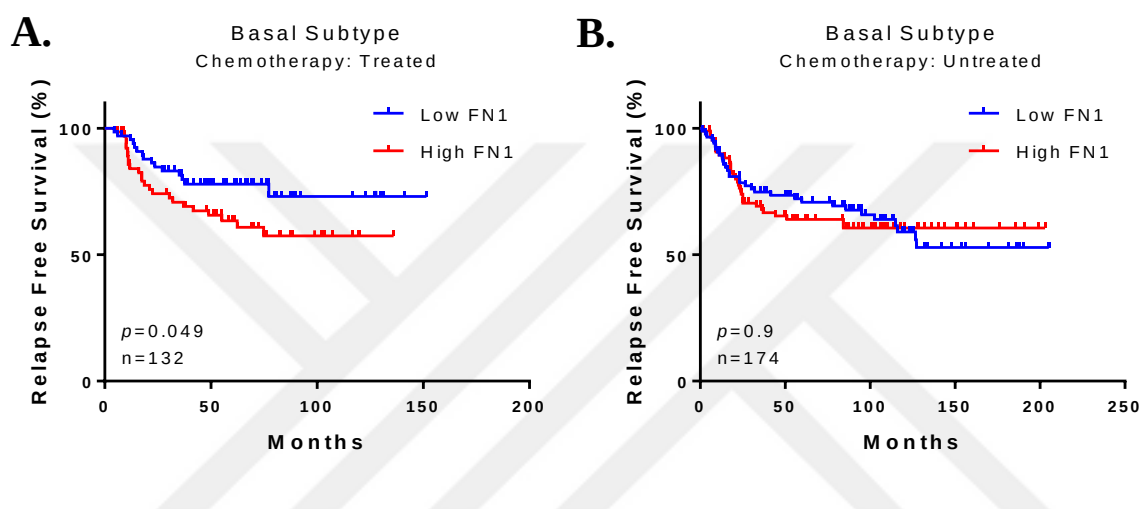


Figure 4. 5. The refined miRNA-mRNA interaction network underlying TNBC chemoresistance and activity flow analysis. (A) FN1-centered network comprising mRNAs, miRNAs and upstream regulators involved in doxorubicin resistance. Red and green nodes represent upregulated and down regulated mRNAs or miRNAs, respectively, where grey nodes represent upstream regulators. Black and blue edges indicate activation and inhibition interactions, respectively. The size of each node and related edges were determined by its activity flow score from C. **(B)** Cellular localization of functional proteins and miRNAs from chemotherapy resistance network depicting in panel A. **(C)** Graphs showing 5 genes with the highest activity flow score. The complete activity flow score from all genes in the network can be seen in the Appendix.

4.2. Fibronectin (FN1) mediates doxorubicin resistance in TNBC cells

Our *in silico* analyses using IPA and survival analyses over differentially expressed transcriptome indicated FN1 as a central gene in doxorubicin resistance network. Fibronectin is a glycoprotein functioning in ECM to serve multiple roles in many biological processes, such as cell adhesion, growth, and differentiation [104, 105]. KM-Plotter survival analysis of

chemotherapy-treated basal patients with high expression of FN1 was associated with poor relapse free survival (RFS) compared to those with low FN1 expression (**Figure 4.4.** and **Figure 4.6A**). Most importantly, we found that separation of RFS curves based on FN1 expression was specific to chemotherapy-treated basal patients. This was not observed neither in untreated basal subtype nor in other subtypes (**Figure 4.6B** and **C**), suggesting the specific role of FN1 in the development of chemotherapy resistance in TNBC.



C. KM-Plotter – Breast cancer relapse free survival analysis for FN1

Subtype	Chemotherapy					
	-			+		
	n	p-value	HR	n	p-value	HR
Basal	173	0.9	1.03	132	0.049	1.88
Luminal A	520	0.63	1.08	83	0.70	0.84
Luminal B	274	0.33	1.2	145	0.58	1.18
ER+	503	0.36	1.14	116	0.63	0.84
HER2+	42	0.45	1.5	61	0.32	1.51

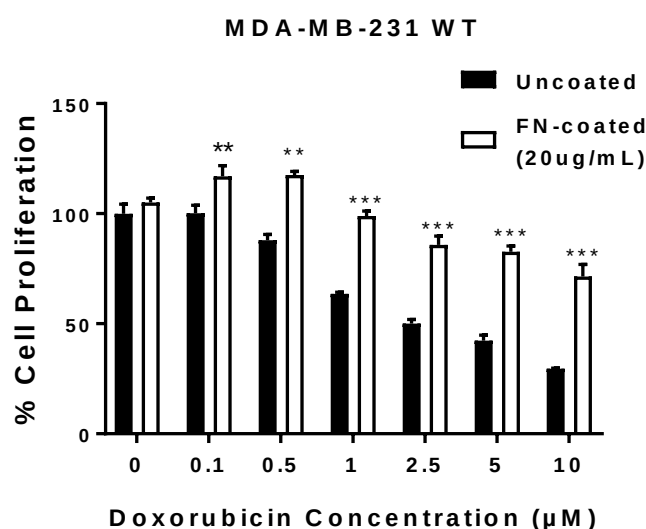
Figure 4. 6. FN1 is associated with poor RFS specifically in chemotherapy-treated TNBCs.

(A and B) Kaplan-Meier survival curves showing RFS percentage in chemotherapy-treated basal subtype (n=132) **(A)** and untreated basal subtype (n=173) **(B)** based on FN1 expression.

(C) Table summarizing the FN1 expression-based Kaplan-Meier RFS analysis from different breast cancer subtypes, either untreated or chemotherapy-treated.

The survival analyses on breast cancer patients based on the FN1 expression convinced us to further investigate the role of FN1 in mediating doxorubicin resistance *in vitro*. We tried to mimic the upregulation of fibronectin in the tumor's ECM by coating the cell culture plates with fibronectin. Then, MDA-MB-231 cells were seeded onto fibronectin-coated wells (along with uncoated ones as control) within serum-free media since serum might interfere fibronectin coating set up. Then, the cells seeded on FN-coated wells were significantly more viable and less responsive to increasing doses of doxorubicin than the ones seeded onto the uncoated wells (**Figure 4.7A**). In addition to viability tests, we also checked relative apoptosis levels in the same experimental settings using cleaved PARP as apoptosis marker since PARP is a protein that functions in DNA repair and its proteolysis by caspases and other proteases is involved in cell death processes [106, 107]. We found that apoptosis was clearly induced in the cells seeded onto uncoated wells compared to those seeded onto FN-coated wells in a doxorubicin dose-dependent manner (**Figure 4.7B**), supporting the idea that fibronectin plays pivotal role in inducing resistance against doxorubicin in TNBC cells.

A.



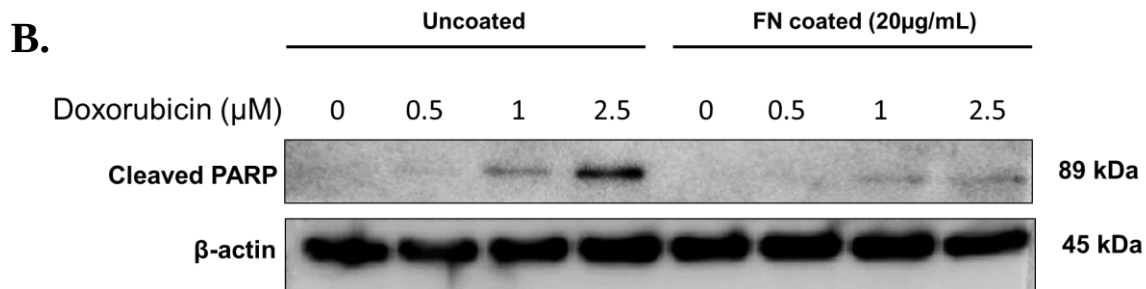


Figure 4. 7. Doxorubicin response of MDA-MB-231 cells seeded on uncoated and FN-coated wells. (A) Bar graph showing percent cell proliferation in MDA-MB-231 cells grown on uncoated or FN-coated plates following doxorubicin treatment at different doses. ** $p < 0.01$; *** $p < 0.001$. **(B)** Western Blot analysis showing apoptosis marker, cleaved PARP, levels in cells cultured on uncoated or FN coated wells with increased doxorubicin dose treatment. Beta-actin was utilized as loading control.

4.3. miR-326 inhibits fibronectin-mediated doxorubicin resistance *in vitro*

We next wanted to test miRNA candidates discovered in our doxorubicin resistance network (**Figure 4.5A**). Two downregulated miRNAs, i.e. miR-326 and miR-342-3p, were found to target some potential upregulated mRNAs interacting with FN1 in the doxorubicin resistance network. We compared the expression of these miRNAs between doxorubicin sensitive and doxorubicin resistant tumors using qRT-PCR and validated their downregulation in doxorubicin resistant tumors (**Figure 4.8**).

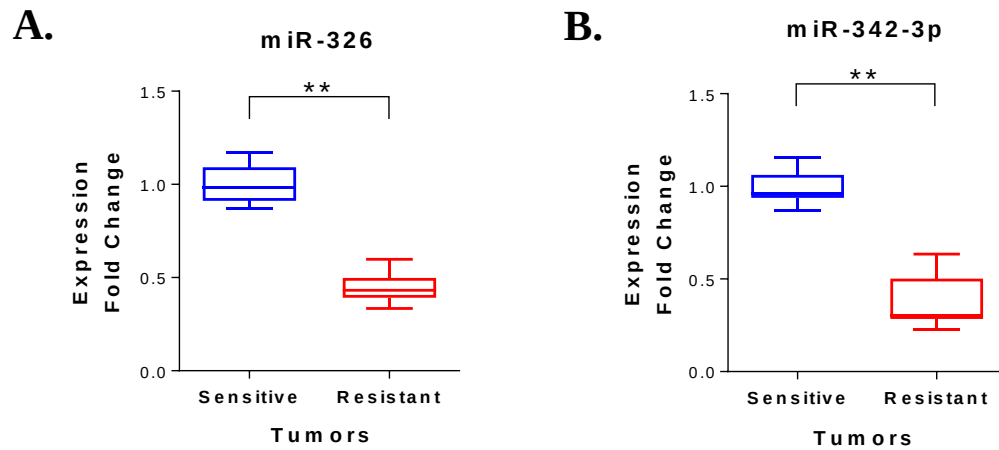


Figure 4. 8. Validation of downregulated miRNA candidates identified in doxorubicin resistance network. qRT-PCR results showing the expression levels of miR-326 (**A**) and miR-342-3p (**B**) in doxorubicin resistant tumors compared to doxorubicin sensitive tumors. ** $p < 0.01$.

Next, we tested whether increased expression of these miRNAs is mitigating or reversing doxorubicin resistance. We separately transfected MDA-MB-231 cells grown on fibronectin-coated wells with miR-326 or miR-342-3p mimics either alone or in combination with doxorubicin. We observed sensitization effect in miR-326 transfected cells when combined with doxorubicin treatment, but not in miR-342-3p transfected ones (**Figure 4.9**).

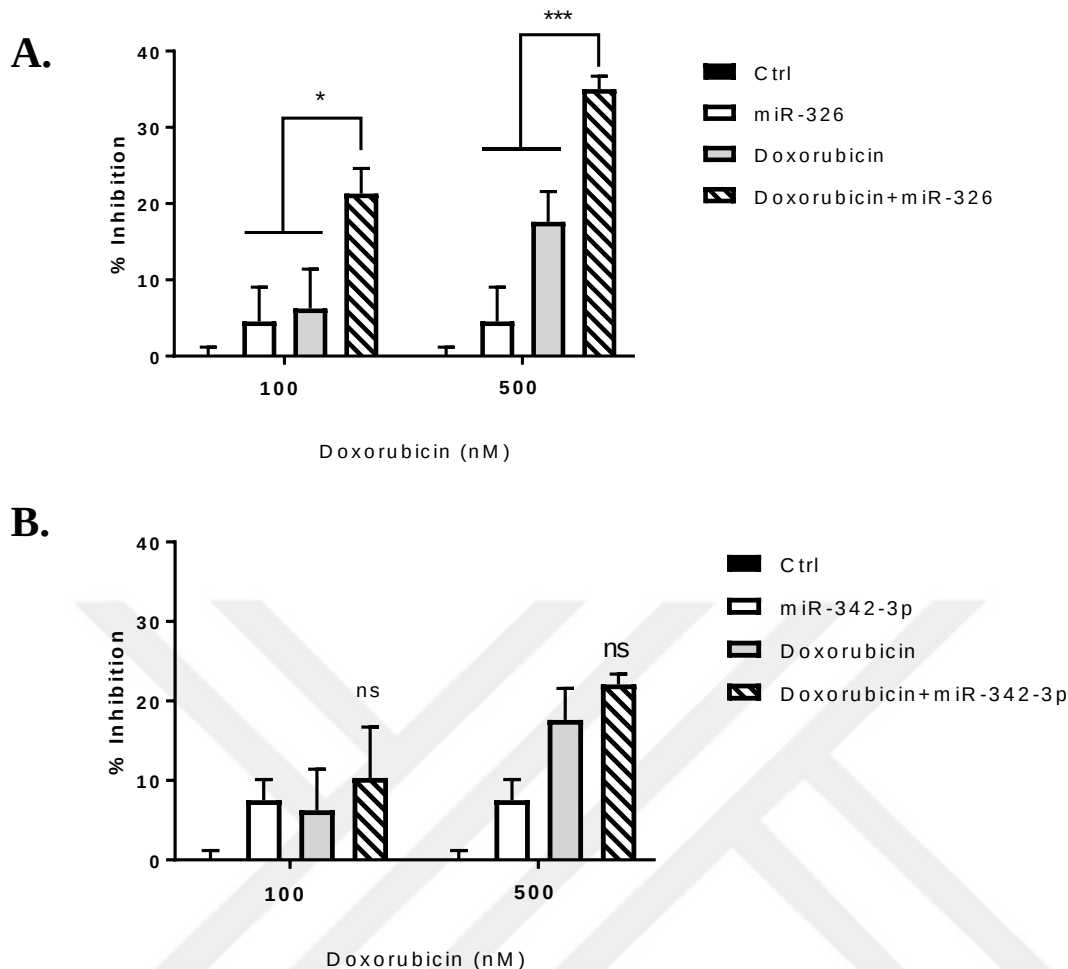


Figure 4. 9. Sensitization of fibronectin-mediated doxorubicin resistance using miRNA mimics. Bar graphs showing percent growth inhibition of MDA-MB-231 cells grown on fibronectin-coated wells upon transfection with miR-326 mimics **(A)** or miR-342-3p mimics **(B)** with or without doxorubicin treatment in two different concentrations. * $p < 0.05$; *** $p < 0.001$; ns, not significant.

Moreover, elevated levels of cleaved PARP was observed in the combination group of doxorubicin and miR-326 when compared to single treatment or control groups (**Figure 4.10**), indicating an apoptosis induction as a combination effect of the treatments.

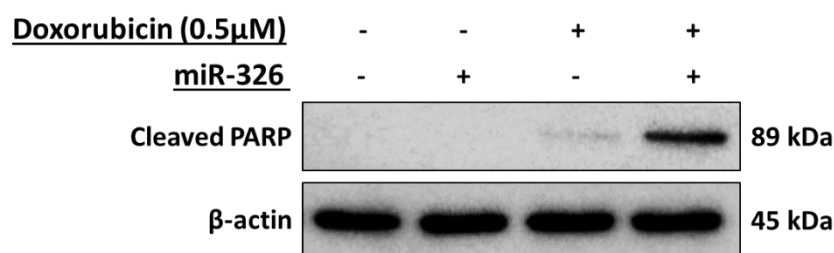


Figure 4. 10. Western blots showing cleaved PARP levels of MDA-MB-231 cells. Cells were grown on fibronectin-coated wells and they were treated with doxorubicin and/or transfected with miR-326 mimic either alone or in combination. Beta actin was utilized as loading control.

Additionally, to see if miR-326 potential in enhancing doxorubicin response is not exclusively occurred in MDA-MB-231, we transfected miR-326 mimic to another TNBC cell line, MDA-MB-436 cells, that also grown on fibronectin-coated wells either alone or in combination with doxorubicin. As a result, we observed increased growth inhibition in the combination group of miR-326 and doxorubicin treatment compared to single treatment or control group (**Figure 4.11**), suggesting that the sensitization function of miR-326 was not limited to MDA-MB-231 cells. Taken together, these findings showed that miR-326 overexpression could sensitize TNBC cells to doxorubicin and encouraged us to further examine the function and sensitization mechanism of miR-326 in the context of fibronectin-mediated doxorubicin resistance.

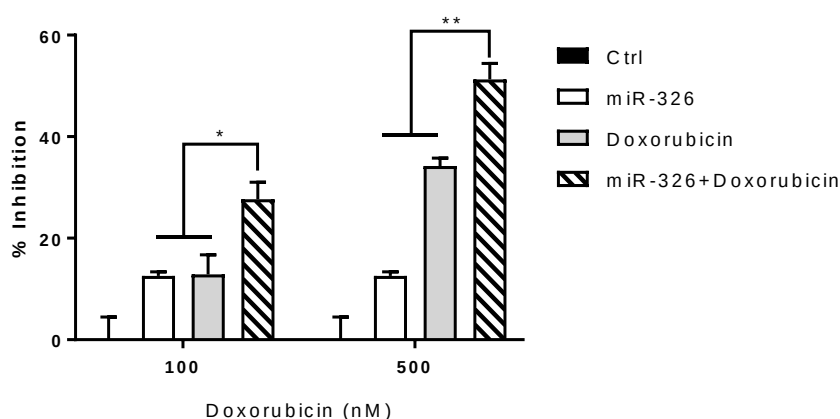


Figure 4. 11. Cell viability of MDA-MB-436 cells upon doxorubicin and/or miR-326 treatment on fibronectin-coated plates. Bar graphs showing percent growth inhibition of MDA-MB-436 cells grown on fibronectin-coated wells upon transfection with miR-326 mimics with or without doxorubicin treatment in two different concentrations. * $p < 0.05$; ** $p < 0.01$.

4.4. miR-326 overcomes FN1-mediated doxorubicin resistance via targeting ITGA5 and repressing integrin-associated signaling pathways

We demonstrated the potential function of miR-326 in sensitizing fibronectin-mediated doxorubicin resistance. To elucidate the detailed mechanism of miR-326 in this process, we re-evaluated our doxorubicin resistance network (**Figure 4.5A**) and examined any genes interacting with miR-326. Integrin alpha 5 or shortly ITGA5, known as a subunit of heterodimer receptor of fibronectin along with integrin beta 1 (ITGB1) [108], was predicted to be one of the miR-326 targets. To confirm the interaction between miR-326 and ITGA5, we transfected MDA-MB-231 cells with either miRNA mimic negative control (miR-Ctrl) or miR-326 mimic, and measured the mRNA and protein levels of ITGA5 using qRT-PCR and Western blot, respectively (**Figure 4.12**). Both mRNA and protein levels of ITGA5 were reduced upon miR-326 transfection, suggesting the inverse correlation between miR-326 and ITGA5. In other words, higher miR-326 expression causes ITGA5 downregulation.

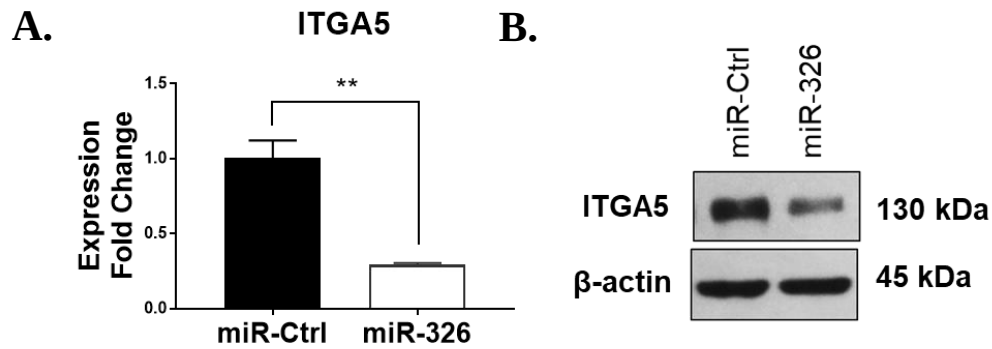


Figure 4. 12. ITGA5 mRNA and protein levels upon miR-326 mimic transfection in MDA-MB-231 cells. (A) Bar graphs showing mRNA level of ITGA5 upon transfection with miR-Ctrl or miR-326. Data was normalized according to miR-Ctrl (negative control). ** $p < 0.01$. **(B)** Western blot showing protein levels of ITGA5 upon transfection with miR-Ctrl or miR-326. Beta-actin was utilized as loading control.

Then, we examined the direct binding between miR-326 and ITGA5 through miRNA target prediction tool, TargetScan v7.2. We found that seed sequence of miR-326 was complementary to 3'-UTR of ITGA5 at 124-131 nucleotide sequence (**Figure 4.13A**). We tested this binding *in vitro* by co-transfecting either miR-Ctrl or miR-326 along with reporter construct containing 3'-UTR of ITGA5 and measuring the luciferase expression. A significant decrease in luciferase expression was observed in miR-326 transfected cells compared to those transfected with miR-Ctrl, confirming ITGA5 as a direct target of miR-326 (**Figure. 4.13B**).

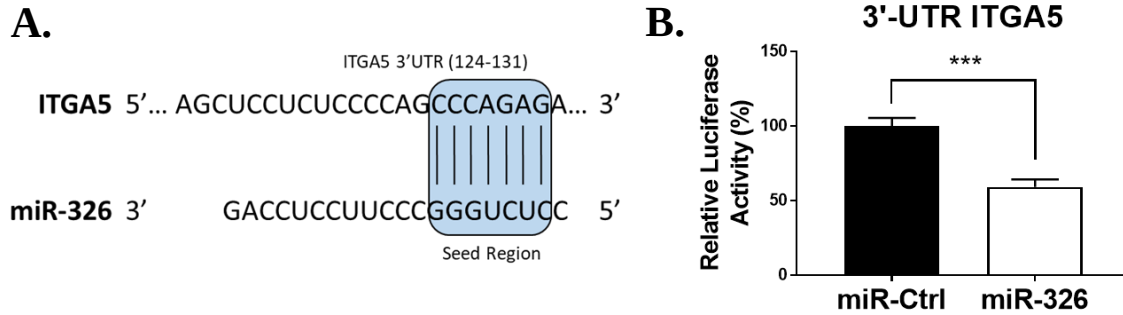


Figure 4. 13. Validation of direct binding of miR-326 to ITGA5. (A) Graphical representation of sequence complementarity between miR-326 seed region and binding site of ITGA5 3'-UTR retrieved from TargetScan v7.2. (B) *In vitro* luciferase activity assay using the 3'-UTR of ITGA5 cloned in reporter construct those were co-transfected with either miR-Ctrl or miR-326. Data was normalized according to miR-Ctrl. *** $p < 0.001$.

Since ITGA5 is a subunit of heterodimeric receptor recognizing fibronectin and a direct target of miR-326, we decided to further investigate its role in the chemoresistance context. Therefore, we first examined the role of ITGA5 expression on the survival of breast cancer patients. Forest plot of patient survival analyses had indicated that ITGA5 is significantly associated with poor RFS, OS and MFS in TNBC (**Figure 4.4**). In addition, survival analysis using KM-Plotter suggested that high expression of ITGA5 was significantly associated with poor RFS in chemotherapy-treated basal patients, but not in untreated basal patients (**Figure 4.14A and B**). Interestingly, significant correlation between ITGA5 expression and RFS was not observed in patients with other breast cancer subtypes, independent of their therapy status (**Figure 4.14C**). These results emphasize the importance of ITGA5 in the development of chemotherapy resistance in basal or TNBC patients.

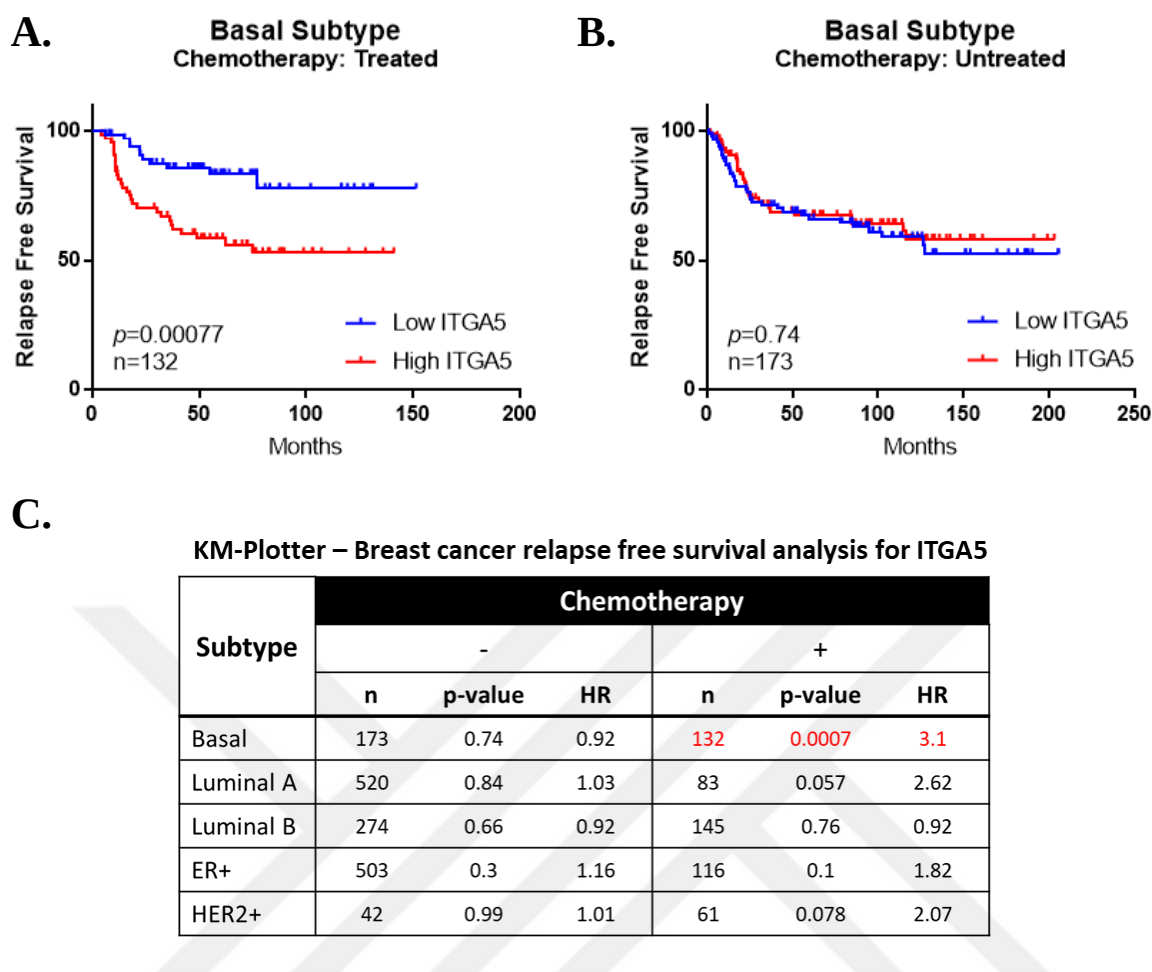


Figure 4. 14. ITGA5 is correlated with poor RFS in chemotherapy-treated basal patients.

(A and B). Kaplan Meier survival curves showing the percent RFS in chemotherapy-treated (n=132) **(A)** and untreated basal patients (n=173) **(B)** based on ITGA5 expression. **(C)** Table summing up the ITGA5 expression-based Kaplan-Meier RFS analysis from different breast cancer subtypes, either untreated or chemotherapy-treated groups.

Having showed the role of ITGA5 levels in patient survival, we tested its involvement in the fibronectin-mediated doxorubicin resistance. Therefore, we inhibited the ITGA5 expression using an siRNA (siITGA5) in MDA-MB-231 cells grown on fibronectin-coated wells either alone or in combination with doxorubicin. We observed that cells are more vulnerable to doxorubicin when ITGA5 is knocked down, meaning that its downregulation led to doxorubicin

sensitization (**Figure 4.15**). Overall, these findings suggest that miR-326 might sensitize fibronectin-mediated doxorubicin resistant cells through suppression of ITGA5.

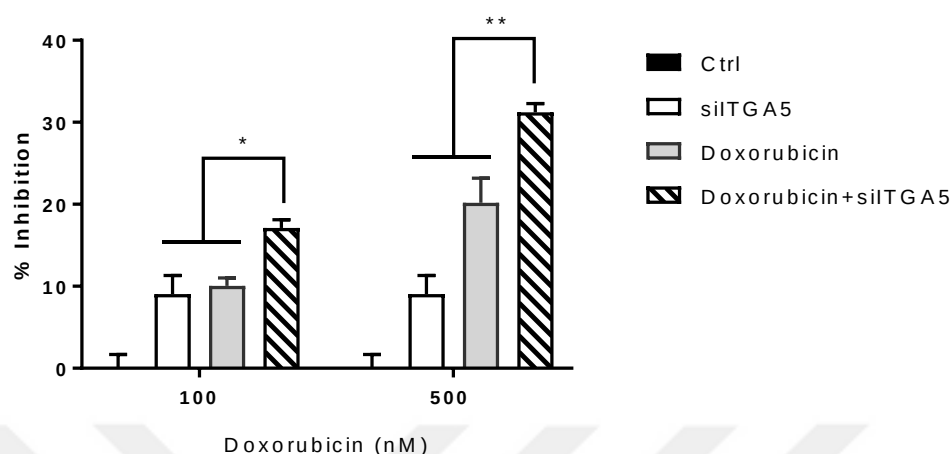


Figure 4. 15. Cell viability response of MDA-MB-231 cells upon doxorubicin and/or siITGA5 treatment on fibronectin-coated plates. Bar graphs showing percent growth inhibition of MDA-MB-231 cells grown on fibronectin-coated wells upon treatment using siITGA5 and doxorubicin either alone or in combination. Graph was normalized to control group. * $p < 0.05$; ** $p < 0.01$.

Next, we examined the signaling pathways potentially regulated by miR-326 that leads to chemosensitization. In order to do this, we determined the whole target portfolio of miR-326 using miRWalk 2.0 program that allows comprehensive prediction of miRNA-target interactions by combining different miRNA target prediction platforms [100] (for details, see Methods). From this analysis, we found 674 genes predicted to be miR-326 targets, which subsequently taken into KEGG pathway enrichment analysis using DAVID 6.8 online tool [101]. The result of KEGG pathway analysis showed signaling pathways that are potentially regulated by miR-326. Among them, we observed three crucial pathways that might be involved in the development of chemotherapy resistance: focal adhesion, MAPK signaling pathway, and PI3K-AKT signaling pathway (**Figure 4.16**). Focal adhesion is a core pathway activated by

integrins whereas MAPK and PI3K-AKT signaling pathways are the common dysregulated pathways in breast cancer that also reported as downstream of focal adhesion [50, 109, 110]. Therefore, we were encouraged to further investigate the potential of miR-326 to regulate these pathways *via* targeting ITGA5.

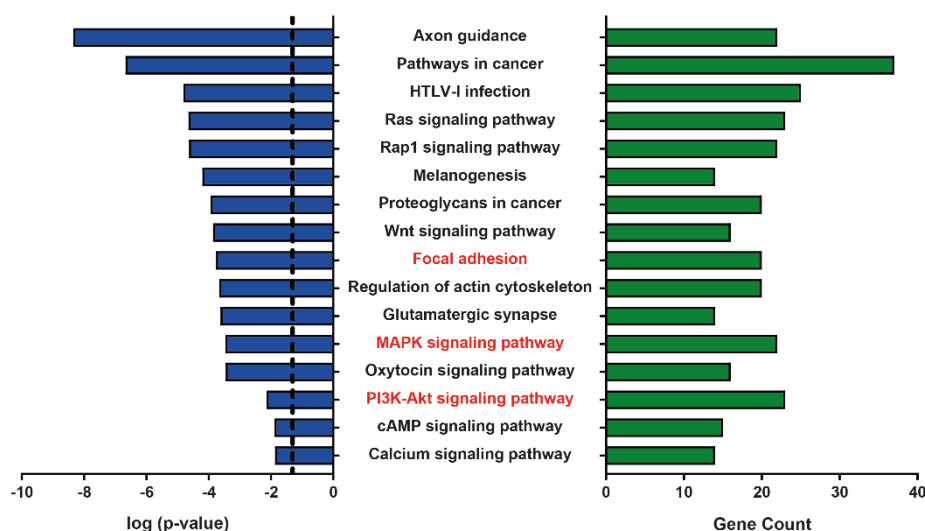


Figure 4. 16. KEGG pathway enrichment analysis of miR-326 targets using DAVID 6.8 tool. Left bar graph indicates log p-value while right bar graph indicates gene count of each pathway. Dashed line in the left bar graph showed significance threshold ($p = 0.05$). Pathways denoted in red are pathways that we focused on.

Before examining the known markers of these three pathways by Western blot to confirm our *in silico* results above, we generated the following cell line models. 231.Luc2.GFP cells were first transfected with inducible miR-326 mimic for its overexpression (hereinafter referred as 231.miR-326 cells). The overexpression was induced with doxycycline and confirmed by qRT-PCR (**Figure 4.17A**). In addition to miR-326, we also created 231.Luc2.GFP cells that inducibly express shRNA inhibiting ITGA5 (referred as 231.shITGA5 cells). ITGA5 mRNA expression was markedly reduced upon doxycycline induction in 231.shITGA5 cells as

confirmed by qRT-PCR (**Figure 4.17B**). These stable-inducible cell lines were also used for the *in vivo* experiments described later.

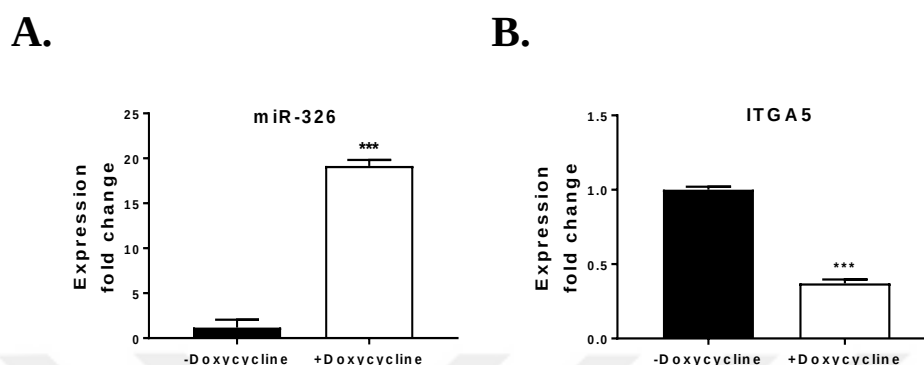
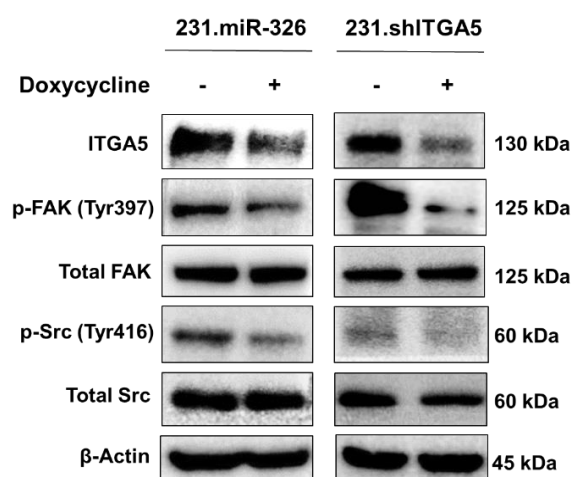


Figure 4. 17. Relative measurement of miR-326 and ITGA5 mRNA expression in 231.Luc2 GFP stable cells using qRT-PCR. Bar graphs showing the levels of miR-326 (**A**) and ITGA5 mRNA (**B**) upon doxycycline induction in 231.miR-326 cells and 231.shITGA5 cells, respectively. Each data was normalized to non-induced groups*** p < 0.001.

After overexpression and knockdown of the corresponding genes were validated in these stable cells, we next tested the known markers of focal adhesion, MAPK and PI3K pathways by Western blot. Upon miR-326 induction with doxycycline, focal adhesion signaling (p-FAK Y397, p-Src Y416), MAPK signaling (p-ERK T202/Y204), and PI3K-AKT signaling (pAKT-T308, pAKT-S473) were reduced with a concomitant decrease of ITGA5 protein level. Similarly, when ITGA5 was knocked down upon doxycycline induction of shITGA5, the levels of these signaling proteins were also decreased (**Figure 4.18**). These data further validated that miR-326 might sensitize TNBC cells toward doxorubicin through inhibiting ITGA5 expression and subsequently reducing integrin-associated intracellular signaling.

A.



B.

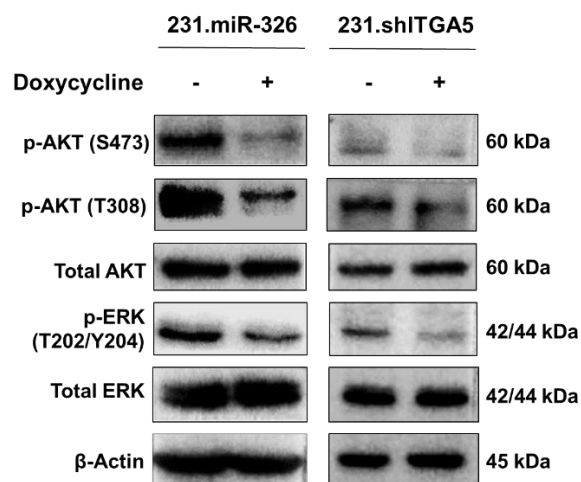


Figure 4. 18. Regulation of signaling pathways upon miR-326 overexpression or ITGA5 inhibition. Western blot analysis showing phospho-protein and total protein levels of focal adhesion signaling **(A)**, PI3K-AKT signaling and MAPK signaling pathways **(B)** in 231.Luc2.GFP stable cells inducibly overexpressing miR-326 and shRNA for ITGA5. Beta-actin was utilized as loading control.

4.5. Hypoxia regulates miR-326 expression via HIF1A

miR-326 was downregulated in doxorubicin resistance tumors compared to their sensitive counterparts. Next, we wanted to identify the upstream regulators that possibly downregulate miR-326 in doxorubicin resistant TNBC. The network of doxorubicin resistance (**Figure 4.5A**) predicted that miR-326 is inhibited by a transcription factor, Hypoxia-inducible factor 1-alpha (HIF1A). *HIF1A* is a gene that encodes protein subunit which, together with HIF1B protein subunit, forms heterodimeric transcription factor called HIF1 [111]. As the name suggests, HIF1 is activated by O₂ insufficiency or hypoxia within the cells, and subsequently initiates many downstream signaling and biological processes including cell proliferation and survival [111, 112]. The presence of HIF1A in our network indicated the involvement of hypoxia-related

signaling in the development of doxorubicin resistance. To test this hypothesis, we performed GSEA in our doxorubicin sensitive vs doxorubicin resistant expression data obtained from tumors (**Figure 4.2A**). Four different hypoxia-related datasets showed that the genes upregulated upon hypoxia are enriched in resistant tumors compared to sensitive ones (**Figure 4.19**), supporting our hypothesis about the involvement of hypoxia response in the development of chemoresistance.

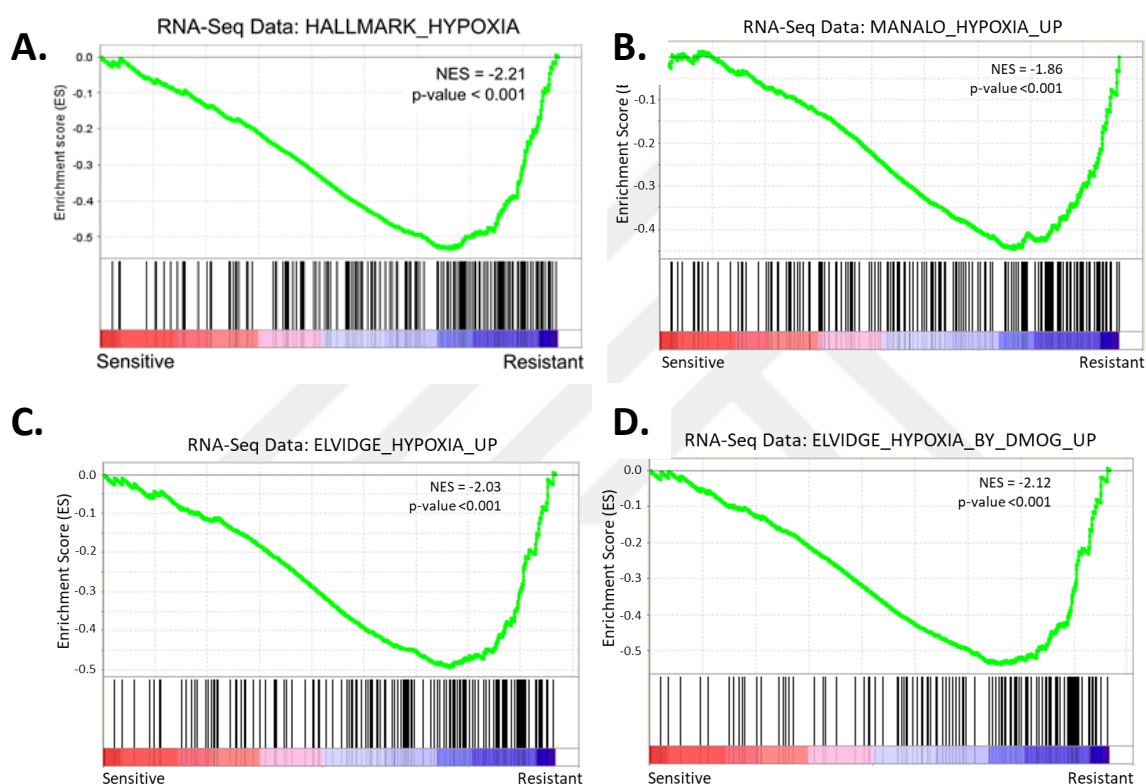


Figure 4. 19. GSEA using four different hypoxia-related gene sets in RNA-seq data from doxorubicin sensitive and resistant tumors. Enrichment plot of RNA-Seq data from doxorubicin sensitive and doxorubicin resistant tumors. Upregulated genes responding to low oxygen levels (**A**), upregulated genes upon hypoxia and activation of HIF1A (**B**), upregulated genes under hypoxia conditions in breast cancer (**C**), and upregulated genes in breast cancer upon hypoxia mimetic DMOG treatment (**D**) are highly and significantly enriched in doxorubicin resistant tumors. NES: Normalized Enrichment Score.

Specifically, expression of HIF1A was found to be significantly negatively correlated with miR-326 expression in two breast cancer patient datasets; GSE19536 and GSE19783 (**Figure 4.20**), suggesting the possibility of miR-326 downregulation by HIF1A. These *in silico* analysis results convinced us to further investigate miR-326 regulation through hypoxia/HIF1A axis.

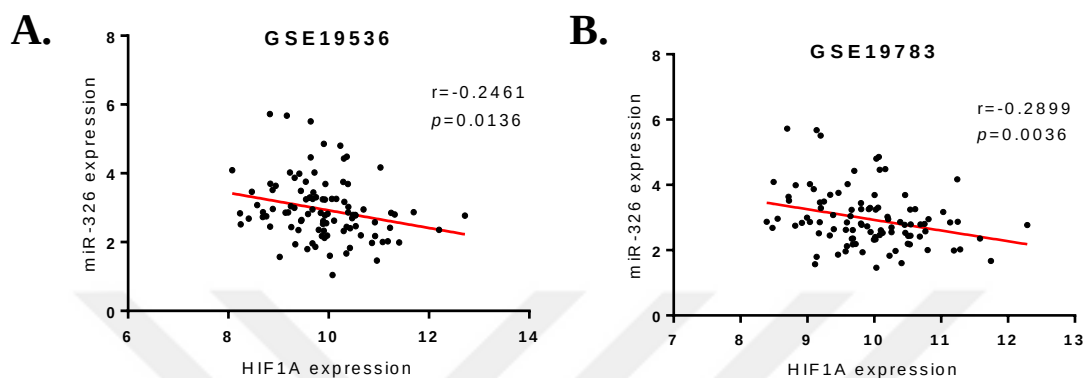


Figure 4. 20. Expression correlation between HIF1A and miR-326 in patient datasets. Dot plots showing the negative correlation between HIF1A and miR-326 expression from two breast cancer patient datasets, GSE19536 (**A**) and GSE19783 (**B**).

We then set out to validate miR-326 regulation associated with hypoxia. To do this, we incubated MDA-MB-231 cells in hypoxia chamber for 12-24 hours. Then, we quantified miR-326 levels from each treatment group using qRT-PCR and observed a significant decrease in miR-326 levels of cells maintained under hypoxia (**Figure 4.21A**). In line with this, we also asked whether the downregulation of miR-326 is mediated by HIF1A. To test this, we inhibited HIF1A using an siRNA targeting HIF1A (siHIF1a) and observed significantly increased miR-326 level (**Figure 4.21B**). Overall, these results suggest that miR-326 is potentially negatively regulated by hypoxia *via* HIF1A.

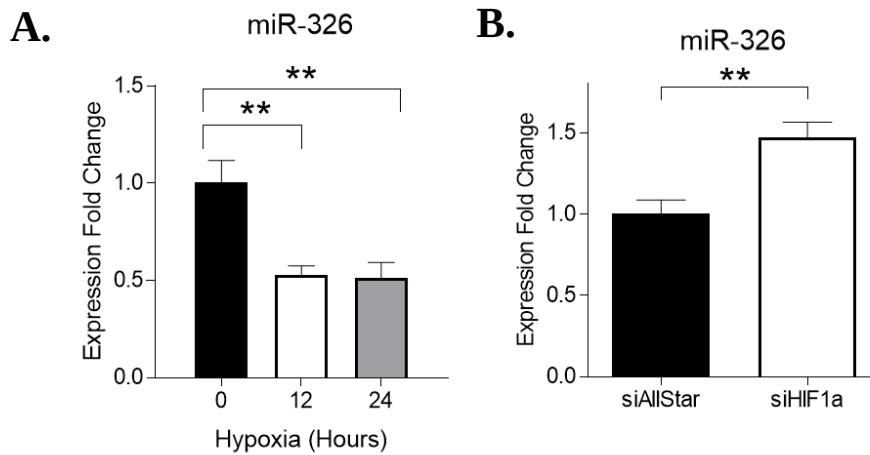


Figure 4. 21. Negative regulation of miR-326 by hypoxia via HIF1A. (A) Bar graphs showing quantification of miR-326 expression levels in MDA-MB-231 cells after incubation in hypoxia condition compared to those grown in normoxia condition. (B) Bar graph showing quantification of miR-326 expression levels in MDA-MB-231 cells upon inhibition of HIF1A using siRNA. siAllstar is used as negative control. ** $p < 0.01$.

4.6. Overexpression of miR-326 sensitizes TNBCs to doxorubicin *in vivo*

After confirming the role and the mechanisms of miR-326 in doxorubicin resistance *in vitro*, we next investigated its function in *in vivo* xenograft models and tested whether it potentiates the efficacy of chemotherapy in TNBC xenografts. We used inducible 231.miR-326 stable cells to develop primary tumor xenografts in female nude mice. These engineered TNBC cells were injected into MFPS of mice and tumor growth was monitored regularly. When the tumors reached around 100 mm³, doxorubicin and/or doxycycline was given to mice through tail vein injection and in drinking water, respectively (for details, see Methods section). Since the 231.miR-326 stable cells also harbor vector expressing inducible red fluorescent protein (RFP) upon doxycycline treatment, we monitored the RFP signals from tumors of doxycycline-treated mice using IVIS instrument (**Figure 4.22**). The presence of the RFP signals from these tumors

ensured that the inducible system was properly working and miR-326 was inducibly overexpressed.

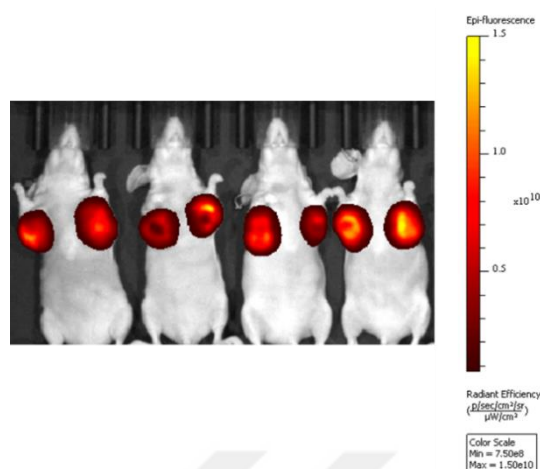
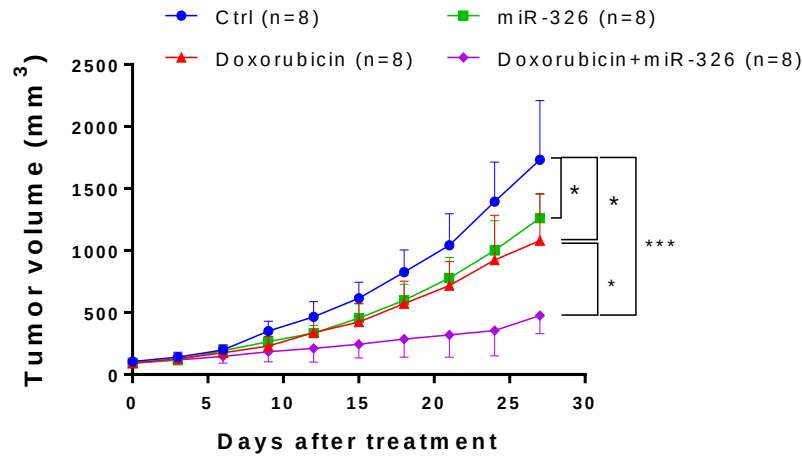


Figure 4. 22. *In vivo* validation of doxycycline-induced stable cells overexpressing miR-326. IVIS imaging of RFP expression of 231.miR-326 stable cells upon induction with doxycycline.

As the tumors reached to 100 mm^3 , mice were randomly allocated into four treatment groups; 1) vehicle, 2) doxorubicin-treated, 3) miR-326-treated, and 4) combination groups. After regular measurement of tumor growth for around 4 weeks, we observed that combination treatment using doxorubicin and miR-326 overexpression synergistically suppressed tumor growth and resulted in noticeably lower tumor volumes compared to individual treatment groups and control group (**Figure 4.23A**). Moreover, this treatment setting did not change mice body weight significantly in all treatment groups during therapy (**Figure 4.23B**), indicating that the treatment is less toxic and considerably safe for mice.

A.



B.

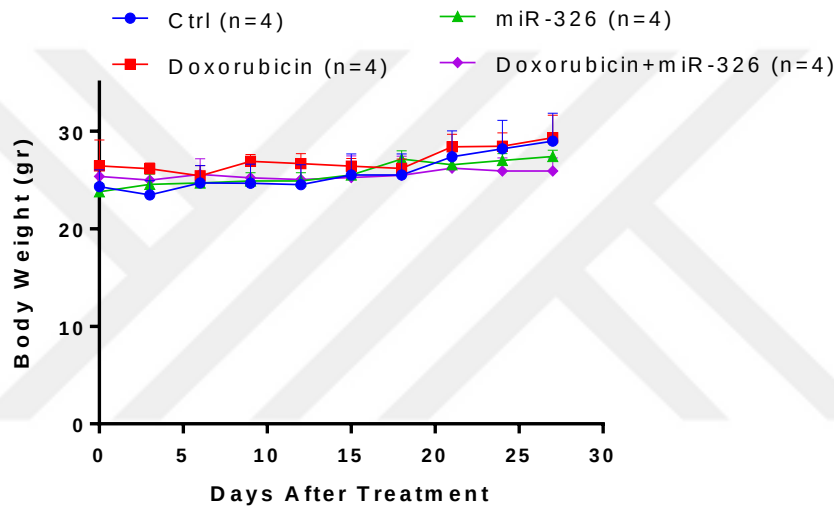


Figure 4. 23. Effect of miR-326 overexpression on doxorubicin response and mice body weight. (A) Changes in tumor volumes of 231.miR-326 xenografts for 27 days after the first treatment with vehicle (control) or doxorubicin and/or miR326 induction. Treatments and induction of miR-326 expression were started once tumors reached to 100 mm³. n indicates tumor number. * p < 0.05; *** p < 0.001. **(B)** Body weight of mice harboring 231.miR-326 tumor from all groups measured during the treatment period. n indicates mouse number.

Since 231.miR-326 cells also express *luciferase* enzyme as these cells were labelled using a Luc2 construct, we were able to observe the luciferase activity of these tumors when injected with D-luciferin, substrate of the enzyme, intraperitoneally (i.p.). Luciferase activity of tumors

were visualized using IVIS at the end of the experiment. Total flux was measured and compared between treatment groups (**Figure 4.24A and B**). Together with tumor mass measurements (**Figure 4.24C and D**), we showed that tumor growth in the combination group was significantly impaired when compared to either single treatment or control groups (**Figure 4.24**).

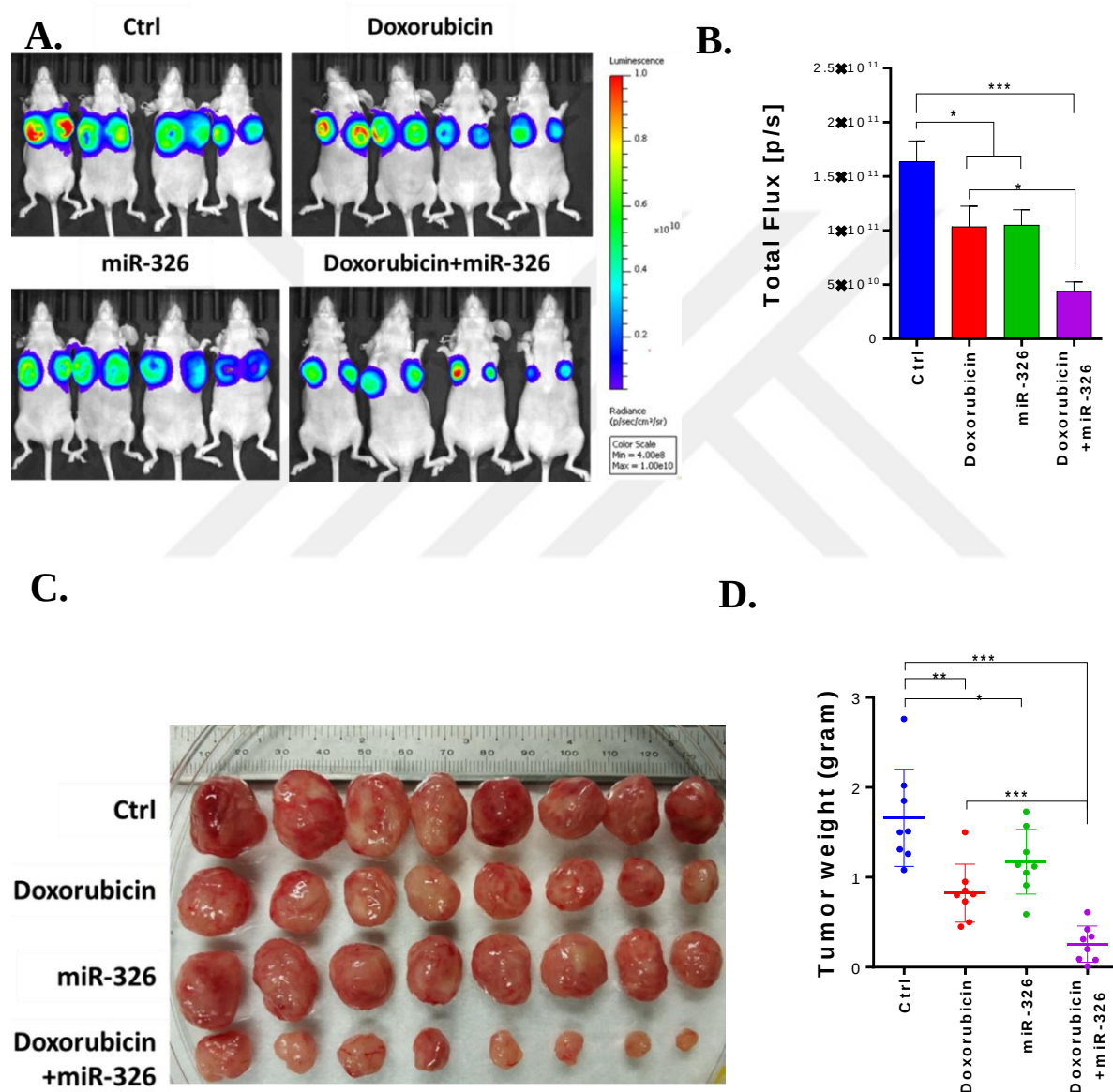


Figure 4. 24. End-point measurement of tumors upon treatment with doxorubicin and miR-326 overexpression either alone or in combination. (A) IVIS images of mice taken on the last day of the experiment. Mice were injected with D-luciferin i.p. in order to measure the

luciferase activity of cells. **(B)** Bar graph showing quantification of luciferase intensity from each tumor group in panel (A) and represented as total flux [p/s]. **(C)** Tumor images taken after mice were sacrificed and arranged per group. **(D)** Dot plots comparing the weights of tumors from each group shown in panel (C). ** $p < 0.01$; *** $p < 0.001$.

4.7. Inhibiting ITGA5 potentiates doxorubicin response *in vivo*.

As in case of miR-326, we also tested the inhibitory effect of shITGA5 and doxorubicin together in *in vivo* xenografts since we showed that ITGA5 is a functional target of miR-326 as well as the major receptor of fibronectin [113, 114]. We injected 231.shITGA5 stable cells into the MFPs of female nude mice and measured tumor volumes regularly. Similar to the miR-326, doxorubicin and/or doxycycline was given to mice through tail vein injection or in drinking water, respectively. Since 231.shITGA5 stable cells also harbor vector expressing inducible RFP upon doxycycline induction, we monitored the RFP signals from tumors of doxycycline-treated mice using IVIS (**Figure 4.25**). The presence of RFP signals from these mice tumors indicated that shRNA targeting ITGA5 was expressed.

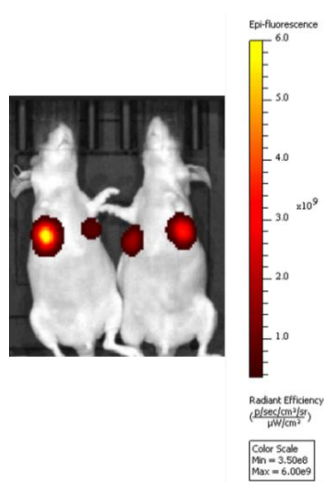
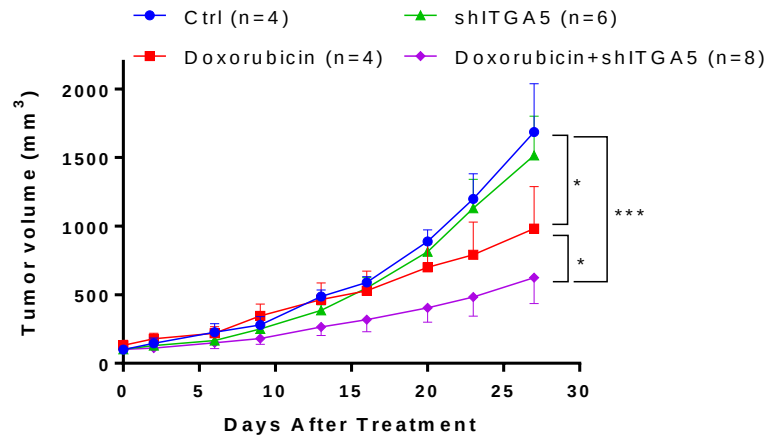


Figure 4. 25. *In vivo* validation of doxycycline-induced stable cells overexpressing shITGA5. Upon doxycycline induction, RFP expression was tested in mice injected with 231.shITGA5 stable cells.

Next, mice were randomly allocated into four groups; 1) control, 2) doxorubicin-treated, 3) shITGA5-treated, and 4) combination groups. After 4 weeks, we demonstrated that ITGA5 suppression together with doxorubicin treatment led to better inhibition of tumor growth compared to individual treatments or control group (**Figure 4.26A**). In addition, this treatment setting also did not change mice body weight significantly in all treatment groups during therapy (**Figure 4.26B**), suggesting that the treatment has less side-effect and considerably safe for mice.

A.



B.

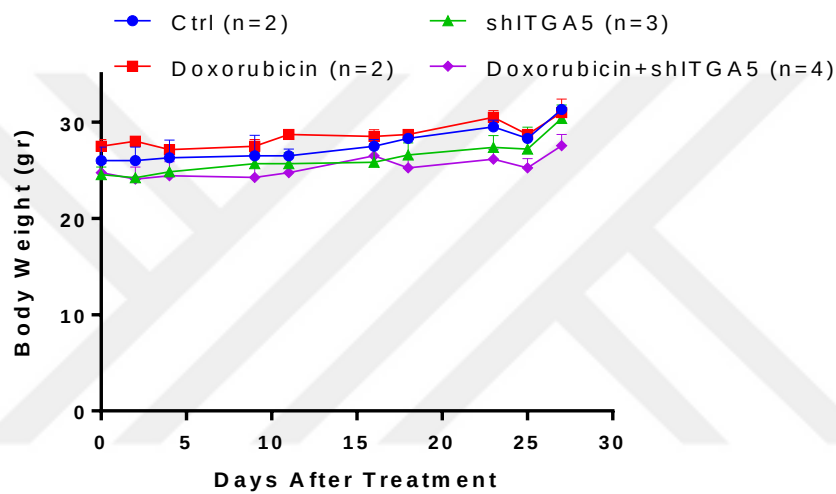


Figure 4. 26. Effect of ITGA5 inhibition on doxorubicin response and mice body weight.

(A) Changes in tumor volumes of 231.shITGA5 xenografts for 27 days after the treatment with vehicle (control) or doxorubicin and/or shITGA5 induction. Treatments were started once the tumors reached 100 mm³. n indicates tumor number. * p < 0.05; *** p < 0.001. **(B)** Body weight of mice harboring 231.shITGA5 tumor from all groups measured during the treatment period. n indicates mouse number.

In addition, mice were injected with D-luciferin and tumor volumes on the last day of the experiment were visualized using IVIS instrument. The signal intensity was quantified and represented as total flux (**Figure 4.27A and B**). In the combination group, greater effect on

tumor volume reduction could be clearly seen by tumor images and tumor weights (**Figure 4.27C and D**). These experiments further corroborate the oncogenic function of ITGA5 in the development of chemotherapy resistance in TNBC.

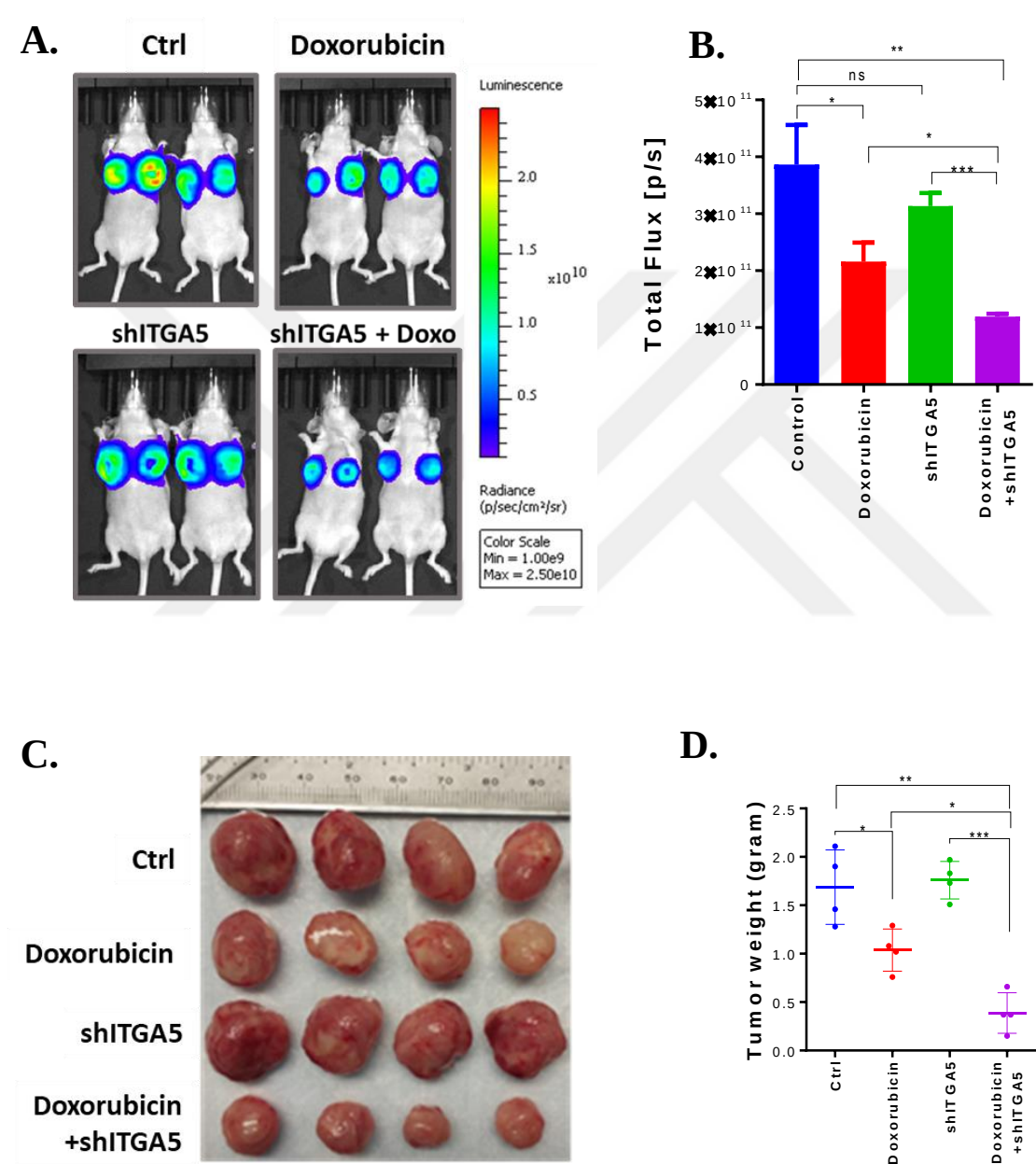


Figure 4. 27. End-point measurement of tumors upon treatment with doxorubicin and ITGA5 inhibition either alone or in combination. (A) IVIS images of mice taken on the last day of the experiment. Mice were injected with D-luciferin i.p. in order to measure the

luciferase activity of cells **(B)** Bar graphs showing quantification of luciferase intensity from each tumor group in panel (A). **(C)** Image of tumors isolated after mice were sacrificed and arranged per group. **(D)** Dot plots comparing the weights of tumors from each group shown in panel (C). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



Chapter 5

Discussion

Resistance to chemotherapy is common in breast cancer patients, especially those with TNBCs. Anthracyclines, such as doxorubicin are still the most preferred chemotherapy agents that are usually applied as adjuvant either in a combinatorial or sequential manner [115]. Despite the wide-use of anthracyclines for early-stage breast cancer, breast tumors frequently develop resistance by the time of disease relapse and hence decreases the rate of 5-year patient survival. In some cases, chemotherapy resistance is observed within 6 months of treatment whereas some patients exhibit tumor progression even during the treatment [116]. Previously, it has been reported that around half of the aggressive breast cancer patients treated with neo-adjuvant chemotherapy had considerable residual cancer burden (RCB-II and RCB-III). While patients with pCR displayed excellent RFS of 94%, those with RCB-II and RCB-III showed poor RFS of 62% and 26% at 5 years, respectively [26, 117]. Additionally, treatment options may become limited once refractory disease exists. For instance, the administration of capecitabine as second-line treatment was only responded by approximately 25% of anthracyclines or taxanes resistant patients [118, 119]. Another chemotherapeutic used to treat anthracycline- and taxane-relapsed diseases includes vinorelbine, yet it only gave response rate of 16% with limited survival [120, 121]. Therefore, there is an urgent need to decipher the mechanisms of chemotherapy resistance and put forward better treatment alternatives for enhancing chemotherapy response in TNBCs.

To work towards this urgent and unmet clinical need, in my thesis, I aimed at delineating the molecular underpinnings of chemotherapy resistance in TNBCs. TNBC is the most aggressive and deadliest subtype of breast cancer with chemotherapy being the major therapy option to

treat patients, though an immunotherapy agent (PDL-1 inhibitor) was very recently approved by the FDA [122]. Here, we made use of *in silico*, *in vitro* and *in vivo* assays to identify the possible miRNA- mRNA interactions underlying doxorubicin resistance. From our miRNA- mRNA network of doxorubicin resistance, we inferred that ***HIF1A/miR-326/ITGA5-FN1 axis*** is involved in the chemoresistance development in TNBCs. Mechanistically; hypoxic tumor microenvironment leads to HIF1A activation, which then inhibits the expression of tumor suppressor miR-326. Then, the expression of the direct target of miR-326, ITGA5, gets upregulated. Together with increased FN1 levels, which is the major substrate for ITGA5 receptor, it initiates activation of downstream survival signaling cascades, adhesion kinase (FAK/Src), PI3K-Akt and MAPK signaling pathways. This then leads to increased cell survival and resistance to chemotherapy. (**Figure 5.1A**). Therefore, I propose that overexpressing miR-326 via decreasing ITGA5 expression and the activation of downstream pathways sensitize cells to chemotherapy. (**Figure 5.1B**).

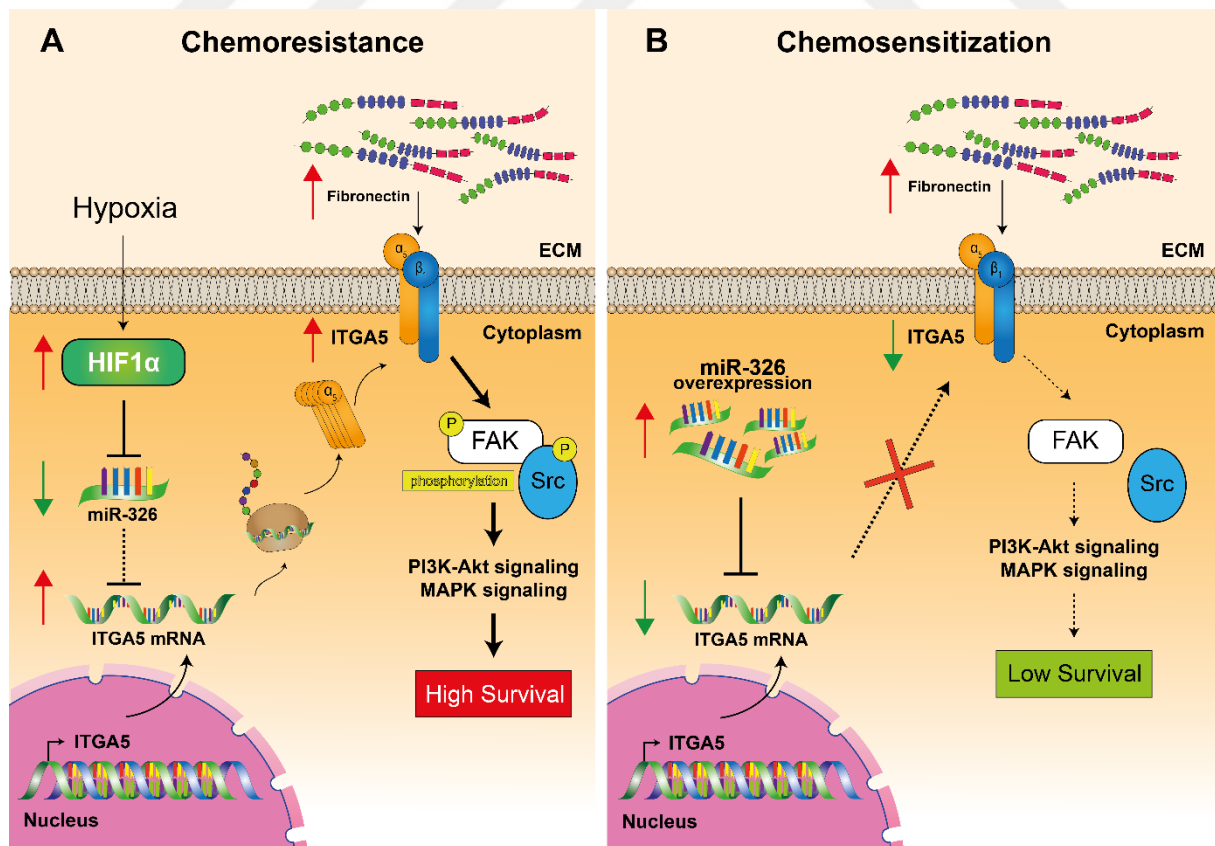


Figure 5. 1. Schematic illustration of HIF1A/miR-326/ITGA5-FN1 axis mediated doxorubicin resistance in TNBCs and chemosensitization upon miR-326 modulation. (A)

In the chemoresistance model, hypoxia-induced HIF1A inhibits miR-326 and thereby, prevents the suppression of ITGA5 expression. The upregulation of ITGA5 is accompanied by upregulation of its ligand, FN1, which consequently induces focal adhesion pathway and other downstream signaling including PI3K-Akt and MAPK pathways. This will eventually lead to increased cell survival. **(B)** In the chemosensitization model, on the other hand, overexpression of miR-326 sensitizes cells to drug by blocking ITGA5 expression and hence, integrin signaling with its downstream pathways. This causes a decrease in survival rates. Dotted arrows indicate reduced or inhibited biological process while solid arrows indicate activated or induced biological process.

5.1. miRNA-mRNA interaction network as a tool to identify key regulations in TNBC chemoresistance

Tumor progression as a multifactorial and complex process requires integration of different - omics data with network analysis tools to elucidate interconnected processes [123]. Based on the transcriptomics of chemo-resistant and –sensitive tumors, here we constructed a miRNA-mRNA interaction network predicting the mechanisms of chemoresistance in TNBCs. Using that network as reference, we verified the possible interactions using appropriate experimental settings. We showed that not all of the interactions shown in the network essentially contribute to the development of chemoresistance, at least in our model. For example, although miR-342-3p has been shown to be downregulated in chemoresistant tumors, its overexpression could not reverse the response of cells to doxorubicin. Perhaps, the reason for this is because of each node within the network was captured using statistical analysis with certain degree of threshold and

significance score. Meanwhile, both the applied technology and its statistical property always have technical bias and give some probabilities on having false positive or false negative results. For instance, the threshold for differentially expressed transcriptomics can be increased to get more stringently chosen genes (potentially less false positives), yet at the same time we might mistakenly eliminate some genes that actually contribute to the biological process (more false negatives) [124]. Therefore, our chemoresistance network is considered as a predictive tool that should be experimentally validated. Despite those technical drawbacks, the use of miRNA-mRNA interaction networks and other similar system biology-based analyses are useful for identifying novel candidates and still outperform the predictive power of single molecule analysis [92, 125].

5.2. Fibronectin and chemotherapy resistance

Fibronectin (gene: *FN1*) is a fibrillary protein that connects cells into ECM and acts as a mechanical support for cells owing to its elastic characteristics. Structurally, fibronectin consists of multiple domains for interacting with other ECM components, integrin receptors, and other fibronectin proteins [126]. Notably, association of fibronectin and integrin attracts much interest in the context of tumorigenesis since it serves not only physical platform for cells, but also exerts a bidirectional signaling process between ECM and the intracellular compartments [127]. The recognition of integrin by Arg-Gly-Asp (RGD) motif on fibronectin enables the cellular reorganization, such as cytoskeleton reformation and protein signaling that are important for cancer progression [128].

The role of fibronectin in cancer has been linked to many processes related to cell survival, such as cell proliferation, migration, invasion, and angiogenesis. For instance, upregulation of SOX2

in ovarian cancer has been shown to increase migration and invasion through induction of fibronectin synthesis [129]. Also, co-localization of fibronectin and lysyl oxidase (LOX) enzyme was reported in the lung pre-metastatic niche prior to the recruitment of metastasis facilitating cells, CD11b⁺ myeloid [130]. A study in glioma showed that fibronectin organization initiated by CD93 is required for tumor angiogenesis [131]. In fact, fibronectin is well-established marker for epithelial-mesenchymal transition (EMT), which is a key process for tumor metastasis [132]. In addition to metastasis and contribution to tumor angiogenesis, fibronectin has been also shown to play role in chemotherapy resistance. Yu and his colleagues recently demonstrated p53-mediated apoptosis was suppressed by FN, making Glioma stem-like cells more chemoresistant to alkylating agents, such as carmustine [133]. Consistent with the literature, our data suggests that fibronectin is upregulated in chemotherapy resistant tumors and plays a crucial role in doxorubicin resistance. Therefore, FN1 might be a common driver gene to initiate cancer metastasis as well as to catalyze chemotherapy resistance in TNBCs. Indeed, both phenomena often appear in parallel and thus constitute the major clinical challenges of aggressive breast cancer.

Despite its strategic role in the tumor aggressiveness, targeting fibronectin might not be the best approach to overcome chemoresistance. One reason is that fibronectin is a very essential component of ECM regulating tissue biogenesis and integrity [134, 135], and its targeting may result in undesired side effects on normal cells. Second, fibronectin also possesses many isoforms expressed from a single gene [136], and it undergoes dynamic assembly from soluble plasma protein into insoluble fibril [126], making its targeting difficult and complicated. Therefore, we propose to target integrins, serving as fibronectin receptor to minimize the toxicity while sensitizing cancer cells to chemotherapy.

5.3. Tumor suppressor miR-326 as potential chemo-sensitizer in TNBCs

miR-326 deregulation has previously been reported in many different cancer types. It has been known to act as a tumor suppressor in cervical cancer by inhibiting ELK1 expression and decreasing cell survival, migration and invasion [137]. Similarly, miR-326 has been found to be downregulated in glioblastoma, whose overexpression resulted in the reduction of cell proliferation as well as colony formation capacity by targeting PI3K pathway [138]. While in lung cancer, miR-326 was suggested to suppress cell growth through CCND1 inhibition and halt cell migration and invasion through MMP suppression [139]. Furthermore, miR-326 was shown to regulate multidrug resistance-associated protein and confer sensitivity to VP-16 and doxorubicin in ER-positive breast cancer cells [140].

In line with the literature, our study demonstrates the tumor suppressor role of miR-326 in sensitizing TNBCs to doxorubicin both *in vitro* and *in vivo*, most likely by targeting ITGA5. Later, KEGG pathway enrichment analysis with the predicted targets of miR-326 revealed significant enrichment of the focal adhesion, PI3K and MAPK pathways. This bioinformatics analysis was then validated with *in vitro* Western blot experiments where we showed overexpression of miR-326 resulted in the decrease of pFAK and pSrc (focal adhesion), pAKT-T308 and pAKT-S473 (PI3K pathway) and pERK-T202/Y204 (MAPK pathway). Supporting this, miR-326 has also been reported to inhibit PI3K and MAPK in melanoma through targeting KRAS [141]. Therefore, miR-326 could be a potential candidate in overcoming chemotherapy resistance in TNBC through modulation of PI3K, MAPK and focal adhesion pathways.

5.4. ITGA5 and integrin associated signaling in the context of chemoresistance

Integrins are the major ECM receptors that consist of 18 α and 8 β integrin subunits, combining into 24 transmembrane heterodimer protein families [142]. The attachment of integrin extracellular domains to the ECM and the intracellular domain into the cytoskeleton provides the cell adhesion and plays role in homeostasis, tissue maintenance as well as tissue repair [143]. Particularly, ITGA5, which we identified in the chemoresistance network, encodes integrin alpha 5 protein together with integrin beta 1, forming the specific receptor for fibronectin, $\alpha 5\beta 1$ integrin. The presence of integrin alpha 5 in this heterodimer receptor ensures the high specificity in fibronectin recognition [108, 113]. Meanwhile, the intracellular domain of $\alpha 5\beta 1$ integrin is connected to adaptor proteins, bridging bidirectional “inside-out” as well as “outside-in” signaling. Inside-out signaling is often mediated by talin that induces the conformational change of $\alpha 5\beta 1$ integrin into its active form, favoring the binding to fibronectin. Whereas, outside-in signaling is initiated with the recruitment of focal adhesion to the cytoplasmic tail of $\alpha 5\beta 1$ integrin [127, 144]. Focal adhesion kinase (FAK) is phosphorylated following the engagement of fibronectin-integrin. In turn, phosphorylated FAK might localize to the nucleus to promote transcription or recruit other protein kinases, such as Src kinase [145, 146]. Activation of integrin through FAK-Src axis is also believed to induce PI3K and MAPK signaling and promote cancer survival [147]. This corroborates our findings that ITGA5 suppression with miR-326 or an shRNA reduces the phosphorylation of FAK (pFAK and pSrc), PI3K (pAKT-T308 and pAKT-S473), and MAPK (pERK-T202/Y204) signaling proteins.

Here, our study suggests that upregulation of ITGA5 in parallel to the overexpression of fibronectin leads to development of chemotherapy resistance, most likely through integrin and focal adhesion signaling activation. Moreover, we also showed that *in vitro* and *in vivo* suppression of ITGA5 using shRNAs sensitized TNBCs into doxorubicin, indicating the crucial role of ITGA5 in chemoresistance development. In general, integrins and their associated

pathways have been frequently linked to chemotherapy or drug resistance. For example, integrin beta 1 has been shown to protect breast cancer from paclitaxel and vincristine through activation of PI3K pathway [148]. The integrin beta 1 that can dimerize with other types of alpha subunit, such as integrin alpha 3 and 6 to recognize laminin, also mediates trastuzumab and lapatinib resistance in HER2+ breast cancer *via* induction of downstream signaling, especially FAK, PI3K and MAPK signaling [149]. Similarly, the integrin signaling axis of FAK, PI3K and MAPK pathways is reported to be responsible for resistance development of BRAF-mutated melanoma to PLX4720 drug [150].

Only few studies specifically linked ITGA5 to chemotherapy or drug resistance. For example, a study carried out in aggressive glioma indicated that ITGA5 blocked p53 pathway and decreased the responsiveness to temozolomide [151]. In contrast, ITGA5 is suggested to be potential metastasis biomarker in many cancers, including breast [152], ovarian [153], melanoma [154] and kidney cancer [155]. Similar to fibronectin, ITGA5 has been also shown to play an important role during EMT process, and its expression is regulated by EMT inducer, ZEB2 [156] whose targeting attenuated breast cancer stemness and metastasis [157]. Therefore, our study meets its novelty as we highlight ITGA5 function as a prominent marker for chemotherapy resistance in TNBCs and augment the evidence of its role in breast cancer aggressiveness. Finally, targeting ITGA5 using miR-326 or inhibitory RNA could be a viable approach to overcome chemotherapy resistance in TNBCs, and perhaps TNBC metastasis.

5.5. Hypoxia modulation in chemoresistance

Tumor needs a vascularization system for oxygen supply and its sustainable growth. However, as tumor cells grow rapidly and turns into a solid tumor mass, the core of the tumor becomes

less vasculated and often experiences oxygen deprivation, a condition called as tumor hypoxia [158]. Unlike healthy tissues, cancer cells might adapt better to hypoxia and evade apoptosis by undergoing genetic and metabolic changes. In fact, hypoxia contributes to the cancer aggressiveness and resistance development. Hypoxia is first observed in radiotherapy resistance since treatment using radiation takes advantage of radical oxygen to kill cancer cells, yet those in hypoxic area tend to be resistant to treatment [159]. Later, it is now understood that hypoxia also causes not only radiotherapy but also chemotherapy resistance by driving extracellular and intracellular change through main regulator of hypoxia, hypoxia-inducible factor (HIF) [160].

HIF is a heterodimeric transcription factor comprising of a β subunit and one of two α subunits: HIF-1 α and HIF-2 α . While β subunit is stably expressed in the cells, α subunit is degraded in the presence of oxygen. In contrast to HIF-2 α , HIF-1 α is expressed in almost all human tissues and plays general role for hypoxia-dependent functions, especially those related with tumorigenesis process [161]. The pro-survival role of HIF-1 α has been shown in many cancer types, including breast cancer [162], colorectal cancer [163] and gastric cancer [164]. HIF-1 α also exhibits variability or protein polymorphisms that might impact on the pathology risk of individual [165]. Interestingly, a study by Chiavarina et al. showed HIF-1 α as tumor suppressor in breast cancer by activating autophagy system and self-degradation [166]. On the other hand, HIF-1 α -activated autophagy also induces oncogenic activities, such as promoting survival in mesenchymal stem cells under radiotherapy [167], protecting lung cancer cells from silver nanoparticle-triggered apoptosis [168] and conferring cisplatin resistance to lung cancer by elevating the expression of BNIP3 and Beclin-1 [169]. Collectively, despite the dual role of HIF-1 α in mediating cell death/survival, which can be cancer type and experimental setting dependent, it is generally accepted that hypoxia and HIF-1 α lead cancers to survive and develop resistance [170].

In our study, HIF1A, a gene encoding HIF-1 α protein was identified in the chemoresistance network as the most prominent upstream regulator. Therefore, we included hypoxia and HIF in our chemotherapy resistance concept for doxorubicin and TNBC. According to GSEA analyses performed using RNA-Seq data of doxorubicin sensitive and resistant tumors, hypoxia-related gene sets were significantly enriched in our resistant samples although the mRNA expression of HIF1A was not changed in our doxorubicin resistant xenografts compared to sensitive ones. Later, our *in vitro* analysis with MDA-MB-231 cells showed that miR-326 level was decreased in hypoxic condition and increased upon HIF1A suppression. Considering HIF1 α 's role as a transcription factor and its negative regulation on miR-326 expression, we speculated that HIF-1 α represses miR-326 host gene transcriptionally, probably through promoter binding. According to our preliminary computational analysis on miR-326 host gene (ARRB1) promoter, we found 6 hypoxia response elements (HREs) where HIF-1 α potentially binds to and regulates the associated gene. Thus, it supports our hypothesis that HIF-1 α binds to ARRB1 promoter and inhibits co-expression of ARRB1 and miR-326.

HIF-1 α has been shown to regulate various miRNAs that can be used to create a miRNA signature of hypoxia [171]. Reciprocally, some miRNAs also regulate HIF-1 α expression [172], creating a miRNA-HIF1 α feedback loop interaction. Many hypoxia-inducible miRNAs harbor HRE sequence in their promoter and is directly regulated by HIF-1 α binding [171]. Interestingly, upregulated miRNAs in hypoxic condition such as miR-24, miR-27, and miR-107 were shown to be pro-survival and induce tumorigenesis [171, 173], indicating that the oncogenic role of hypoxia can be exerted through overexpression of these miRNAs. Likewise, chemoresistance might occur as hypoxia inhibit tumor suppressor miRNAs, like miR-326 in our TNBC case.

Chapter 6

Conclusion and Future Perspectives

In this thesis, I identified a mechanism of chemotherapy resistance in TNBC that involves ***HIF1A/miR-326/ITGA5-FN1 axis*** and presented preclinical data for overcoming chemotherapy resistance using miR-326. We started with the generation of *in vivo* acquired chemotherapy resistance model through continuous treatment with doxorubicin. The whole transcriptome sequencing analysis (mRNA and miRNA) was performed for doxorubicin sensitive and doxorubicin resistant tumors to identify differentially expressed mRNAs and miRNAs. Further, we established and refined a miRNA-mRNA interaction network using differentially expressed mRNAs and miRNAs regulating chemoresistance, where FN1 appears to be a hub or central protein. FN1 induces chemoresistance by activating integrin signaling through its binding to ITGA5. Tumor suppressor miR-326, downregulated by hypoxia *via* HIF-1 α , sensitizes FN1-induced chemoresistant TNBCs by inhibiting ITGA5.

FN1 is an ECM protein that acts both as physical support for cells and a ligand for activating many downstream signals *via* integrin receptor [127]. Our findings on FN1 upregulation in TNBC chemoresistance and its role in conferring resistance to chemotherapy can further be validated by overexpressing FN1 in TNBC cells and measure its potency on chemotherapy resistance. In addition, we can extend the experiment setting by adding other TNBC cell lines, such as MDA-MB-157 or HCC1143 cells to confirm that FN1-induced chemoresistance occurs in wide-range of TNBCs. Other chemotherapy agents can also be used to test whether FN1-induced chemoresistance is restricted to doxorubicin (anthracycline) or it is a general chemoresistance phenomenon encompassing other types of chemotherapy agents. Similarly, miR-326 can be combined with other chemotherapy agents of distinct classes to examine if

another type of chemoresistance might be modulated and reversed by miR-326. Furthermore, FN1 is a well-established mesenchymal marker during EMT process [132]. Therefore, the upregulation of FN1 in the chemoresistant TNBC tumors may also indicate that they have higher potential to proceed to metastasis compared to their wild type counterparts. As miR-326 could sensitize FN1-induced chemoresistant TNBC, it would be interesting to test miR-326 potential in limiting TNBC metastasis as well.

Mechanistically, we propose that downregulation of miR-326 occurs due to hypoxic tumor microenvironment and HIF-1 α activation. Supporting our data in this thesis, the direct regulation of miR-326 by HIF-1 α can be further validated by performing luciferase-based reporter assay. As we hypothesized that HIF-1 α might bind to the predicted HREs in the promoter of miR-326 host gene, it will be necessary to clone the promoter of ARBB1 host gene into promoterless luciferase vector construct and check the promoter activity under hypoxic condition or treatment with siRNA targeting HIF1A. Furthermore, we observed that chemosensitization using miR-326 involves targeting ITGA5 by miR-326. Similarly, suppressing ITGA5 with shRNAs also augmented the effect of doxorubicin on TNBC cells. In order to further confirm this, ITGA5 inhibitor such as ATN161 [174] can be used in combination with doxorubicin both *in vitro* and *in vivo* setup. Here, we expect to see results similar to what we observed in miR-326 overexpression and ITGA5 suppression using shRNAs.

In vivo experiments in this thesis were conducted using human TNBC cell lines that do not directly reflect the full spectrum of patient tumor heterogeneity in the clinic. Therefore, it will be critical to perform combination treatment of doxorubicin and miR-326 overexpression or ITGA5 inhibition using chemoresistant TNBC patient derived xenograft (PDX) models. These results will better represent the actual framework of chemotherapy resistance and provide

supporting preclinical data. Importantly, for therapeutic applications of targets we identified, namely miR-326 or its target ITGA5, it is important to design the drug delivery system for miR-326 mimics or siITGA5. As oligonucleotides are often prone to degradation in the body, lipid nanoparticle encapsulation can be good choice for carrier system. Importantly, Food and Drug Administration (FDA) recently approved RNAi-based liposomal delivered therapeutics for its immune-safe behavior [175], encouraging the future study to develop miRNA and siRNA-based cancer therapeutics. In summary, even though there are many advanced studies need to be done, the strategy to overcome chemoresistance using miR-326 can be a viable approach in future as a therapeutic approach.

Bibliography

1. Bray, F., et al., *Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries*. CA Cancer J Clin, 2018. **68**(6): p. 394-424.
2. Colditz, G.A., T.A. Sellers, and E. Trapido, *Epidemiology - identifying the causes and preventability of cancer?* Nat Rev Cancer, 2006. **6**(1): p. 75-83.
3. de Ronde, J.J., et al., *Concordance of clinical and molecular breast cancer subtyping in the context of preoperative chemotherapy response*. Breast Cancer Res Treat, 2010. **119**(1): p. 119-26.
4. Perou, C.M., et al., *Molecular portraits of human breast tumours*. Nature, 2000. **406**(6797): p. 747-52.
5. 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): p. 10869-74.
6. Nicholson, R.I. and S.R. Johnston, *Endocrine therapy--current benefits and limitations*. Breast Cancer Res Treat, 2005. **93 Suppl 1**: p. S3-10.
7. Press, M.F., et al., *Her-2/neu expression in node-negative breast cancer: direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease*. Cancer Res, 1993. **53**(20): p. 4960-70.
8. Ross, J.S., et al., *Targeted therapy in breast cancer: the HER-2/neu gene and protein*. Mol Cell Proteomics, 2004. **3**(4): p. 379-98.
9. Vargo-Gogola, T. and J.M. Rosen, *Modelling breast cancer: one size does not fit all*. Nat Rev Cancer, 2007. **7**(9): p. 659-72.
10. Abramson, V.G., et al., *Subtyping of triple-negative breast cancer: implications for therapy*. Cancer, 2015. **121**(1): p. 8-16.
11. Dent, R., et al., *Triple-negative breast cancer: clinical features and patterns of recurrence*. Clin Cancer Res, 2007. **13**(15 Pt 1): p. 4429-34.
12. Mayer, I.A., et al., *New strategies for triple-negative breast cancer--deciphering the heterogeneity*. Clin Cancer Res, 2014. **20**(4): p. 782-90.
13. Cancer Genome Atlas, N., *Comprehensive molecular portraits of human breast tumours*. Nature, 2012. **490**(7418): p. 61-70.
14. Lehmann, B.D., et al., *Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies*. J Clin Invest, 2011. **121**(7): p. 2750-67.
15. Hirshfield, K.M. and S. Ganesan, *Triple-negative breast cancer: molecular subtypes and targeted therapy*. Curr Opin Obstet Gynecol, 2014. **26**(1): p. 34-40.
16. Chabner, B.A. and T.G. Roberts, Jr., *Timeline: Chemotherapy and the war on cancer*. Nat Rev Cancer, 2005. **5**(1): p. 65-72.
17. Wani, M.C. and S.B. Horwitz, *Nature as a remarkable chemist: a personal story of the discovery and development of Taxol*. Anticancer Drugs, 2014. **25**(5): p. 482-7.
18. Sriram, D., et al., *Camptothecin and its analogues: a review on their chemotherapeutic potential*. Nat Prod Res, 2005. **19**(4): p. 393-412.
19. Arcamone, F., et al., *Adriamycin, 14-hydroxydaunomycin, a new antitumor antibiotic from S. peucetius var. caesius*. Biotechnol Bioeng, 1969. **11**(6): p. 1101-10.
20. Rosenberg, B., L. Vancamp, and T. Krigas, *Inhibition of Cell Division in Escherichia Coli by Electrolysis Products from a Platinum Electrode*. Nature, 1965. **205**: p. 698-9.
21. De Giorgi, U., et al., *High-dose chemotherapy for triple negative breast cancer*. Ann Oncol, 2007. **18**(1): p. 202-3.
22. Ramirez, L.Y., et al., *Potential chemotherapy side effects: what do oncologists tell parents?* Pediatr Blood Cancer, 2009. **52**(4): p. 497-502.
23. Luqmani, Y.A., *Mechanisms of drug resistance in cancer chemotherapy*. Med Princ Pract, 2005. **14 Suppl 1**: p. 35-48.
24. Ismail-Khan, R. and M.M. Bui, *A review of triple-negative breast cancer*. Cancer Control, 2010. **17**(3): p. 173-6.

25. Prowell, T.M. and R. Pazdur, *Pathological complete response and accelerated drug approval in early breast cancer*. N Engl J Med, 2012. **366**(26): p. 2438-41.
26. Liedtke, C., et al., *Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer*. J Clin Oncol, 2008. **26**(8): p. 1275-81.
27. Housman, G., et al., *Drug resistance in cancer: an overview*. Cancers (Basel), 2014. **6**(3): p. 1769-92.
28. Longley, D.B. and P.G. Johnston, *Molecular mechanisms of drug resistance*. J Pathol, 2005. **205**(2): p. 275-92.
29. Gilbert, L.A. and M.T. Hemann, *Chemotherapeutic resistance: surviving stressful situations*. Cancer Res, 2011. **71**(15): p. 5062-6.
30. Cree, I.A. and P. Charlton, *Molecular chess? Hallmarks of anti-cancer drug resistance*. BMC Cancer, 2017. **17**(1): p. 10.
31. Friedman, R., *Drug resistance missense mutations in cancer are subject to evolutionary constraints*. PLoS One, 2013. **8**(12): p. e82059.
32. Blagosklonny, M.V., *Oncogenic resistance to growth-limiting conditions*. Nat Rev Cancer, 2002. **2**(3): p. 221-5.
33. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer*. Cell, 2000. **100**(1): p. 57-70.
34. Egeblad, M., E.S. Nakasone, and Z. Werb, *Tumors as organs: complex tissues that interface with the entire organism*. Dev Cell, 2010. **18**(6): p. 884-901.
35. Bremnes, R.M., et al., *The role of tumor stroma in cancer progression and prognosis: emphasis on carcinoma-associated fibroblasts and non-small cell lung cancer*. J Thorac Oncol, 2011. **6**(1): p. 209-17.
36. Balkwill, F.R., M. Capasso, and T. Hagemann, *The tumor microenvironment at a glance*. J Cell Sci, 2012. **125**(Pt 23): p. 5591-6.
37. Lu, P., V.M. Weaver, and Z. Werb, *The extracellular matrix: a dynamic niche in cancer progression*. J Cell Biol, 2012. **196**(4): p. 395-406.
38. Schwartz, M.A., *Integrins and extracellular matrix in mechanotransduction*. Cold Spring Harb Perspect Biol, 2010. **2**(12): p. a005066.
39. Senthilane, D.A., et al., *The Role of Tumor Microenvironment in Chemoresistance: To Survive, Keep Your Enemies Closer*. Int J Mol Sci, 2017. **18**(7).
40. Naba, A., et al., *Extracellular matrix signatures of human mammary carcinoma identify novel metastasis promoters*. Elife, 2014. **3**: p. e01308.
41. Lee, S., et al., *Differentially expressed genes regulating the progression of ductal carcinoma in situ to invasive breast cancer*. Cancer Res, 2012. **72**(17): p. 4574-86.
42. Pouyssegur, J., F. Dayan, and N.M. Mazure, *Hypoxia signalling in cancer and approaches to enforce tumour regression*. Nature, 2006. **441**(7092): p. 437-43.
43. Hockel, M. and P. Vaupel, *Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects*. J Natl Cancer Inst, 2001. **93**(4): p. 266-76.
44. Semenza, G.L., *Targeting HIF-1 for cancer therapy*. Nat Rev Cancer, 2003. **3**(10): p. 721-32.
45. Graham, K. and E. Unger, *Overcoming tumor hypoxia as a barrier to radiotherapy, chemotherapy and immunotherapy in cancer treatment*. Int J Nanomedicine, 2018. **13**: p. 6049-6058.
46. Wouters, A., et al., *Review: implications of in vitro research on the effect of radiotherapy and chemotherapy under hypoxic conditions*. Oncologist, 2007. **12**(6): p. 690-712.
47. Barkan, D., et al., *Metastatic growth from dormant cells induced by a col-I-enriched fibrotic environment*. Cancer Res, 2010. **70**(14): p. 5706-16.
48. Zahir, N., et al., *Autocrine laminin-5 ligates alpha6beta4 integrin and activates RAC and NFkappaB to mediate anchorage-independent survival of mammary tumors*. J Cell Biol, 2003. **163**(6): p. 1397-407.
49. Weaver, V.M., et al., *beta4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium*. Cancer Cell, 2002. **2**(3): p. 205-16.
50. Pylayeva, Y., et al., *Ras- and PI3K-dependent breast tumorigenesis in mice and humans requires focal adhesion kinase signaling*. J Clin Invest, 2009. **119**(2): p. 252-66.

51. Huanwen, W., et al., *Intrinsic chemoresistance to gemcitabine is associated with constitutive and laminin-induced phosphorylation of FAK in pancreatic cancer cell lines*. Mol Cancer, 2009. **8**: p. 125.
52. Kozomara, A. and S. Griffiths-Jones, *miRBase: integrating microRNA annotation and deep-sequencing data*. Nucleic Acids Res, 2011. **39**(Database issue): p. D152-7.
53. Friedman, R.C., et al., *Most mammalian mRNAs are conserved targets of microRNAs*. Genome Res, 2009. **19**(1): p. 92-105.
54. Di Leva, G., M. Garofalo, and C.M. Croce, *MicroRNAs in cancer*. Annu Rev Pathol, 2014. **9**: p. 287-314.
55. Kim, V.N., J. Han, and M.C. Siomi, *Biogenesis of small RNAs in animals*. Nat Rev Mol Cell Biol, 2009. **10**(2): p. 126-39.
56. Mogilyansky, E. and I. Rigoutsos, *The miR-17/92 cluster: a comprehensive update on its genomics, genetics, functions and increasingly important and numerous roles in health and disease*. Cell Death Differ, 2013. **20**(12): p. 1603-14.
57. Yuan, X., et al., *Clustered microRNAs' coordination in regulating protein-protein interaction network*. BMC Syst Biol, 2009. **3**: p. 65.
58. Mehlich, D., F. Garbicz, and P.K. Wlodarski, *The emerging roles of the polycistronic miR-106b approximately 25 cluster in cancer - A comprehensive review*. Biomed Pharmacother, 2018. **107**: p. 1183-1195.
59. Lee, Y., et al., *MicroRNA genes are transcribed by RNA polymerase II*. EMBO J, 2004. **23**(20): p. 4051-60.
60. Lee, Y., et al., *The nuclear RNase III Drosha initiates microRNA processing*. Nature, 2003. **425**(6956): p. 415-9.
61. Lund, E., et al., *Nuclear export of microRNA precursors*. Science, 2004. **303**(5654): p. 95-8.
62. Zhang, H., et al., *Single processing center models for human Dicer and bacterial RNase III*. Cell, 2004. **118**(1): p. 57-68.
63. Cheloufi, S., et al., *A dicer-independent miRNA biogenesis pathway that requires Ago catalysis*. Nature, 2010. **465**(7298): p. 584-9.
64. Ameres, S.L. and P.D. Zamore, *Diversifying microRNA sequence and function*. Nat Rev Mol Cell Biol, 2013. **14**(8): p. 475-88.
65. Lujambio, A. and S.W. Lowe, *The microcosmos of cancer*. Nature, 2012. **482**(7385): p. 347-55.
66. Cai, Y., et al., *A brief review on the mechanisms of miRNA regulation*. Genomics Proteomics Bioinformatics, 2009. **7**(4): p. 147-54.
67. Seitz, H., *Redefining microRNA targets*. Curr Biol, 2009. **19**(10): p. 870-3.
68. Mutlu, M., et al., *miR-564 acts as a dual inhibitor of PI3K and MAPK signaling networks and inhibits proliferation and invasion in breast cancer*. Sci Rep, 2016. **6**: p. 32541.
69. 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): p. 4297-306.
70. Calin, G.A., 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): p. 15524-9.
71. Cimmino, A., et al., *miR-15 and miR-16 induce apoptosis by targeting BCL2*. Proc Natl Acad Sci U S A, 2005. **102**(39): p. 13944-9.
72. Peng, Y. and C.M. Croce, *The role of MicroRNAs in human cancer*. Signal Transduct Target Ther, 2016. **1**: p. 15004.
73. Raver-Shapira, N., et al., *Transcriptional activation of miR-34a contributes to p53-mediated apoptosis*. Mol Cell, 2007. **26**(5): p. 731-43.
74. Yamakuchi, M. and C.J. Lowenstein, *MiR-34, SIRT1 and p53: the feedback loop*. Cell Cycle, 2009. **8**(5): p. 712-5.
75. Hayashita, Y., et al., *A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation*. Cancer Res, 2005. **65**(21): p. 9628-32.
76. Walz, A.L., et al., *Recurrent DGCR8, DROSHA, and SIX homeodomain mutations in favorable histology Wilms tumors*. Cancer Cell, 2015. **27**(2): p. 286-97.

77. Rupaimoole, R. and F.J. Slack, *MicroRNA therapeutics: towards a new era for the management of cancer and other diseases*. Nat Rev Drug Discov, 2017. **16**(3): p. 203-222.
78. Reid, G., et al., *Restoring expression of miR-16: a novel approach to therapy for malignant pleural mesothelioma*. Ann Oncol, 2013. **24**(12): p. 3128-35.
79. van Zandwijk, N., et al., *Response to "An innovative mesothelioma treatment based on mir-16 mimic loaded EGFR targeted minicells (TargomiRs)"*. Transl Lung Cancer Res, 2018. **7**(Suppl 1): p. S60-S61.
80. Ma, L., et al., *Therapeutic silencing of miR-10b inhibits metastasis in a mouse mammary tumor model*. Nat Biotechnol, 2010. **28**(4): p. 341-7.
81. Gabriely, G., et al., *Human glioma growth is controlled by microRNA-10b*. Cancer Res, 2011. **71**(10): p. 3563-72.
82. Ravi Kumar, M., et al., *Nanoparticle-mediated gene delivery: state of the art*. Expert Opin Biol Ther, 2004. **4**(8): p. 1213-24.
83. Wiggins, J.F., et al., *Development of a lung cancer therapeutic based on the tumor suppressor microRNA-34*. Cancer Res, 2010. **70**(14): p. 5923-30.
84. Lam, J.K., et al., *siRNA Versus miRNA as Therapeutics for Gene Silencing*. Mol Ther Nucleic Acids, 2015. **4**: p. e252.
85. Laubenbacher, R., et al., *A systems biology view of cancer*. Biochim Biophys Acta, 2009. **1796**(2): p. 129-39.
86. Raza, U., et al., *The miR-644a/CTBP1/p53 axis suppresses drug resistance by simultaneous inhibition of cell survival and epithelial-mesenchymal transition in breast cancer*. Oncotarget, 2016. **7**(31): p. 49859-49877.
87. Karakosta, A., et al., *Genetic models of human cancer as a multistep process. Paradigm models of colorectal cancer, breast cancer, and chronic myelogenous and acute lymphoblastic leukaemia*. J Exp Clin Cancer Res, 2005. **24**(4): p. 505-14.
88. Huang, T., et al., *Noncoding RNAs in cancer and cancer stem cells*. Chin J Cancer, 2013. **32**(11): p. 582-93.
89. Hansen, A.R. and P.L. Bedard, *Clinical application of high-throughput genomic technologies for treatment selection in breast cancer*. Breast Cancer Res, 2013. **15**(5): p. R97.
90. Hornberg, J.J., et al., *Cancer: a Systems Biology disease*. Biosystems, 2006. **83**(2-3): p. 81-90.
91. Lee, S. and X. Jiang, *Modeling miRNA-mRNA interactions that cause phenotypic abnormality in breast cancer patients*. PLoS One, 2017. **12**(8): p. e0182666.
92. Uhlmann, S., et al., *Global microRNA level regulation of EGFR-driven cell-cycle protein network in breast cancer*. Mol Syst Biol, 2012. **8**: p. 570.
93. Dragomir, M., et al., *Using microRNA Networks to Understand Cancer*. Int J Mol Sci, 2018. **19**(7).
94. Harden, M.E., et al., *Modulation of microRNA-mRNA Target Pairs by Human Papillomavirus 16 Oncoproteins*. MBio, 2017. **8**(1).
95. Li, J., et al., *Network-based identification of microRNAs as potential pharmacogenomic biomarkers for anticancer drugs*. Oncotarget, 2016. **7**(29): p. 45584-45596.
96. 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): p. 542-59.
97. Jezequel, P., et al., *Gene-expression molecular subtyping of triple-negative breast cancer tumours: importance of immune response*. Breast Cancer Res, 2015. **17**: p. 43.
98. Dancik, G.M. and D. Theodorescu, *Robust prognostic gene expression signatures in bladder cancer and lung adenocarcinoma depend on cell cycle related genes*. PLoS One, 2014. **9**(1): p. e85249.
99. Györfy, B., et al., *An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients*. Breast Cancer Res Treat, 2010. **123**(3): p. 725-31.
100. Dweep, H. and N. Gretz, *miRWalk2.0: a comprehensive atlas of microRNA-target interactions*. Nat Methods, 2015. **12**(8): p. 697.
101. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources*. Nat Protoc, 2009. **4**(1): p. 44-57.

102. Enerly, E., et al., *miRNA-mRNA integrated analysis reveals roles for miRNAs in primary breast tumors*. PLoS One, 2011. **6**(2): p. e16915.
103. Shannon, P., et al., *Cytoscape: a software environment for integrated models of biomolecular interaction networks*. Genome Res, 2003. **13**(11): p. 2498-504.
104. Oskarsson, T., *Extracellular matrix components in breast cancer progression and metastasis*. Breast, 2013. **22 Suppl 2**: p. S66-72.
105. Pankov, R. and K.M. Yamada, *Fibronectin at a glance*. J Cell Sci, 2002. **115**(Pt 20): p. 3861-3.
106. Soldani, C. and A.I. Scovassi, *Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update*. Apoptosis, 2002. **7**(4): p. 321-8.
107. Chaitanya, G.V., A.J. Steven, and P.P. Babu, *PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration*. Cell Commun Signal, 2010. **8**: p. 31.
108. Schaffner, F., A.M. Ray, and M. Dontenwill, *Integrin alpha5beta1, the Fibronectin Receptor, as a Pertinent Therapeutic Target in Solid Tumors*. Cancers (Basel), 2013. **5**(1): p. 27-47.
109. Thompson, K.N., et al., *The combinatorial activation of the PI3K and Ras/MAPK pathways is sufficient for aggressive tumor formation, while individual pathway activation supports cell persistence*. Oncotarget, 2015. **6**(34): p. 35231-46.
110. Adeyinka, A., et al., *Activated mitogen-activated protein kinase expression during human breast tumorigenesis and breast cancer progression*. Clin Cancer Res, 2002. **8**(6): p. 1747-53.
111. Lee, J.W., et al., *Hypoxia-inducible factor (HIF-1)alpha: its protein stability and biological functions*. Exp Mol Med, 2004. **36**(1): p. 1-12.
112. Majmundar, A.J., W.J. Wong, and M.C. Simon, *Hypoxia-inducible factors and the response to hypoxic stress*. Mol Cell, 2010. **40**(2): p. 294-309.
113. Aota, S., M. Nomizu, and K.M. Yamada, *The short amino acid sequence Pro-His-Ser-Arg-Asn in human fibronectin enhances cell-adhesive function*. J Biol Chem, 1994. **269**(40): p. 24756-61.
114. Nam, J.M., et al., *Breast cancer cells in three-dimensional culture display an enhanced radioresponse after coordinate targeting of integrin alpha5beta1 and fibronectin*. Cancer Res, 2010. **70**(13): p. 5238-48.
115. Dean-Colomb, W. and F.J. Esteva, *Emerging agents in the treatment of anthracycline- and taxane-refractory metastatic breast cancer*. Semin Oncol, 2008. **35**(2 Suppl 2): p. S31-8; quiz S40.
116. Thomas, E.S., et al., *Ixabepilone plus capecitabine for metastatic breast cancer progressing after anthracycline and taxane treatment*. J Clin Oncol, 2007. **25**(33): p. 5210-7.
117. Symmans, W.F., et al., *Long-Term Prognostic Risk After Neoadjuvant Chemotherapy Associated With Residual Cancer Burden and Breast Cancer Subtype*. J Clin Oncol, 2017. **35**(10): p. 1049-1060.
118. Blum, J.L., et al., *Multicenter, Phase II study of capecitabine in taxane-pretreated metastatic breast carcinoma patients*. Cancer, 2001. **92**(7): p. 1759-68.
119. Wist, E.A., et al., *Oral capecitabine in anthracycline- and taxane-pretreated advanced/metastatic breast cancer*. Acta Oncol, 2004. **43**(2): p. 186-9.
120. Degardin, M., et al., *Vinorelbine (navelbine) as a salvage treatment for advanced breast cancer*. Ann Oncol, 1994. **5**(5): p. 423-6.
121. Seidman, A.D., *Monotherapy options in the management of metastatic breast cancer*. Semin Oncol, 2003. **30**(2 Suppl 3): p. 6-10.
122. *Atezolizumab Combo Approved for PD-L1-positive TNBC*. Cancer Discov, 2019. **9**(5): p. OF2.
123. Rai, A., et al., *Understanding cancer complexome using networks, spectral graph theory and multilayer framework*. Sci Rep, 2017. **7**: p. 41676.
124. Stumpf, M.P., C. Wiuf, and R.M. May, *Subnets of scale-free networks are not scale-free: sampling properties of networks*. Proc Natl Acad Sci U S A, 2005. **102**(12): p. 4221-4.
125. Peng, Q., et al., *Screening candidate microRNA-mRNA regulatory pairs for predicting the response to chemoradiotherapy in rectal cancer by a bioinformatics approach*. Sci Rep, 2017. **7**(1): p. 11312.
126. Singh, P., C. Carraher, and J.E. Schwarzbauer, *Assembly of fibronectin extracellular matrix*. Annu Rev Cell Dev Biol, 2010. **26**: p. 397-419.

127. Hamidi, H., M. Pietila, and J. Ivaska, *The complexity of integrins in cancer and new scopes for therapeutic targeting*. Br J Cancer, 2016. **115**(9): p. 1017-1023.
128. Nieberler, M., et al., *Exploring the Role of RGD-Recognizing Integrins in Cancer*. Cancers (Basel), 2017. **9**(9).
129. Lou, X., et al., *SOX2 targets fibronectin 1 to promote cell migration and invasion in ovarian cancer: new molecular leads for therapeutic intervention*. OMICS, 2013. **17**(10): p. 510-8.
130. Erler, J.T., et al., *Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche*. Cancer Cell, 2009. **15**(1): p. 35-44.
131. Lugano, R., et al., *CD93 promotes beta1 integrin activation and fibronectin fibrillogenesis during tumor angiogenesis*. J Clin Invest, 2018. **128**(8): p. 3280-3297.
132. Park, J. and J.E. Schwarzbauer, *Mammary epithelial cell interactions with fibronectin stimulate epithelial-mesenchymal transition*. Oncogene, 2014. **33**(13): p. 1649-57.
133. Yu, Q., et al., *Fibronectin Promotes the Malignancy of Glioma Stem-Like Cells Via Modulation of Cell Adhesion, Differentiation, Proliferation and Chemoresistance*. Front Mol Neurosci, 2018. **11**: p. 130.
134. Bonnans, C., J. Chou, and Z. Werb, *Remodelling the extracellular matrix in development and disease*. Nat Rev Mol Cell Biol, 2014. **15**(12): p. 786-801.
135. Rozario, T. and D.W. DeSimone, *The extracellular matrix in development and morphogenesis: a dynamic view*. Dev Biol, 2010. **341**(1): p. 126-40.
136. White, E.S. and A.F. Muro, *Fibronectin splice variants: understanding their multiple roles in health and disease using engineered mouse models*. IUBMB Life, 2011. **63**(7): p. 538-46.
137. Zhang, P., et al., *MicroRNA-326 suppresses the proliferation, migration and invasion of cervical cancer cells by targeting ELK1*. Oncol Lett, 2017. **13**(5): p. 2949-2956.
138. Nawaz, Z., et al., *PI3 kinase pathway regulated miRNome in glioblastoma: identification of miR-326 as a tumour suppressor miRNA*. Mol Cancer, 2016. **15**(1): p. 74.
139. Sun, C., et al., *Hsa-miR-326 targets CCND1 and inhibits non-small cell lung cancer development*. Oncotarget, 2016. **7**(7): p. 8341-59.
140. Liang, Z., et al., *Involvement of miR-326 in chemotherapy resistance of breast cancer through modulating expression of multidrug resistance-associated protein 1*. Biochem Pharmacol, 2010. **79**(6): p. 817-24.
141. Kang, K., et al., *MicroRNA-326 inhibits melanoma progression by targeting KRAS and suppressing the AKT and ERK signalling pathways*. Oncol Rep, 2018. **39**(1): p. 401-410.
142. Hamidi, H. and J. Ivaska, *Every step of the way: integrins in cancer progression and metastasis*. Nat Rev Cancer, 2018. **18**(9): p. 533-548.
143. Calderwood, D.A., S.J. Shattil, and M.H. Ginsberg, *Integrins and actin filaments: reciprocal regulation of cell adhesion and signaling*. J Biol Chem, 2000. **275**(30): p. 22607-10.
144. Gupton, S.L., et al., *Mena binds alpha5 integrin directly and modulates alpha5beta1 function*. J Cell Biol, 2012. **198**(4): p. 657-76.
145. Serrels, A., et al., *Nuclear FAK controls chemokine transcription, Tregs, and evasion of anti-tumor immunity*. Cell, 2015. **163**(1): p. 160-73.
146. Nader, G.P., E.J. Ezratty, and G.G. Gundersen, *FAK, talin and PIPKIgamma regulate endocytosed integrin activation to polarize focal adhesion assembly*. Nat Cell Biol, 2016. **18**(5): p. 491-503.
147. Ghatak, S., et al., *Periostin induces intracellular cross-talk between kinases and hyaluronan in atrioventricular valvulogenesis*. J Biol Chem, 2014. **289**(12): p. 8545-61.
148. Aoudjit, F. and K. Vuori, *Integrin signaling inhibits paclitaxel-induced apoptosis in breast cancer cells*. Oncogene, 2001. **20**(36): p. 4995-5004.
149. Lesniak, D., et al., *Beta1-integrin circumvents the antiproliferative effects of trastuzumab in human epidermal growth factor receptor-2-positive breast cancer*. Cancer Res, 2009. **69**(22): p. 8620-8.
150. Hirata, E., et al., *Intravital imaging reveals how BRAF inhibition generates drug-tolerant microenvironments with high integrin beta1/FAK signaling*. Cancer Cell, 2015. **27**(4): p. 574-88.

151. Janouskova, H., et al., *Integrin alpha5beta1 plays a critical role in resistance to temozolomide by interfering with the p53 pathway in high-grade glioma*. *Cancer Res*, 2012. **72**(14): p. 3463-70.
152. Ju, J.A., et al., *Hypoxia Selectively Enhances Integrin alpha5beta1 Receptor Expression in Breast Cancer to Promote Metastasis*. *Mol Cancer Res*, 2017. **15**(6): p. 723-734.
153. Sawada, K., et al., *Loss of E-cadherin promotes ovarian cancer metastasis via alpha 5-integrin, which is a therapeutic target*. *Cancer Res*, 2008. **68**(7): p. 2329-39.
154. Qian, F., et al., *Interaction between integrin alpha(5) and fibronectin is required for metastasis of B16F10 melanoma cells*. *Biochem Biophys Res Commun*, 2005. **333**(4): p. 1269-75.
155. Tani, N., et al., *Expression level of integrin alpha 5 on tumour cells affects the rate of metastasis to the kidney*. *Br J Cancer*, 2003. **88**(2): p. 327-33.
156. Nam, E.H., et al., *ZEB2 upregulates integrin alpha5 expression through cooperation with Sp1 to induce invasion during epithelial-mesenchymal transition of human cancer cells*. *Carcinogenesis*, 2012. **33**(3): p. 563-71.
157. Li, Y., et al., *In vivo beta-catenin attenuation by the integrin alpha5-targeting nano-delivery strategy suppresses triple negative breast cancer stemness and metastasis*. *Biomaterials*, 2019. **188**: p. 160-172.
158. Challapalli, A., L. Carroll, and E.O. Aboagye, *Molecular mechanisms of hypoxia in cancer*. *Clin Transl Imaging*, 2017. **5**(3): p. 225-253.
159. Harris, A.L., *Hypoxia--a key regulatory factor in tumour growth*. *Nat Rev Cancer*, 2002. **2**(1): p. 38-47.
160. Rohwer, N. and T. Cramer, *Hypoxia-mediated drug resistance: novel insights on the functional interaction of HIFs and cell death pathways*. *Drug Resist Updat*, 2011. **14**(3): p. 191-201.
161. Bertout, J.A., S.A. Patel, and M.C. Simon, *The impact of O2 availability on human cancer*. *Nat Rev Cancer*, 2008. **8**(12): p. 967-75.
162. Liu, Z.J., G.L. Semenza, and H.F. Zhang, *Hypoxia-inducible factor 1 and breast cancer metastasis*. *J Zhejiang Univ Sci B*, 2015. **16**(1): p. 32-43.
163. Zhang, W., et al., *HIF-1alpha Promotes Epithelial-Mesenchymal Transition and Metastasis through Direct Regulation of ZEB1 in Colorectal Cancer*. *PLoS One*, 2015. **10**(6): p. e0129603.
164. Chen, L., et al., *HIF-1 alpha overexpression correlates with poor overall survival and disease-free survival in gastric cancer patients post-gastrectomy*. *PLoS One*, 2014. **9**(3): p. e90678.
165. Li, Y., et al., *The association between the rs11549465 polymorphism in the hif-1alpha gene and cancer risk: a meta-analysis*. *Int J Clin Exp Med*, 2015. **8**(2): p. 1561-74.
166. Chiavarina, B., et al., *HIF1-alpha functions as a tumor promoter in cancer associated fibroblasts, and as a tumor suppressor in breast cancer cells: Autophagy drives compartment-specific oncogenesis*. *Cell Cycle*, 2010. **9**(17): p. 3534-51.
167. Lv, B., et al., *Hypoxia-inducible factor 1 alpha protects mesenchymal stem cells against oxygen-glucose deprivation-induced injury via autophagy induction and PI3K/AKT/mTOR signaling pathway*. *Am J Transl Res*, 2017. **9**(5): p. 2492-2499.
168. Jeong, J.K., et al., *Hypoxia-mediated autophagic flux inhibits silver nanoparticle-triggered apoptosis in human lung cancer cells*. *Sci Rep*, 2016. **6**: p. 21688.
169. Wu, H.M., et al., *Hypoxia-induced autophagy mediates cisplatin resistance in lung cancer cells*. *Sci Rep*, 2015. **5**: p. 12291.
170. Sullivan, R., et al., *Hypoxia-induced resistance to anticancer drugs is associated with decreased senescence and requires hypoxia-inducible factor-1 activity*. *Mol Cancer Ther*, 2008. **7**(7): p. 1961-73.
171. Kulshreshtha, R., et al., *A microRNA signature of hypoxia*. *Mol Cell Biol*, 2007. **27**(5): p. 1859-67.
172. Serocki, M., et al., *miRNAs regulate the HIF switch during hypoxia: a novel therapeutic target*. *Angiogenesis*, 2018. **21**(2): p. 183-202.
173. Ge, X., et al., *MicroRNA-421 regulated by HIF-1alpha promotes metastasis, inhibits apoptosis, and induces cisplatin resistance by targeting E-cadherin and caspase-3 in gastric cancer*. *Oncotarget*, 2016. **7**(17): p. 24466-82.

174. Stoeltzing, O., et al., *Inhibition of integrin alpha5beta1 function with a small peptide (ATN-161) plus continuous 5-FU infusion reduces colorectal liver metastases and improves survival in mice*. Int J Cancer, 2003. **104**(4): p. 496-503.
175. Mullard, A., *FDA approves landmark RNAi drug*. Nat Rev Drug Discov, 2018. **17**(9): p. 613.



Appendix

Appendix 1.

Survival analysis of TNBC patients using KM-plotter and GSE58812 datasets based on 69 differentially expressed mRNAs and 13 upstream regulators.

	Gene	RFS - KM-Plotter (n=132)		OS - GSE58812 (n=107)		MFS - GSE58812 (n=107)	
		HR	p-value	HR	p-value	HR	p-value
Upregulated mRNAs	ADAM19	0.790	0.460	1.340	0.289	1.540	0.141
	AREG	1.610	0.140	1.890	0.322	2.040	0.049
	ARID3A	1.150	0.660	1.120	0.105	1.280	0.211
	BRAT1	2.400	0.081	2.177	0.051	1.593	0.196
	CAPZA1	1.060	0.850	1.270	0.994	1.480	0.733
	CITED2	1.210	0.550	1.720	0.976	1.880	0.705
	COL1A2	2.260	0.012	1.420	0.499	1.130	0.834
	DCTN3	0.910	0.780	1.810	0.062	1.530	0.278
	DDIT4	1.510	0.200	2.630	0.018	3.420	0.002
	EDAR	0.660	0.200	1.820	0.143	1.350	0.480
	ELF5	0.590	0.100	0.890	0.673	0.890	0.652
	EMP1	2.370	0.009	2.490	0.019	3.440	0.048
	FBLIM1	1.520	0.410	1.560	0.268	2.070	0.063
	FLNC	1.660	0.120	1.890	0.118	1.850	0.121
	FN1	1.880	0.049	3.170	0.016	2.600	0.041
	HGF	0.660	0.180	1.610	0.669	1.820	0.243
	IRAK4	1.010	0.990	0.470	0.037	0.560	0.081
	ITGA5	3.100	0.001	3.090	0.001	3.430	0.0004
	LCP1	1.650	0.110	0.390	0.008	0.530	0.061
	LNK2	1.370	0.530	2.100	0.108	2.110	0.085
	MMP1	2.040	0.026	0.780	0.611	1.050	0.919
	MOAP1	1.100	0.750	1.600	0.353	1.370	0.573
	NR4A2	0.990	0.970	1.640	0.337	1.370	0.567
	NUP210	1.020	0.950	1.260	0.151	1.090	0.142
	PARK2	0.890	0.720	1.340	0.874	1.360	0.908
	PGK1	0.600	0.320	1.920	0.078	2.060	0.042
	PIK3CG	0.600	0.310	0.920	0.288	0.720	0.186
	PODXL	0.860	0.620	0.600	0.335	0.600	0.221
	PSMD5	1.290	0.420	0.850	0.495	1.120	0.701
	RIPK4	1.920	0.047	1.290	0.329	1.770	0.905
	RPS6KA2	2.840	0.002	1.840	0.814	1.590	0.678
	SERPINB5	1.250	0.490	0.970	0.901	1.260	0.561

	SLC18A2	1.030	0.920	1.250	0.591	1.090	0.854
	SLC43A3	1.200	0.560	1.130	0.851	1.280	0.578
	STRADA	1.370	0.320	2.330	0.025	1.710	0.176
	TFAP2C	0.650	0.190	0.770	0.399	0.920	0.992
	TGM2	0.970	0.930	2.530	0.024	1.830	0.069
	TPM4	0.930	0.890	1.350	0.903	1.200	0.543
	TRAF5	0.980	0.950	0.990	0.877	0.740	0.328
	ZNF20	0.860	0.630	1.194	0.635	1.242	0.043
	ZNF488	1.050	0.920	0.920	0.730	0.920	0.719
Downregulated mRNAs	AKT1S1	1.560	0.380	0.610	0.571	0.690	0.121
	APOBEC3D	0.350	0.040	2.300	0.014	1.880	0.041
	ATG4C	1.020	0.970	0.650	0.213	0.880	0.243
	BAHD1	1.210	0.560	2.190	0.053	1.900	0.081
	BCAN	0.590	0.100	0.710	0.832	0.720	0.000
	BRCA1	1.140	0.680	0.760	0.671	0.940	0.061
	CDC6	0.670	0.210	1.230	0.980	1.260	0.085
	CREB5	0.640	0.170	0.750	0.349	0.750	0.919
	E2F1	0.670	0.210	1.210	0.248	1.080	0.573
	GIN51	1.000	0.990	1.210	0.609	1.220	0.567
	HIST1H4F	0.930	0.810	0.950	0.780	0.610	0.142
	HIST1H4L	1.460	0.230	0.770	0.409	1.160	0.908
	KIT	0.900	0.750	0.690	0.328	0.920	0.042
	MARK2	0.840	0.580	0.740	0.586	0.720	0.186
	MCM10	0.980	0.940	0.760	0.453	0.830	0.221
	MMP10	1.010	0.990	1.340	0.508	1.550	0.701
	MYL1	0.480	0.022	1.230	0.666	1.410	0.905
	PLS1	1.890	0.049	1.560	0.124	1.760	0.678
	RAPGEF4	1.550	0.170	0.840	0.889	0.720	0.561
	SLC1A3	0.700	0.260	0.820	0.517	0.940	0.854
	TAP1	0.420	0.009	0.620	0.167	0.650	0.578
	TFPI2	1.000	0.990	0.580	0.020	0.440	0.176
	TIMELESS	1.920	0.043	1.250	0.299	1.240	0.992
	TNFRSF9	0.660	0.200	0.580	0.354	0.640	0.069
	TP63	0.600	0.110	0.640	0.185	0.660	0.543
	TRAF1	0.630	0.140	0.640	0.188	0.790	0.328
	TRAF2	0.780	0.410	0.580	0.918	0.690	0.053
	USP2	0.570	0.410	0.890	0.830	0.810	0.719
Upstream Regulators	CBFB	0.590	0.100	0.770	0.380	0.900	0.732
	EPAS1	2.010	0.030	1.640	0.956	2.530	0.817
	EREG	1.350	0.340	1.810	0.747	2.070	0.928
	ETS1	2.300	0.110	1.190	0.260	1.140	0.082
	HIF1A	1.510	0.190	2.170	0.025	1.380	0.076
	HTT	1.260	0.460	1.490	0.309	1.540	0.355
	IRF3	0.750	0.370	1.520	0.142	1.650	0.114
	IRF7	1.030	0.930	0.990	0.926	0.770	0.667

	NUPR1	1.190	0.570	1.270	0.722	1.260	0.843
	PTGS2	1.000	1.000	0.940	0.653	1.260	0.580
	SREBF1	3.430	0.023	2.580	0.006	1.940	0.040
	TAL1	1.020	0.950	1.610	0.948	1.170	0.242
	VEGF	2.560	0.004	2.910	0.044	2.950	0.017



Appendix 2.

List of mRNAs, miRNAs, and upstream regulators with the activity flow score in the chemoresistance network

Name	Activity Flow Score
FN1	3.1451654
COL1A2	1.0483886
ITGA5	1.0483886
TGM2	1.0483886
MYL1	0.52419436
APOBEC3D	0.52419436
PGK1	0.52419436
MMP1	3.22E-24
VEGF	1.86E-24
HIF1A	0
TFPI2	0
FBLIM1	0
SREBF1	0
miR-342-3p	0
STRADA	0
AREG	0
EPAS1	0
DDIT4	0
miR-326	0
ZNF20	0
miR-1291	0
miR-30b-3p	0

Appendix 3.

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