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Nashwa Kabil

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by

Nashwa Kabil MD.

APPROVED:

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Bulent Ozpolat M.D. Ph.D.
Advisory Professor

_____

Gabriel Lopez-Berestein M.D.

_____

George A. Calin M.D. Ph.D

_____

Zahid H. Siddik Ph.D.

_____

Prahlad Ram Ph.D.

APPROVED:

_____

Dean, The University of Texas M.D. Anderson Cancer Center
UT Health Graduate School of Biomedical Sciences at Houston

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A

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BY:

Nashwa Kabil M.D.

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miR-484 Functions as an Onco-miR in Triple Negative Breast Cancer

Nashwa Kabil M.D.

Supervisory Professor: Bulent Ozpolat M.D. Ph.D

Abstract:

Triple negative breast cancer (TNBC) is a highly aggressive subtype of breast cancer (BC), with a poor prognosis with currently used therapies, and thus represents an unmet therapeutic challenge. Lack of molecular targets (i.e. ER, PR, HER2) and significant genetic heterogeneity are the major reasons contributing to early relapse and high mortality rates. Numerous studies have indicated that microRNAs (miRs) have an important role in BC progression, invasion, angiogenesis, and metastasis. We analyzed miRNA expression profiles of BC patient data bases and identified that miR-484 is highly upregulated in all subtypes of BC patients, with the highest expression in TNBC patients. miR-484 was found to be associated with significantly shorter patient survival, while inhibition of miR-484 in TNBC cells led to significant reduction of cell proliferation, motility and invasion, and induced cell cycle arrest and apoptosis. Furthermore, we found that miR-484 is inversely correlated with levels of *HOXA5* in patients' tumors and demonstrated that miR-484 directly binds to the 3′-untranslated region (3′-UTR) of *HOXA5* mRNA to suppress its expression. Moreover, HOXA5 over-expression recapitulated the effects of miR-484 inhibition. *In vivo* therapeutic targeting of miR-484 by systemic administration of anti-miR-484 nanoparticles significantly induced HOXA5 expression and suppressed tumor growth and progression in orthotopic xenograft mouse models of TNBC. Thus, our findings provide new insights about the oncogenic role of miR-484 and suggest that miR-484 represents a novel therapeutic target in TNBC.

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List of Abbreviations:

3′-UTR	3'-untranslated region
AGO	Argonaute2
AMOS	Anti-micro RNAs
APAF-1	Apoptotic protease activating factor
BC	Breast cancer
BCL-2	B-cell lymphoma2
BL1	Basal-like-1
BL2	Basal-like-2
CDKS	Cyclin dependent kinases
CEA	Carcinoembryonic antigen
CLL	Chronic lymphocytic leukemia
CRC	Colorectal cancer
DCGR8	Digeorge critical region 8
DFS	Disease-free survival
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal-transition
ER	Estrogen receptor
FAK	Focal adhesion kinase
FGFR	Fibroblast growth factor receptor
FOXM1	Forkhead box protein M1
FOXO3A	Forkhead box-O3
HCV	Hepatitis C virus

HER2	Human epidermal growth factor receptor 2
HIF-1A	Hypoxia inducible factor1
HMGA2	High-mobility group AT-hook 2
HOXA5	Homeobox A5
IM	Immunomodulatory
LAR	Luminal and androgen receptor
LNAS	Locked nucleic acids
M	Mesenchymal
miRNAs	Micro RNAs
mRNAS	Messenger RNA
MSL	Mesenchymal stem–like
MTS	[3-4,5-dimethylthiazol-2-yl-5-3-carboxymethoxyphenyl-2-4-sulfophenyl-2Htetrazolium]
NSCLC	Non-small cell lung cancer
OS	Overall survival
PARP	Poly ADP ribose polymerase
PCR	Pathologic complete response
PD-1	Programmed death 1
PDCD4	Programmed cell death 4
PDGFR	Platelet-derived growth factor receptor
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
RCC	Renal cell carcinoma

RHOC	Ras homolog gene family member C
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RPPA	Reverse phase protein array
SNPS	Single nucleotide polymorphisms
SOCS1	Suppressor of cytokine signaling 1
STAT3	Signal transducer and activator of transcription
TCGA	The cancer genome atlas
TGF-β	Transforming growth factor beta
TNBC	Triple negative breast cancer
TPM1	Tropomyosin1
TSP-2	Thrombospondin-2
TWIST1	Twist related protein1
XPO5	Exportin 5
ZEB1	Zinc finger E-box-binding homeobox1

CHAPTER I:
INTRODUCTION

Breast Cancer Statistics

It is estimated that about 1 out of 8 women in the U.S. (about 12.4%) will develop invasive breast cancer (BC) throughout their lifetime. Over a quarter of a million new cases of invasive BC are expected to be diagnosed by the end of 2018 in women in the U.S, with an estimated 41,000 deaths (1). Worldwide, BC still remains a global burden, with the latest reported statistics by the global cancer project (GLOBOCAN 2012), estimating more than 1.5 million newly diagnosed cases of BC, and over 500,000 deaths. Despite the advancement in BC management, it still remains the most common cancer in women, accounting for more than a quarter of all cancer cases (2). The incidence of BC is higher in developed countries (western world), while relative mortality is greater in developing countries (2). This discrepancy can be largely attributed to differences in socio-economic status, availability of early screening and detection programs, and access for treatment (2).

The Heterogeneity of Breast Cancer

BC is a highly heterogenous disease, composed of multiple subtypes, with each subtype displaying specific morphological features, which can account for differences in tumor behaviors, as well as therapeutic response to treatment (3). Historically, BC has been classified according to the expression of three molecular markers: estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2) (4). These molecules also serve as druggable targets for specific therapies (5). BC tumors that lack ER, PR, and HER-2 receptors are classified as triple-negative breast cancer (TNBC); a highly aggressive and metastatic subtype of BC, with poor responses to targeted therapies (6, 7). Subsequent studies utilizing gene expression-profiling

revealed that BC is highly heterogeneous and subtyping was expanded beyond the original ER/PR/HER2 classification (4, 8, 9). Other clinico-pathological variables such as, tumor size, tumor grade, and lymph node status, are also used to predict patient prognosis and management (10, 11).

Gene expression profiling and intrinsic molecular subtypes

In the era of the human genome project, the emergence of microarrays, and other gene expression profiling platforms has led to the development of an intrinsic subtyping system using multiple genes in order to classify BC (4, 8). Perou and colleagues conducted the first study that classified the molecular subtypes of BC into the following 4 subtypes: estrogen receptor positive (ER+)/luminal-like, basal-like, receptor tyrosine kinase positive (HER2/neu+), and normal breast (4). According to Perou and colleagues, most of the triple-negative breast cancers (TNBCs) were included in the basal-like subtype (4, 12). Subsequently, Sorlie and colleagues identified five major molecular types, that included luminal A, luminal B, HER2 over-expression, basal, and normal-like tumors (4, 8, 9). Each of these subtypes harbors specific histopatholoical features that can affect clinical progression and treatment outcome (13-18). Other gene signatures were introduced later, including the PAM50 classification which depends on the expression of hormone receptors, in addition to proliferation related genes, and genes exhibiting myoepithelial and basal features (19-21). These markers were found to be clinically significant as having prognostic value and help in predicting therapeutic outcome (22).

Luminal A tumors (ER+/PR+) are frequently low-grade tumors and respond well to ER targeted therapies such as tamoxifen and aromatase inhibitors (17, 23). Luminal B

tumors (ER+ with or without HER2+ and Ki67 overexpression) tend to be more aggressive subtype that may respond to hormonal therapies, but are also frequently associated with recurrence and poorer response (22, 24, 25). HER2+ tumors (ERBB2/HER2 amplified) while they are regarded as an aggressive subtype, are sensitive to anti-HER2 therapies such as monoclonal antibodies (eg. trastuzumab and pertuzumab) or the small-molecule kinase inhibitor lapatinib (25, 26).

Basal tumors

Basal tumors do not express ER, PR, or HER2R and display expression profiles similar to basal epithelial cells, as wells as normal breast myoepithelial cells (4). They also have high expression of basal markers such as keratins 5, 6, 14, 17, and epidermal growth factor receptor (EGFR), and proliferation related genes (4, 27). These tumors are more frequently associated with low BRCA1 expression (28) and TP53 mutations (8, 29). Basal tumors, which account for 60% to 90% of triple negative tumors (13, 30), tend to follow an aggressive clinical course, with more likelihood to metastasize to distant organs, with the exception to bone, and lymph nodes (31). Given their lack of expression of hormonal receptors, basal tumors are not sensitive to anti-hormonal targeted therapeutics, leaving conventional chemotherapies as their only therapeutic option (32).

Triple Negative Breast Cancer

Characteristics & Risk Factors

Approximately 15% to 20% of all diagnosed BC cases are TNBC. These tumors share considerable molecular similarities with basal-like cancers (up to 70% overlap). However, TNBC and basal subtypes are histo-pathologically and clinically distinct, and thus these two subtypes are mutually exclusive (33, 34). TNBCs more prevalent in African-American or Hispanic women of younger age (<40 years) (4, 6, 35). Other risk factors for TNBC include multiple and early pregnancies, as well as lack of breast feeding (36, 37). At stage of presentation, TNBCs are mainly poorly differentiated invasive ductal carcinoma with a tendency to metastasize to the lung and brain (38, 39). However, unlike other BC subtypes, the correlation between TNBC tumor size and lymph node status is not clearly defined (40-42). TNBCs have the worse prognosis compared with other BC subtypes, with an estimated 5 year survival rate of 70% (3). This survival rate is much lower for patients with advanced metastasis (~12 month survival) (43, 44).

Molecular Heterogeneity of TNBC

TNBCs are a highly heterogeneous subtype of BC that is composed of 6 molecular subtypes according to the study by Lehman and colleagues. Each subtype displays distinct oncogenic drivers that can thus be utilized as potential molecular targets. These subtypes include: basal-like (BL1 and BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem–like (MSL), and luminal androgen receptor (LAR). In their study, they also identified TNBC cell line models representing each subtype to be utilized in targeting specific oncogenic pathways identified in the gene expression analysis. These subclasses were found to display distinct therapeutic responses that correlate with

pathologic complete response (pCR) rates following neoadjuvant chemotherapy (NAC) (45). For example, BL1 and BL2 subtypes displayed higher expression of cell cycle and DNA damage proteins, and representative cell lines showed a favorable response to taxane-based therapies. BL1 tumors show the most favorable pCR rates (52%) compared to other subtypes after NAC, whereas BL2 patients display the lowest pCR (46). M and MSL subtypes showed higher expression of epithelial-mesenchymal markers, and growth factor pathways which responded well to a PI3K/mTOR inhibitors and an Abl/Src inhibitors (7). Patients with MSL subtype displayed upregulation of transforming growth factor receptor III (TGFβ-III), a known driver of migration and invasion (47), and showed moderate pCR rates (between 20-30%) (46). The LAR subtype includes patients characterized by androgen receptor (AR) signaling, with frequent display of positive PI3KCA activating mutations (48). LAR subtype patients are less responsive to chemotherapy, with a pCR rate of around 10% (46), but display favorable response to anti-androgen treatments in combination with PI3K inhibitors (48). Finally, the IM subclass display higher expression of immune response signaling proteins, and have a moderate pCR of around 30% (46).

Current Therapeutic Strategies for TNBC

Currently, there are no approved targeted therapeutics available for TNBC, although there are a several drugs in pre-clinical and clinical trials that are being investigated (49, 50). Taxane based therapies (eg. docetaxel or paclitaxel), anthracyclines (eg. doxorubicin or epirubicin) and alkylating agents (eg. Cyclophosphamide) are still considered the gold standard of therapy for TNBC (49). Given that approximately 15-20% of TNBCs harbor BRCA 1/2 mutations (51), platinum based therapies that affect the DNA repair mechanism have been proven to be effective in TNBC patients (50, 52). Also other therapeutic options include poly ADP ribose polymerase inhibitors (PARP inhibitors), Src family kinase inhibitors, EGFR inhibitors, as well as anti-androgens (50). A small percentage of TNBC patients, particularly mesenchymal and luminal androgen receptor (LAR) subtypes have also been shown to benefit from PI3K/AKT/mTOR inhibitors (50). Another emerging concept in TNBC management is the use of immune checkpoint inhibitors, targeting either the programmed death (PD)-1 receptor or its ligand PD-L1, in combination with either cytotoxic chemotherapy or radiotherapy (53, 54).

Drug Resistance in TNBC

TNBC management remains an extensive clinical challenge due to its aggressive course and poor therapeutic outcome (6, 35, 55, 56), compared with other BC subtypes (57-59). Substantial tumor heterogeneity is one of the major reasons for the development of drug resistance, resulting in the selective survival of residual tumor cells that can repopulate the tumor and result in relapse (60). Other evidence suggests, that some cytotoxic agents can promote epithelial-mesenchymal-transition (EMT) and or enrich the tumor initiating cell population to promote metastasis (61).

Due to the lack of effective targeted therapeutics, new interest has emerged in identifying new molecular targets and development of therapeutic strategies against them in order to improve TNBC patient survival and prognosis. In recent years, numerous publications have highlighted the critical role of miRNAs in cancer (62). Extensive research over the years has shown that micro RNAs (miRNAs) are implicated in all stages of BC (63, 64), which has rendered them as valuable diagnostic and prognostic markers (65). Recently, there has been a growing interest in the use of miRNA based therapeutic strategies in BC (66).

Role of miRNA in the Pathogenesis of Breast Cancer

The initial discovery of miRNAs was in early 1990s by Ambros and Lee that found that short non-coding region of lin-4 negatively regulates the expression of lin-14 during larval development of C. elegans (67). Later that year, lin-4 was shown to bind to the lin-14 3' untranslated region (3'-UTR) that harbors multiple conserved sequences complementary to lin-4 (68). As of 2001, these short non-coding RNAs were classified as a new set of genes called micro RNAs (miRNAs) (69-71).

miRNA Biogenesis & Mechanism of Action

miRNA genes reside in either intergenic, or intragenic (intronic or in exonic) regions within the genome. They can be transcribed as a single transcript from its own promoter or several miRNAs can share a promoter and be transcribed as a long polycistronic primary transcript (72-74).

miRNA are primarily transcribed by RNA polymerase II into a long primary transcript called pri-miRNA which can have a nucleotide length up to 1kb. This pri-miRNA is 5'capped and 3' poly-adenylated (72, 75), and then converted into a hair pin structure around 70-80 nct. called pre-miRNA by ribonucleases III enzyme DROSHA and RNA-binding protein Digeorge Critical Region 8 (DGCR8), also known as Pasha (75). Subsequently this pre-miRNA is then transported form the nucleus into the cytoplasm by Exportin 5 (XPO5), to undergo further processing by DICER (RNAse III endonuclease enzyme) into a double stranded miRNA, which is around 18-25 nct in length (76). This double stranded structure is then unwound and single strands, composed of a guide strand and a passenger strand, are then loaded on to the RNA-Induced Silencing Complex (RISC) to its target mRNA (77) (Figure 1).

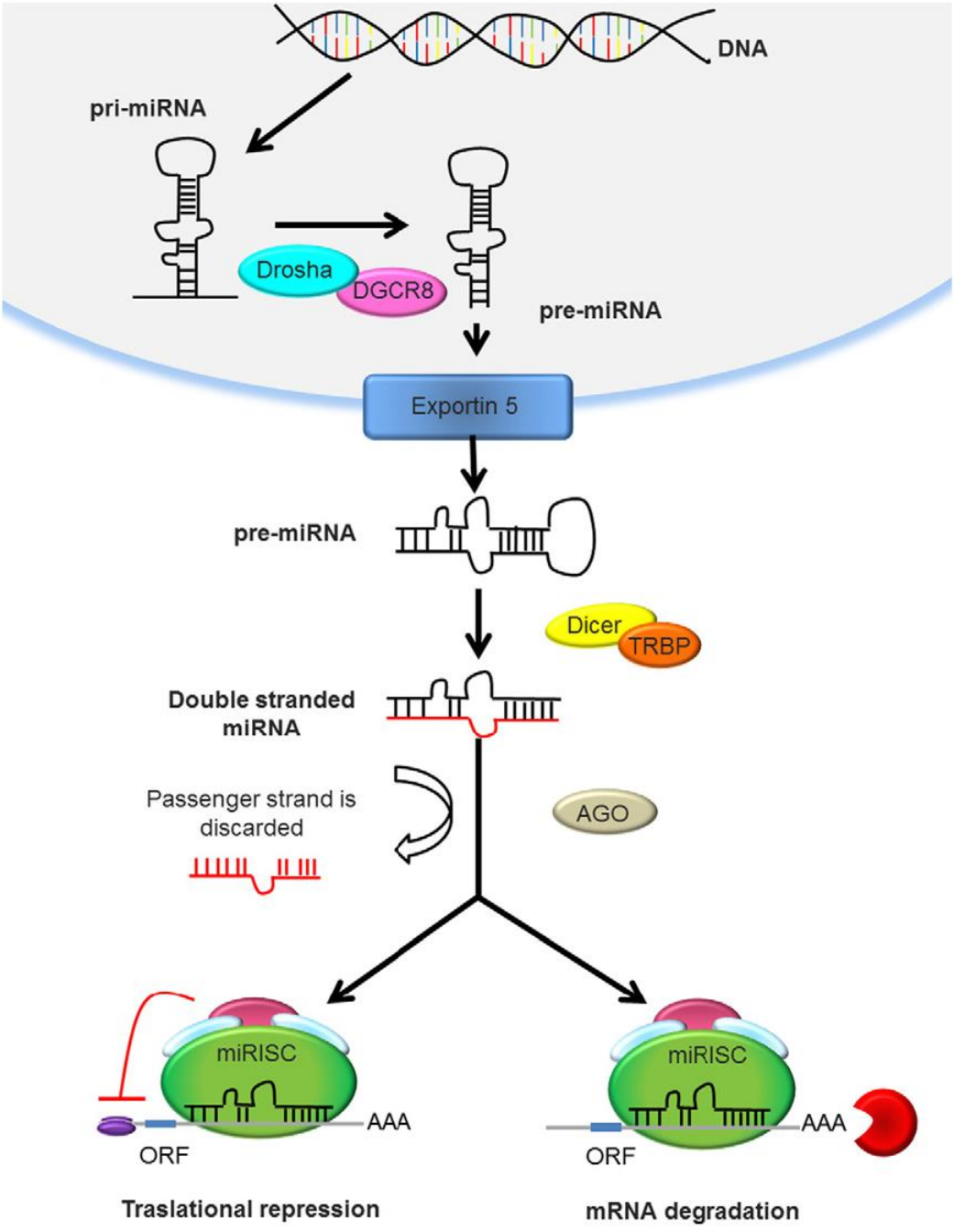

Figure 1: Gene silencing mechanisms of miRNAs

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miRNAs mainly act by regulating gene expression at the post-transcriptional level depending on the degree of sequence complementarity between the miRNA and its target mRNA. They can either lead to mRNA degradation, in the case of perfect complementarity; or translational inhibition, in the case of imperfect complementarity (78). The binding of miRNAs and their target mRNAs mainly occurs by interaction between the 3'-UTR of the mRNA with the miRNA seed sequence (~6–8 nt), which is located near their 5' end, and was found to be highly conserved (79). The 3'-UTR of a single mRNA can bind to multiple miRNAs and any single miRNA can bind to hundreds of targets. Thus, miRNAs have the ability to regulate many signaling pathways simultaneously (80, 81). The binding between miRNAs and their target mRNAs can be computationally predicted using a number of highly accurate predictive algorithms, which can then be experimentally verified (82, 83). Currently, there over 2600 mature human miRNAs according to the miRbase database humans [http://www.mirbase.org/].

Although miRNAs mainly act by binding to the 3'-UTR of their target mRNAs, several other mechanisms have also been proposed as means of their actions. For example, miRNAs can by bind to the 5'-UTR regions to increase mRNA translation (84, 85), as in the case of miR-10b, which was shown to bind the 5'-UTR of ribosomal protein mRNA and increase their translation (85). Other miRNAs, such as let-7 and miR-363, were found to increase mRNA expression by recruitment of specific micro-RNPs (eg. Argonaute 2 (AGO) and fragile X mental retardation-related protein 1 (FXR1), to the AU rich regions in the 3'-UTR of their target mRNA (84). Some studies have also suggested that miRNAs can be translocated to the nucleus to activate the promotor region of their target genes and increase transcription (86). For instance, miR-551b-3p was found to recruit RNA

polymerase II and the transcription factor Twist related protein 1 (TWIST1) to the signal transducer and activator of transcription (STAT3) promoter region, to activate its transcription (86). Additionally, some miRNAs can localize to different subcellular compartments, as in the case of miR-29b, which has a specific hexanucleotide terminal sequence that directs its translocation to the nucleus (87). Also a few miRNAs can bind to RNA-binding proteins and thus inhibit their binding with their target (88). Thus, the mechanism of miRNA-mediated regulation of gene expression is a mutli-facted subject that requires further exploration.

Role of miRNA in Cancer

The role of miRNA in cancer was first described in 2002, where it was found that the chromosomal region 13q14, which is frequently deleted in chronic lymphocytic leukemia (CLL) patients (89, 90), harbors a chromosomal translocation at t(2:13) at a fragile site resulting in the deletion of the miR-15a/16-1 cluster (91), suggesting their potential role as tumor suppressors. The following year in a follow up study, miRNAs were mapped in chromosomal fragile sites, regions of loss of heterozygosity, or regions of amplifications (92). Furthermore, in 2005, another study reported that the miR 17~92 cluster, induced by c-MYC, enhances lymphoma in mouse models of B-CLL, suggesting its possible role as an oncogenic miRNA (93). These discoveries paved the way for a new era of biomedical research in deciphering the role of miRNAs in tumorigenesis, resulting in more than 30,000 publications recorded on PUB MED to date.

Mechanisms Altering miRNA Expression in Cancer

The aberrant expression of miRNAs in cancer can be attributed to many factors discussed below (62).

Genetic Regulation: miRNAs can reside within the chromosomal regions that are proximal to fragile sites, or in regions of loss of heterozygosity, deletions, amplifications, or translocations. Chromosomal regions that harbor miRNAs involved in negatively regulating known tumor suppressors (oncogenic miRNAs), may be amplified, resulting in increased expression of these oncogenic miRNAs and subsequent reduction in the expression of their tumor suppressor genes (94). On the other hand, miRNAs that inhibit oncogenes (tumor suppressor miRNAs) are located at chromosomal fragile sites, where deletion or mutations can decrease their levels, resulting in overexpression of their target oncogenes (94). Such is the case for miR15a/16-1, which were found to be deleted/translocated in the majority of CLL patients (91), and were later revealed to target the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) in CLL (95).

Epigenetic Regulation: The presence of DNA-binding factors can affect the promoter regions of miRNA genes. This can result in the downregulation of miRNA genes by hyper methylation or histone deacetylation of the promoter regions (96). For example, the miR-9-1 gene in BC is downregulated due to hyer-methylation of its promoter regions (97). Other means of epigenetic regulation include histone deacetylation and tri-methylation, as in the case of miR-29 in B-cell lymphoma (98). On the other hand, histone acetylation can lead to the activation of miRNA genes, such as that of miR-224 in hepatocellular carcinoma (99). Other miRNAs can be activated by transcription factors acting at their

promoter region. For example, the tumor suppressor p53 was found to bind and activate the promoter regions of the miR-34a (100).

Regulation of miRNA Biogenesis/Processing: miRNAs expression levels can also be regulated by factors that affect their biogenesis or processing at multiple levels (101). For example, miRNA biogenesis proteins such as DROSHA, DICER, DGCR8, TRBP, XPO5 and AGO can be affected by genetic mutations, post-translational modifications, or binding to regulatory proteins, which can ultimately affect miRNA expression levels (102). Additionally, single nucleotide polymorphisms (SNPs) in a miRNA gene may alter miRNA processing efficiency by changing its stem–loop structure (101).

Given that one miRNA can have up to several hundred mRNA targets, aberrantly expressed miRNAs in cancer may affect multiple transcripts and hence significantly impact numerous cancer signaling pathways (103). For example, factors that lead to increased expression of miRNAs that are frequently over-expressed in cancer would lead to enhanced silencing of tumor suppressor genes. Consequently, this may promote tumor formation by increasing cell proliferation, invasiveness, angiogenesis, or suppressing apoptosis. On the other hand, under expression of tumor suppressor miRNAs in cancers could also promote tumorigenesis through upregulation of their oncogenic target mRNAs (104) (Figure 2).

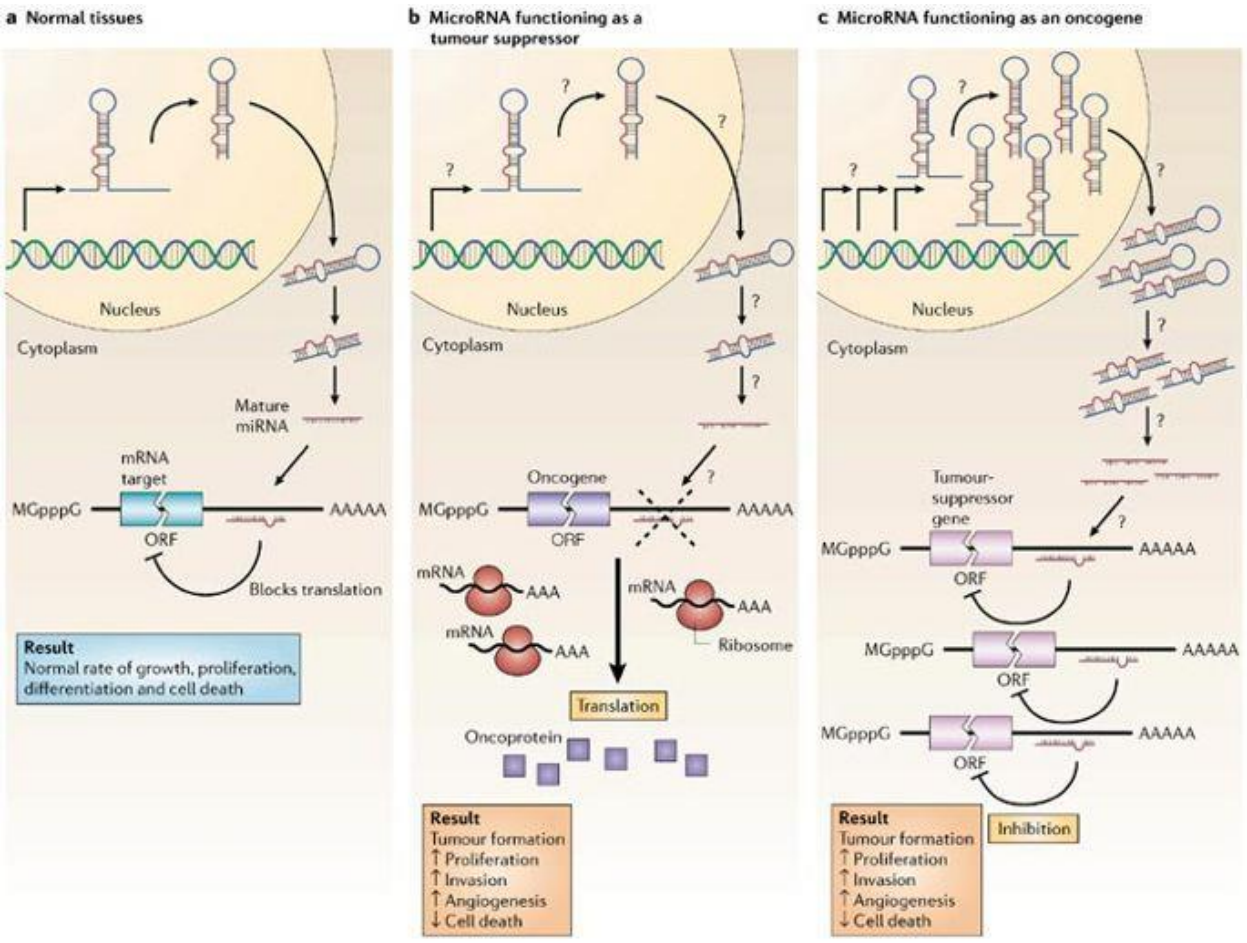

Figure 2: miRNAs can function as tumor suppressors or oncogenes

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MicroRNAs in Breast Cancer

Several platforms have been developed to profile the global expression of miRNAs in normal or diseased tissues. In the context of cancer, these profiling studies have been used to aid in tumor classification and the assessment of diagnosis and prognosis (62). Iorio and colleagues, in 2005, were the first to describe a specific miRNA signature pattern that were differentially expressed in normal vs. BC tissue, and was correlated with tumor grade, disease stage, vascular invasion, proliferation index, and hormone receptor expression (105). Subsequently, several studies revealed that aberrantly expressed miRNAs are able to regulate many process in breast carcinogenesis, thereby acting as either oncogenic or tumor suppressor miRNAs (106). In BC, miRNAs have been shown to regulate many processes such as cell cycle progression, apoptosis, angiogenesis, epithelial-mesenchymal transition, metastasis, and drug resistance (64).

Oncogenic miRNAs in Breast Cancer

Micro RNAs have been demonstrated to be key modulators in controlling the primary tumor growth, as well as in promoting the metastatic process, and modulating the interaction of the tumor with its microenvironment (107, 108). Some of the well described examples of oncogenic miRNAs in BC are discussed below and are listed in Table 1.

miR-21:

Among the differentially expressed miRNAs that were shown to be upregulated in BC patients and speculated to be oncogenic was miR-21, which was later one of the most extensively researched miRNAs with oncogenic properties. Some of its oncogenic properties to promote BC cell survival and proliferation may be attributed to targeting tumor suppressors such as phosphatase and tensin homolog (PTEN) (109), programmed cell death 4 (PDCD4) (110) and tropomyosin 1 (TPM1) (111). Additionally, the clinical significance of miR-21 in BC was demonstrated by studies that found it to be associated with advanced clinical staging, lymph node status, and worse prognosis in BC patients (112, 113).

miR-10b:

miR-10b was shown to be an oncogenic driver of BC, by promoting migration and invasion in metastatic BC cells. Moreover, it was also shown to initiate invasion and metastasis in non-metastatic breast cells. miR-10b expression is enhanced by the transcription factor TWIST1, which binds to its promoter region. miR-10b acts by binding and inhibiting the expression of HOXD10, which then enhances the expression of the pro-metastatic gene, Ras homolog gene family member C (RHOC) protein (114). miR-10b was also shown to be a miRNA of clinical significance as it was found to be positively correlated with BC staging, histological grading, and lymph node metastasis (115). Additionally, miR-10b was shown to target E-cadherin in metastatic BC cells to promote cell invasion (116).

miR-155:

miR-155 is another miRNA that was found to be frequently up-regulated in breast tumor tissue and was found to be associated with clinicopathologic markers, BC subtype, and poor survival rates (105, 117, 118) . miR-155 was found to act via targeting and downregulating the expression forkhead box O3 (*FOXO3a*) to enhance tumor cell sensitivity to chemotherapy and mediate apoptosis (119). Other studies suggested that miR-155 promotes BC oncogenesis by targeting suppressor of cytokine signaling 1 (SOCS1), leading to the activation of the JAK/STAT3 pathway. In that study, miR-155 expression was found to be induced by inflammatory cytokines such as IL-6 and INF-γ, suggesting its possible relationship to inflammation in cancer (120). Additionally, miR-155 was shown to target *caspase-3* in activated macrophages to promote their survival in the inflammatory response (121).

Table1: Oncogenic miRNAs in Breast Cancer

Oncogenic miRNA	Target	Cancer Related Events	References
miR-21	TPM1, PDCD4, TIMP3, PTEN	Cell proliferation, Apoptosis, Invasion	(110, 111, 122, 123)
miR-155	FOXO3a, SOCS1, caspase-3, TP53INP1	Cell proliferation, Apoptosis, Cell cycle progression	(119-121, 124)
miR-10-b	HOXD10, Tiam1	Invasion, Migration, Metastasis	(125, 126)
miR-9	E-cadherin	Cell motility, Invasion, Angiogenesis, Metastasis	(127)
miR-27a	HOXO1, ZBTB10	Cell proliferation, Cell cycle progression, Angiogenesis, Metastasis	(128-130)
miR-181a	Bim	EMT, Migration, Invasion, Metastasis	(131)
miR-182	RECK, MIM, FOXO1	Cell proliferation, Invasion	(129, 132)
miR-221/222	TRPS1, ADIPOR1, p27Kip1	EMT	(133-135)
miR-373/520c	CD44	Migration, Invasion, Metastasis	(136)

Tumor Suppressor miRNAs in Breast Cancer

Let-7 family:

One of the well characterized examples of tumors suppressor miRNAs in BC is the let-7 family. Several groups have reported that the let-7 family are differentially expressed in BC, as well as in other tumors (137-139). Let-7, tumor suppressor microRNA was originally discovered in C. elegans, where it was found to regulate cell differentiation and cell cycle (140). This family has been shown to act as tumor suppressor miRNAs by targeting critical oncogenes such as RAS, high-mobility group AT-hook 2 (HMGA2), c-Myc, and caspase-3 (141-144), as well as several genes involved in stem cell maintenance (145).

miR-34 family:

Another well characterized tumor suppressor miRNA family in BC is the miR-34 family. The miR-34 family is composed of 3 members: miR-34a, which is encoded by its own gene from chromosome 1p36, and miR-34b/c which are co-transcribed from a shared locus on chromosome 11q23 (146). miR-34a is the most extensively studied member in cancer and was found to inhibit many different oncogenic processes relating to tumor cell differentiation, proliferation, migration, and invasion by targeting BCL-2 and SIRIT1 (147, 148); and induce apoptosis and cell cycle arrest (100). Previous studies have shown that miR-34a js transcriptionally activated by tumor suppressor p53, and thereby contributes to p53 mediated downstream effects on cell cycle arrest and induction of apoptosis, by targeting c-MYC, CDK6, and c-MET (146). Other studies have also shown that miR-34a targets NOTCH, epithelial-mesenchymal transition (EMT), and transforming growth factor beta (TGF-β) signaling pathways, as well as elongation factor 2 kinase

(EF2-K) and forkhead box protein M1 (FOXM1) axis, WNT/β-Catenin pathways (146, 149, 150).

miR-200 family:

There are five members of the miR-200 family, which are organized into two clusters. Cluster 1 is composed of miR-200a, miR-200b, and miR-429, located on chromosome1, while, cluster 2 is composed of miR-200c and miR-141 (miR-200c/141) located on chromosome 12 (151). Previous studies have shown the miR-200 family is involved in regulating EMT by zinc finger E-box-binding homeobox 1 (ZEB1) and ZEB2, which are transcriptional repressors of E-cadherin, and thereby maintaining an epithelial like state (152-154). On the other hand, other studies have shown the existence of a reciprocal feedback loop where ZEB1 and ZEB2 also act by repressing miR-200 transcription (155, 156).

Table 2: Tumor Suppressor miRNAs in Breast Cancer

Tumor Suppressor miRNA	Targets	Cancer Related Events	References
Let-7 family	*H-Ras*, *HMGA2*, *PAK1*, *DIAPH2*	Stemness, Cell motility, Migration, Invasion	(157-159)
miR-34 family	*BCL-2, SIRIT1 c-MYC, CDK6, c-MET, NOTCH1, EF-2K, FOXM1*	Cell proliferation, Migration, Invasion, EMT, Cell cycle progression, Apoptosis	(147, 148) (146, 160, 161)
miR-200 family	*ZEB1*, *ZEB2*, *HER3*, *Sec23a*, *SIRT1*	EMT, Stemness, Metastasis	(162-165)
miR-145	*IRS-1*, *ER-α*, *RTKN*, *MUC1*, *OCT4*, *N-Ras*, *VEGF-A*	Cell proliferation, EMT, Invasion, Metastasis, Angiogenesis	(166-171)
miR-205	*ZEB1*, *ZEB2*, *HER3*, *VEGF-A*	EMT, Cell proliferation, Invasion, Stemness	(172-175) (176)
miR-30 family	MTDH, FOXD1, AVEN, VIM, Eya2, Vimentin, KRAS, MAPK, TWFI	Cell proliferation, Cell cycle progression, Apoptosis, Invasion, Chemo-sensitivity	(177-181)
miR-335	*SOX4*, *tenascin C*, *ER-α*, *IGF1*, *RSP1*, *ID4*	Cell proliferation, Apoptosis, Metastasis	(182-184)

miRNAs as Diagnostic & Prognostic Markers in Breast Cancer

Recent evidence has suggested that circulating miRNAs are present in several body fluids including blood, serum, saliva, urine, and breast milk (185-187). Circulating miRNAs are either free or packaged into vesicles such as exosomes, apoptotic bodies, or incorporated with high density lipoproteins, or AGO proteins (188). Thus circulating miRNAs are stable, easily detected by non-invasive measures, making them ideal biomarkers for early cancer detection and predictors of therapeutic outcome (65).

Studies have indicated that miRNAs may be valuable diagnostic markers for early detection of BC. One of the most extensively studied miRNAs in cancer is miR-21, which has been shown in numerous studies to be a useful diagnostic biomarker for BC, as it is significantly overexpressed in either plasma/serum or tissue samples of BC patients compared to normal healthy volunteers (189-192). Furthermore, miR-21 proved to be a highly reliable biomarker, displaying higher sensitivity than other well characterized markers, such as clinical cancer antigen 15-3 (CA153) and carcinoembryonic antigen (CEA) in BC diagnosis (191). Other extensively studied oncogenic miRNAs for BC diagnosis include miR-155 (193-195) and miR-18a (196, 197).

miRNAs could also serve as prognostic tools in BC whereby their expression can predict patient survival and treatment outcome. For example, high miR-21 expression levels were shown to be associated with reduced disease-free survival (DFS) and overall survival (OS), as well as clinical staging and lymph node metastasis in BC patients (112,

198, 199). Furthermore, miR-21 was also shown to have prognostic value as it was demonstrated to be highly expressed in the bone marrow of BC patients (200).

miRNAs as Markers for Therapeutic Response

miRNAs could also be predictive of therapeutic outcome, whereby their expression levels could indicate either sensitivity or resistance to treatment. For instance, high expression of miR-210 in tissues has been associated with poor patient survival and prognosis in ER+ tamoxifen-treated BC patients (201). Similarly, high levels of miR-210 was also found to be correlated with trastuzumab resistance in HER2+ breast tumors (202).

miRNAs as a Novel Class of Targeted Therapeutics

Give the critical role of miRNAs in carcinogenesis, and their ability to simultaneously regulate many targets/pathways, a growing interest in recent years has been in utilizing miRNA based therapies as a therapeutic modality in cancer (203). This can be achieved by either restoring tumor suppressive miRNAs (miRNA mimetics) or by inhibition of oncogenic miRNAs (miRNA inhibitors) (203).

Restoring Tumor Suppressor miRNAs in Breast Cancer

Restoring the expression and function of tumor suppressor miRNAs can be achieved by miRNA mimics which are synthetic oligonucleotides that can also be chemically modified (2′-O′methoxy) to increase their stability (204). By replacing the lost or suppressed tumor suppressor miRNAs, these synthetic molecules can be loaded into the RISC complex to achieve downstream target inhibition (203). Several studies have validated the efficiency of miRNA replacement therapies in many *in vitro* and *in vivo* models of cancer (203),

including BC (205, 206). For example replacement of the tumor suppressor Let-7 miRNA by lenti-viral system lead to decrease cellular proliferation, self-renewal, and metastasis of BC cells (207). Another example is the replacement of miR-145 and miR-205 , which were found to restore functional BRCA1 gene in BC (208). Furthermore, down-regulated tumor suppressor miRNAs such as miR-205, miR-126, miR-335, and miR-451 can be restored through miRNA replacement therapy (157, 209, 210).

Targeting oncogenic miRNAs in Breast Cancer

miRNA inhibitors are single stranded oligonucleotides that are complementary to endogenous miRNAs and have the ability to bind/sequester miRNAs and thereby prevent their processing by the RISC complex. Some examples of miRNA inhibitors include: anti-miRNAs (AMOs), locked nucleic acids (LNAs), antagomirs, and miRNA sponges (203).

Anti-miRNA oligonucleotides (AMOs) are single-stranded, anti-sense oligonucleotides, that can bind to their selected miRNA by Watson Crick interaction, and thus prevent the miRNA from binding to its target (211). AMOs have shown to be successful in suppressing miR-21 levels in BC cells both *in vitro* and *in vivo*. For instance, the use of anti-miR-21 oligonucleotides were found to suppress both MCF-7 cell growth *in vitro* and tumor growth *in vivo* in xenograft mouse models. Furthermore, the effect of miR-21 inhibition in decreasing cell growth was also associated with an increase in apoptosis, in part by downregulation of the anti-apoptotic protein Bcl-2 (212).

AntagomiRs are chemically modified synthetic oligonucleotides that are complementary to miRNAs and can effectively compete with miRNAs for their target mRNAs with a stronger binding affinity (213). AntagomiRs are modified by the addition of 2'-O-methoxy group on the ribose residues, partial replacement of phosphodiester bonds to phosphorothioate, and the addition of a cholesterol motif at 3′ end (213). The 2′-O-methoxy and phosphorothioate modifications help improve their bio-stability, whereas the cholesterol conjugation increases their cell distribution and permeation (214). It has been demonstrated that antagomiR-21 can reduce cell proliferation and lead to induction of apoptosis in BC cells (212, 215). Additionally, miR-21 antagomiRs were found to enhance the response to trastuzumab in resistant BC cells by upregulating PTEN (216).

Locked nucleic acids (LNAs) are modified anti-sense oligonucleotides where the ribose moiety is locked in a C3'-endo conformation by an extra methylene bridge (217). LNAs against miR-10b were found to be effective in inhibiting BC metastasis (218). LNAs packaged in nano-liposomes were also found to prevent lymph node metastasis in orthotopic MDA-MB-231 tumor models (219). Additionally, the use of LNA miR-21 successfully reduced miR-21 expression levels as well as proliferation of BC cells (215).

A miRNA sponge is a construct that encodes a mRNA containing multiple complementary binding sites in its 3′-UTR for the miRNA of interest (220). Sponges can bind from 2-7 specific seed sequences of the miRNAs of interest, and have the ability to bind to miRNAs from the same family (221). Previous studies have shown that miR-9 sponges results in more than 50% reduction of miR-9 activity in 4T1 mammary tumor cells (127). In addition,

miR-10b sponges effectively reduced cell growth, migration, and invasion in MDA-MB-231 and MCF-7 BC cell lines, along with upregulating the expression of the miR-10b target HOXD10 (222) .

Current Challenges in microRNA Delivery

Despite the recent advances in the field of miRNA-based therapies, there are still many challenges to overcome in order to ensure safe and effective miRNA delivery *in vivo*. These obstacles include enzymatic degradation by nucleases, rapid renal clearance, as well the development of immune toxicities, and off-target effects (223, 224). Thus, the use of miRNA modulators is limited due to their poor bioavailability, stability, and tissue permeability (223). Therefore, several miRNA delivery systems have been engineered using viral or non-viral vectors in order to overcome these hurdles (225). Although viral based vectors; made of either lentiviruses, adenoviruses, or adeno-associated viruses; have been shown to efficiently deliver miRNA modulators *in vivo*; their use is limited by their immunogenic effects (226). Hence, the use of non-viral vectors may offer a safer, less toxic alternative (227). One increasingly popular approach for miRNA delivery is the use of nano-carriers which are biocompatible and biodegradable carriers, that are highly versatile with the ability to modify their size and surface in order to enhance tumor-specific delivery (227). Nano-carriers (1-1000nm) can be formed of inorganic materials such as gold or silica; or organic materials such as polymers or lipids; (228, 229) and offer the advantage of increased payload stability, and bioavailability, as well as selective accumulation at the tumor site due to the enhanced permeability and retention effect (228,

230). Additionally, nano-carriers can be modified to express specific ligands for receptors on tumor cells (231).

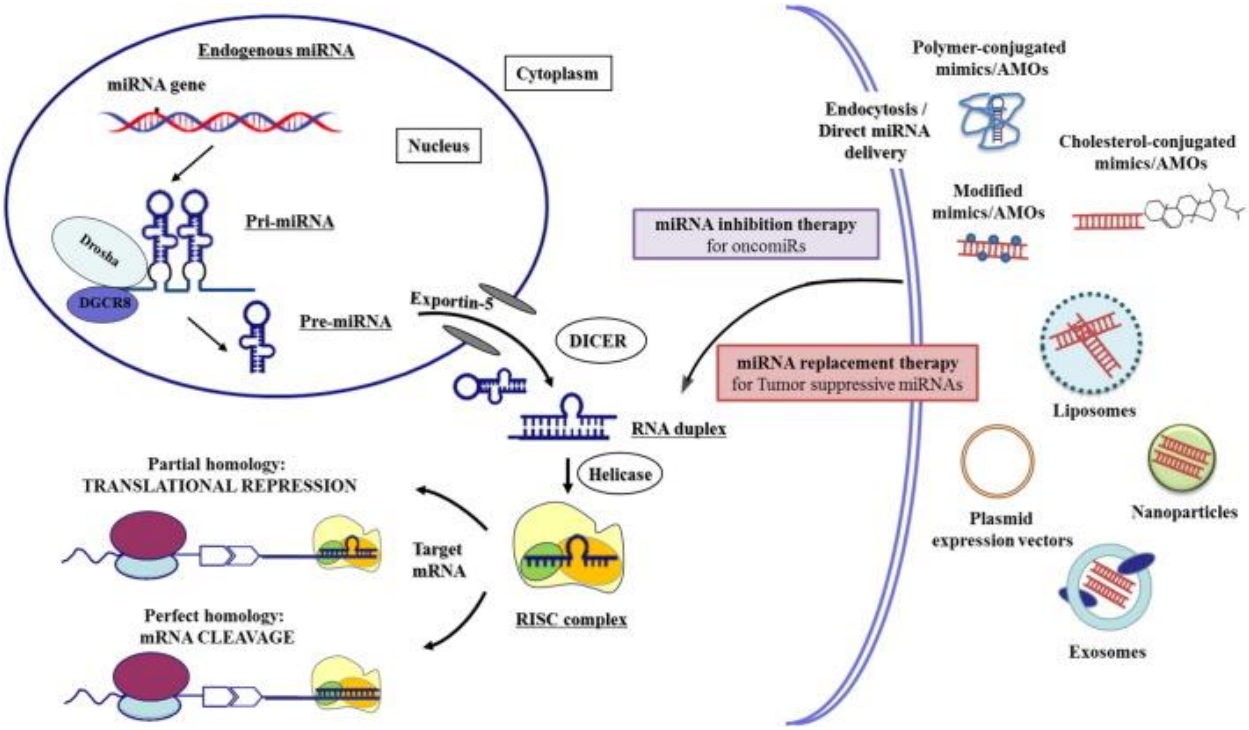


Figure 3: miRNA mechanism and modulation. Canonical biogenesis and processing of miRNAs and mechanism of RNAi-regulated gene silencing.

First miRNA-Based Clinical Trials

Due to the great promise in utilizing miRNAs as therapeutic agents, there are now several ongoing clinical trials on miRNA based therapies in many cancers. For example, the locked nucleic acid (LNA) against miR-122 Miravirsen (SPC3649), developed as treatment of hepatitis C virus (HCV), was found to safe and well tolerated in phase I clinical trial, and effective in Phase II trials, with a significant reduction in HCV RNA levels (232).

Another noteworthy example is the replacement therapy of the tumor suppressor miR-34a by a liposomal mimetic (MRX34), which was evaluated in the first-in-human, phase I study, in patients with advanced solid tumors, including hepatocellular carcinoma, melanoma, and renal cell carcinoma (233). However, this clinical trial was halted by miRNA Therapeutics due to multiple immune-related severe adverse effects that were observed (http://www.businesswire.com). Therefore, dose optimization for miRNA based strategies is highly warranted in order to prevent potential adverse events.

With the great promise that miRNA based therapies hold, there are still some obstacles that need to be overcome such as improving their safety, modes of delivery, and their therapeutic efficacy before their translation from the bench to the clinic. However, a deeper understanding of the biological role of miRNAs could pave the way for a new era in personalized medicine.

Hypothesis & Aims:

TNBCs represent a significant clinical challenge that is largely attributed to lack of effective targeted therapeutics, significant tumor heterogeneity, and poor response to conventional chemotherapies (6). Therefore, better understanding of the biology of the disease and identification of novel molecular targets is crucial for the development of highly effective therapies to eradicate TNBC and improve patient survival. Given the role of miRNAs in initiation, progression, metastasis, and drug resistance in various human cancers including BC, identification of clinically significant miRNAs that are involved in TNBC growth and progression is critical for better understanding of the complex biology of this cancer and for development of miRNA based strategies (106, 225). Recent studies have shown that miRNA 484 was among seven miRNAs that were correlated with OS amongst various clinical and molecular subtypes of invasive ductal carcinoma patients (234). Furthermore, miR-484 was also found to be significantly highly expressed in serum of early BC patients compared to healthy volunteers, suggesting that it may serve as an early diagnostic biomarker (235). However, the role and mechanism of action of miR-484 in TNBC has not been previously elucidated. In light of this, we embarked on identifying clinically significant miRNAs using the The Cancer Genome (TCGA) database, and we identified miR-484, which we found to be clinically and prognostically significant and correlated with poor overall survival (OS) in BC (Fig. 4), supporting the previous findings. We further analyzed miR-484 expression profiles in all clinical BC subtypes including, ER+, ER-, HER2+, and TNBC, which we found to have the highest expression compared with normal breast tissues (Fig. 5). Additionally, we found that miR-484 expression is significantly higher in basal subtype of BC compared to non-basal subtype and matched

normal breast tissues (Fig. 6). Furthermore, we screened a panel of TNBC cells lines for basal miR-484 expression, and found that miR-484 was upregulated in all of our TNBC panel (2-10 folds) compared to normal mammary epithelial cells (MCF-10A) (Fig. 7). .

Overall Hypothesis:

Thus, based on our preliminary data, as well as the recent findings, our overall hypothesis is that miR-484 acts as onco-miR to promote tumor growth & progression in TNBC.

We tested this hypothesis with the following specific aims:

Aim 1: Determine the functional role of miR-484 in TNBC cells *in vitro.*

Aim 2: Determine the mechanism of action of miR-484 in TNBC cells.

Aim 3: Determine the role of miR-484 in TNBC tumorigenesis in orthotopic TNBC mouse models.

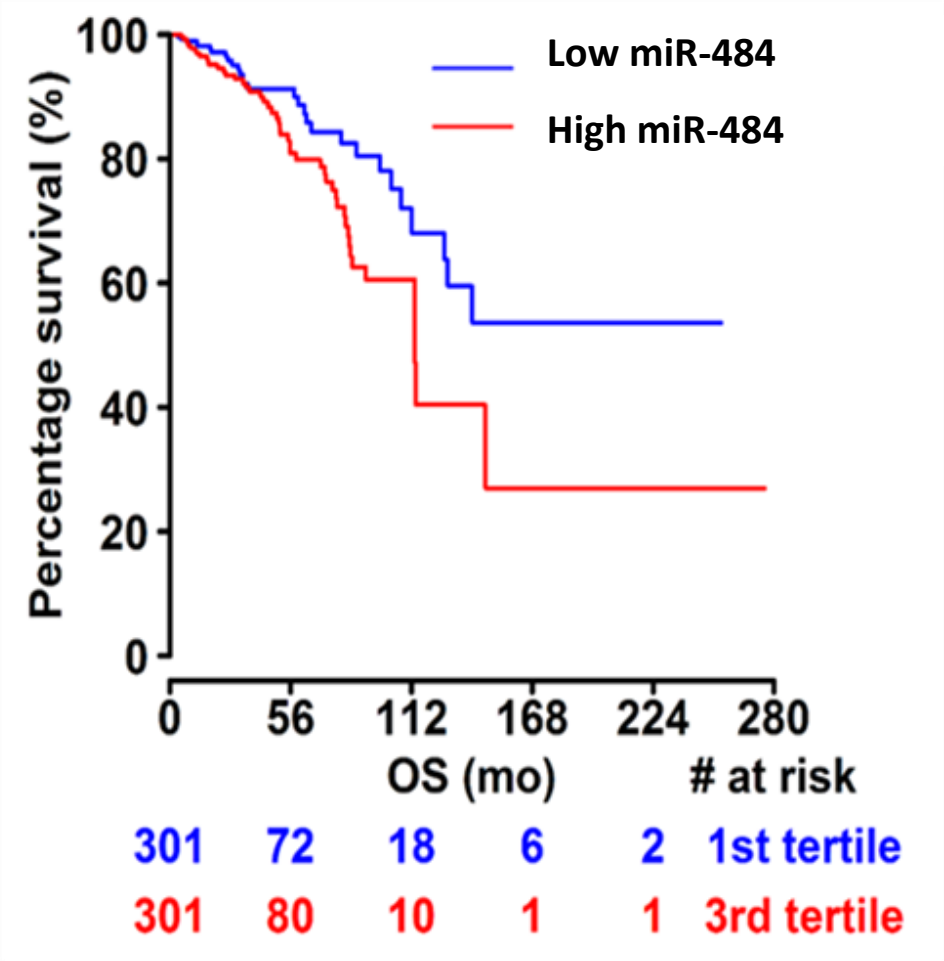


Figure 4: High miR-484 expression is associated with shorter overall survival in breast cancer patients. Kaplan-Meier Survival curves analysis showing high miR-484 expression is associated with shorter overall survival rate in BC patients compared with patients with low miR-484 expression (n=602) (p≤0.001).

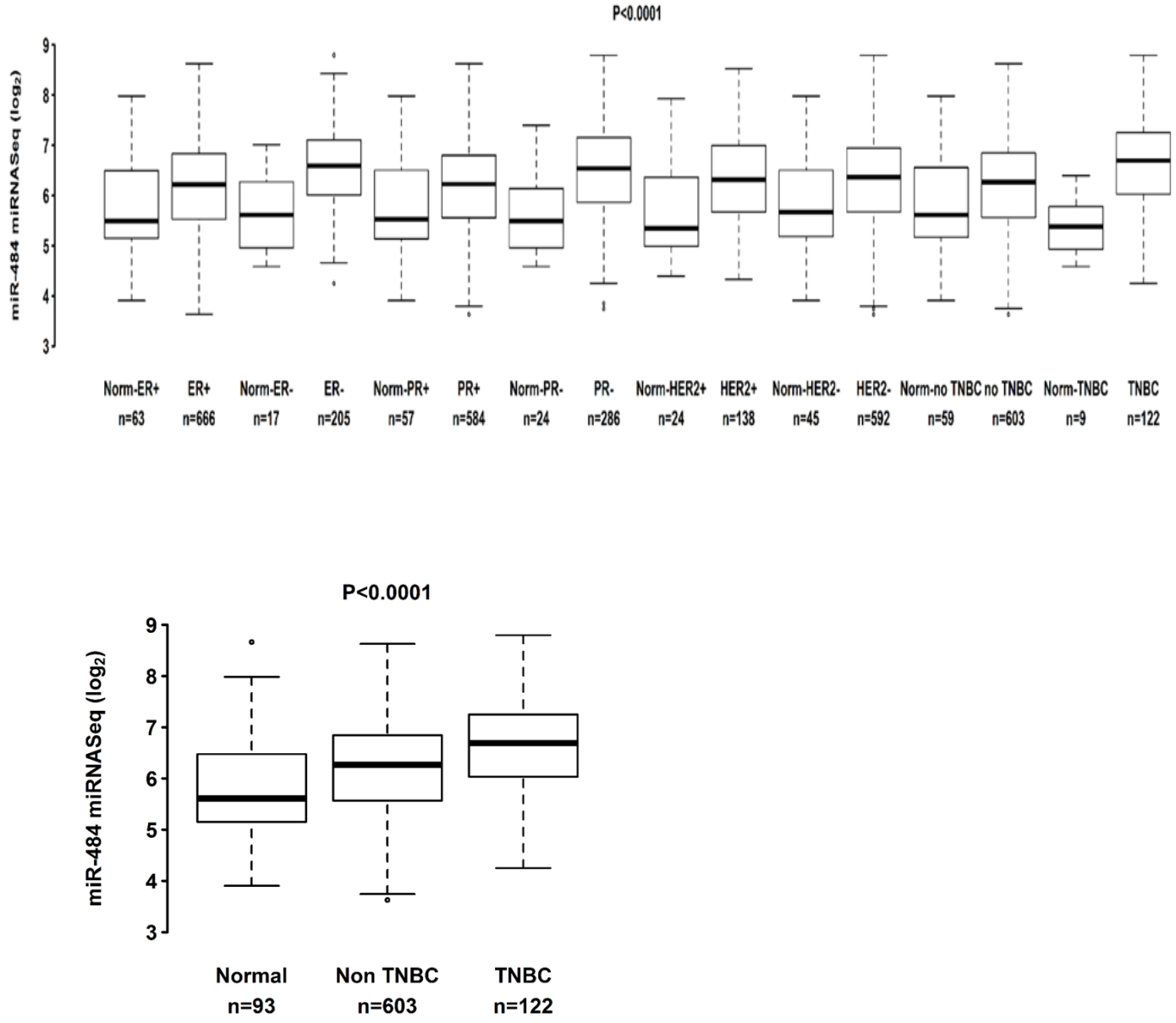


Figure 5: miR-484 expression is significantly higher in TNBC subtypes compared to non-TNBC subtypes and normal tissues. The number of patients is listed at the bottom of the graph (p≤0.001).

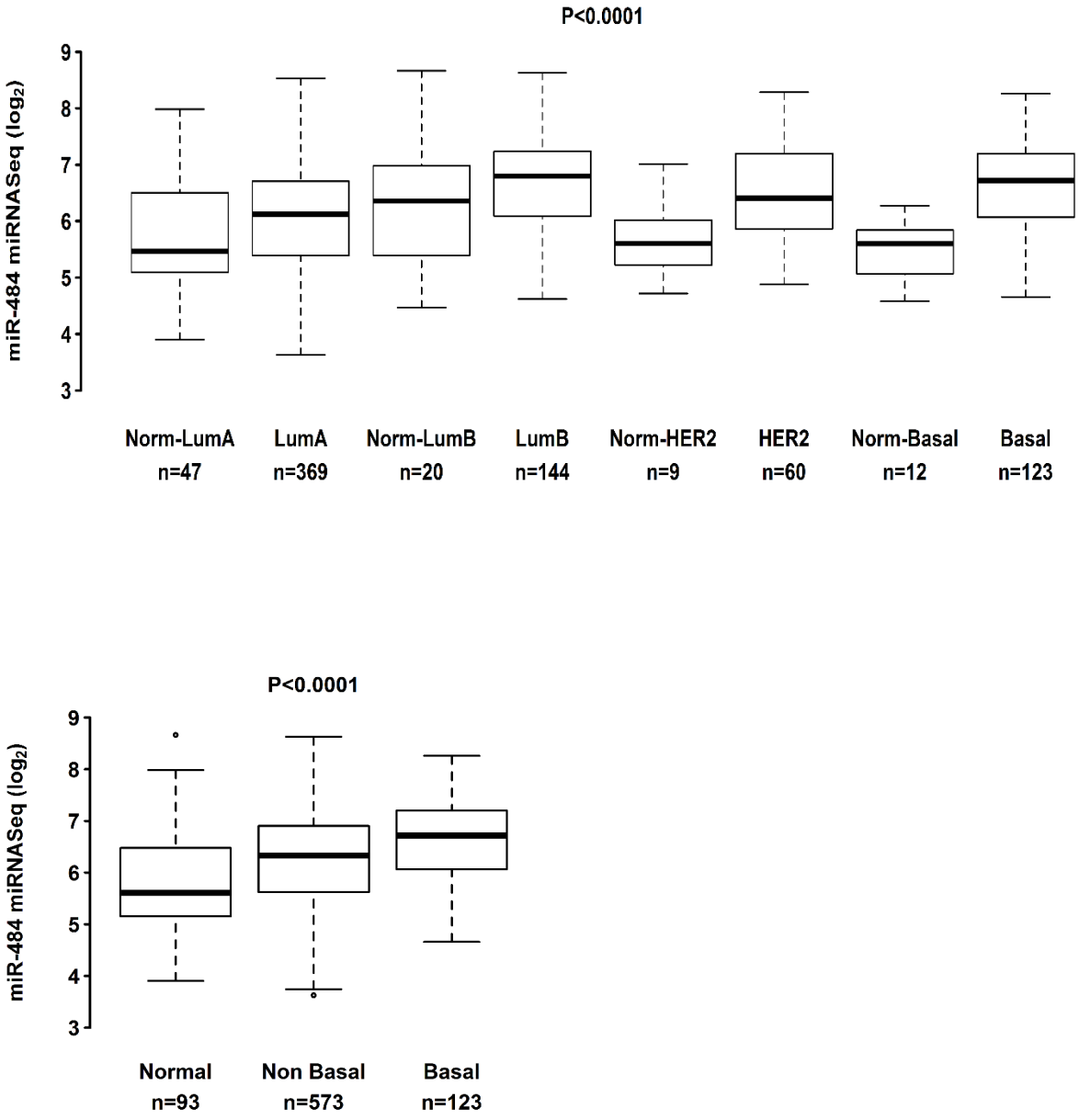


Figure 6: miR-484 expression is significantly higher in basal subtype of BC compared to non-basal subtypes. The number of patients is listed at the bottom of the graph (p≤0.001).

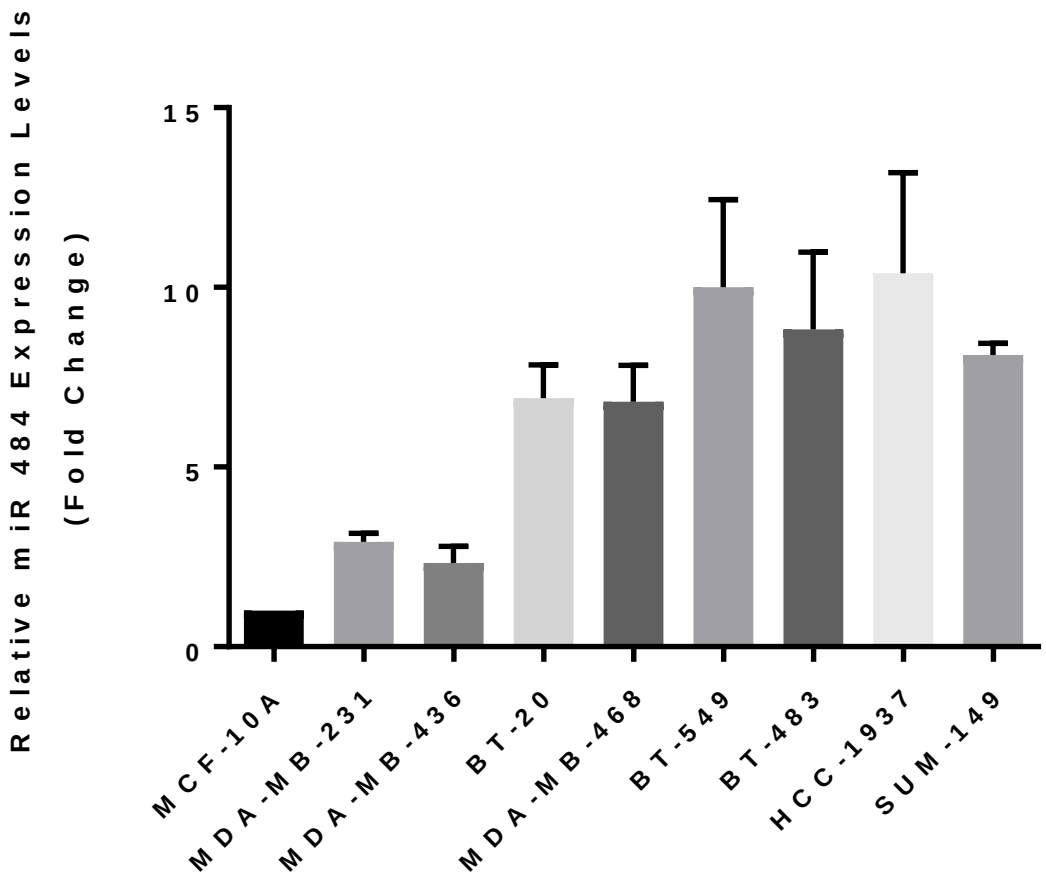


Figure 7: miR-484 levels are upregulated in TNBC cells. Expression levels of miR-484 in TNBC cell lines and normal breast epithelial MCF-10A cells by qRT-PCR. Data was normalized to the expression of U6 as an endogenous control and represent means ± SDs of three independent experiments.

CHAPTER II:

METHODS

Cell Lines and Culture conditions

TNBC cell lines (MDA-MB-231, MDA-MB-436, MDA-MB-453, MDA-MB-468, MDA-MB-361, BT-549, BT-20, BT-483, HCC-1937, and SUM-149) and human mammary epithelial cell lines (MCF-10A, HMEC) and) were purchased from the American Type Culture Collection (Manassas, VA). TNBC cells were cultured in Dulbecco's modified Eagle's medium (DMEM/F12), with the exception of HCC-1937, which was cultured in RPMI1640, and all media were supplemented with fetal bovine serum (FBS 10%) and a penicillin/streptomycin (100-U/ml) (Sigma). MCF-10A cells were cultured in DMEM/F12 media with the addition of horse serum (5%), insulin, hydrocortisone, epidermal growth factor, and cholera toxin. Cultured cells were kept in a water-saturated incubator (95% air–5% CO2) at a temperature of 37°C.

The Cancer Genome Atlas (TCGA) and Bioinformatics Analysis

Statistical analyses were performed in R (version 3.4.1) (http:///www.r-project.org/) and the statistical significance was defined as a p-value less 0.05. We downloaded patient clinical information for the TCGA patients with breast invasive carcinoma from cBioPortal (http://www.cbioportal.org/). For the miRNA-Seq data, we derived the 'reads_per_million_miRNA_mapped' values for the mature form hsa-miR-484 (MIMAT0002174) from the "Isoform Expression Quantification" files from Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov/). The log2-transformation was applied to the data. We analyzed total of 914 invasive BC cases with miRNA data and clinical information available. For 93 cases matched, normal solid tissue was available. To determine the expression difference for miR-484 among normal and tumor tissue of different subtypes, we first employed a Shapiro-Wilk test and verified that the data does

not follow a normal distribution. The Kruskal-Wallis non-parametric test was applied to determine the relationship between miRNA expression and tissue type. Data is represented as box and-whisker plots (Box plot represents first (lower bound) and third (upper bound) quartiles, whiskers represent 1.5 times the interquartile range). Univariate Cox proportional hazards model was fitted to evaluate the association between OS and covariates including miR-484 expression levels (dichotomized at the tertiles to create groups that are “high” or “low”) and available clinical variables (age at diagnosis, stage). Stage, age, and miR-484 were statistically significant factors in the univariate Cox proportional hazards models, and were included in the final multivariable analysis of OS. miR-484 was an independent factor (HR= 2.02, CI(95%)=(1.23, 3.31), Wald test p-value= 0.005). In order to visualize the survival difference the Kaplan-Meier plots were generated for “low” (first tertile) and “high” (last tertile) miR-484 groups. We applied a Spearman's rank-order correlation test to measure the strength of the association between HOXA5 expression and miR-484 expression. We imposed a cut-off of functional relevance on the Spearman correlation coefficient in absolute value of 0.2 based on previously published methodology (236).

miRNA Transfection

MDA-MB-231, MDA-MB-436, and BT-20 cells were plated at a density of 1.5×10^5 cells/well in six-well plates and treated with either miR-484 (100 nM), or control miRNA mimic or inhibitor (100 nM) (Ambion) with the addition of HiPerFect transfection reagent (Qiagen) in Opti-MEM serum free media according to the manufacturer’s instructions. After 6 h of transfection, cultured media was substituted with DMEM supplemented with 10% FBS for up to 48 h.

Cell viability

Cell viability of MDA-MB-231, MDA-MB-436, BT-20, and MCF-10A cells was analyzed using MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium] as previously described (237). Cells were seeded in a density of 1 to 2 × 10^3 cells/well in 96-well plates. After overnight incubation, the cells were treated with miR-484 inhibitor or control inhibitor miRNA (Ambion). We determined the cell viability by measuring the optical density at 490-nm wavelength in a VMax kinetic enzyme-linked immunosorbent assay microplate reader (Molecular Devices) at 24, 48, and 72 hours

Colony formation assay

The effect of miR-484 on TNBC cell proliferation was evaluated by the clonogenic assay. MDA-MB-231, MDA-MB-436, and BT-20 cells were seeded at low density (500 cells/well) in 12-well plates. After overnight incubation, the cells were treated with either control inhibitor miRNA or miR-484 inhibitor and cultured for approximately 10-14 days. Colonies were stained with crystal violet, and quantified with Image J software (National Institutes of Health, Bethesda, MD). Each experiment was independently triplicated.

Cell motility and invasion assays

Cell motility and migration was analyzed by an *in vitro* wound healing assay. TNBC cells (MDA-MB-231, MDA-MB-436, and BT-20) were plated at a density of 1.5 × 10^5 cells/well in six-well plates. The following day cells were transfected with the control miRNA inhibitor or miR-484 inhibitor. After 48h, as the cells reached ~80% confluence, a single scratch was made, and cells were imaged at 0h and subsequent 12h time points, using

a phase contrast microscope (Nikon Eclipse TE-200-U) to measure the wound width. Wound healing was measured as percentage open area of the wound by Image J software. All experiments were independently repeated three times.

We evaluated TNBC cell invasion utilizing matrigel coated transwell inserts (Corning). After 48h transfection with either miR-484 inhibitor or control miRNA inhibitor MDA-MB-231, MDA-MB-436, and BT-20 cells (4×10^4) were collected in serum free medium and added to the upper chamber of the transwell inserts, allowing cell invasion toward the lower chamber which contains serum positive media (10% FBS). After 24h, invaded cells at the bottom of the inserts were fixed, stained with Hema 3 (Thermo Scientific), and counted using a light microscope (Nikon Eclipse TE-200-U) at 10X magnification. Invaded cells were counted in five fields per slide and all experiments triplicated.

Cell cycle analysis

TNBC cells were transfected as previously described with miRNA inhibitors or control inhibitor. After 48h treatment, cells were collected and washed in PBS and fixed in 75% ethanol overnight. The following day cells were centrifuged and resuspended in PBS containing 50 µg/ mL propidium iodide (PI) and 100 U/mL of RNAse A. Samples were incubated in the dark for 30 minutes at a temperature of 37ºC prior to flow cytometry analysis. The number of cells in each phase of the cell cycle was determined by FlowJo Software. All experiments were independently triplicated.

Analysis of apoptosis

Apoptosis was assessed by an Annexin V assay. TNBC cells were seeded in 6 well plates (1.5 X10^5/well) and transfected with either control inhibitor or miR-484 inhibitor (100nM) for 48 h. Cells were then collected and stained with Annexin V/propidium iodide (PI) according to the manufacturer's protocol (BD Pharmingen FITC–Annexin V kit, San Diego, CA). We determined the number of apoptotic cells by flow cytometry using CellQuest Pro software (BD Biosciences). This assay is based on the binding of Annexin V to membrane phospholipids of the apoptotic cells that are translocated from the inner to the outer the membrane in apoptotic cells (238). Apoptosis was also confirmed, by detecting the cleavage of caspase-3, caspase-8, caspase-2, and PARP by Western blotting.

Reverse phase protein array (RPPA)

We performed the RPPA analysis at the Functional Proteomics RPPA Core Facility of The University of Texas MD Anderson Cancer Center according to the method described previously (239). MDA-MB- 231 cells were plated in six well plates at a density of 1.5 × 10^6 cells/well and transfected with either miR-484 mimic or control miRNA (100 nM) for 48h. Cells were collected in 100 µl of lysis buffer supplemented with protease and phosphatase inhibitors (Roche Applied Science). Samples were centrifuged at 14,000 X g for 30 minutes at a temperature of 4°C. Supernatants were collected, and total proteins concentration was determined by Pierce BCA protein assay kit. Protein concentrations were adjusted to a concentration of 1.0 µg/µl by the addition of lysis buffer. 4XSDS Sample Buffer was mixed with β-mercaptoethanol (β-Me) at a ratio of 9:1. Cell lysates

were then mixed with 4× SDS sample buffer + β-Me mixture at a ratio of 3:1. Samples were boiled for 5 minutes and stored at -80ºC prior to submission to the RPPA Core Facility.

Luciferase reporter assay

pEZX-MT06 miRNA reporter vectors containing the binding sites for miR-484 in the 3'-UTR of HOXA5 and the luciferase gene (GeneCopoeia) were transfected into MDA-MB-231 and MDA-MB-436. As a control for target specificity, we transfected pEZX-MT06 miRNA reporter vectors containing one point mutation at the miR-484 binding site (GAGCCTG> GCTACAG) into MDA-MB-231 and MDA-MB-436 cells. Cells were plated (5×10^4 cells/well) in a 24-well plate and incubated overnight. The following day cells were co-transfected with the pEZX-MT06 vector (200 ng) and either 100 nM miR-484 mimic or control miRNA. After 48h, firefly luciferase activity was determined by utilizing Luc-Pair miR Luciferase Assay (GeneCopoeia) and measurements were normalized to Renilla luciferase activity.

Western blot analysis

TNBC cells (MDA-MB-231, MDA-MB-436, and BT-20) were treated with miR-484 inhibitor or control inhibitor miRNA (100nM) and cells were collected after 48h transfection. Lysates were prepared in lysis buffer supplemented with protease and phosphatase inhibitors and samples were centrifuged at 14,000 × g for 30 min at a temperature of 4°C. Supernatants were collected and analyzed for protein concentration by using the Pierce BCA protein assay kit (Thermo Scientific). Protein samples (40µg) were separated by SDS-PAGE on a 4%–15% gradient polyacrylamide gels (Bio-Rad), and subsequently

electro-transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% milk in TBST, rinsed, and then incubated with primary antibodies overnight at 4ºC. The following day membranes were rinsed and incubated with their corresponding HRP-conjugated secondary antibodies. GAPDH expression levels were detected as loading control. Antibodies used in this study are listed in the appendix in Table 3. HyGLO Chemiluminescent Reagent (Denville Scientific) was used to detect the expression levels of the selected proteins and immunoblots were imaged by Fluor Chem 8900 imager and using Alpha Imager software (Alpha Innotech). All experiments were independently triplicated.

RNA isolation and quantitative real time polymerase chain reaction (qRT-PCR)

For mRNA and miRNA detection, first we isolated total RNA using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Subsequently, RNA concentration and purity was determined spectrophotometrically (260 and 280 nm UV absorbance) by Epoch microplate reader (BioTek Instruments). For miRNA expression, 1µg of total RNA was reverse transcribed to complementary DNA (cDNA) using the qScript microRNA cDNA Synthesis Kit (Quanta BioSciences) according to manufacturer's instructions. miR-484 expression was detected by using miRNA primers (Quanta Bio Sciences) by quantitative real time polymerase chain reaction (qRT-PCR) and utilizing the PerfeCTa microRNA Assay Kit (Quanta Bio Sciences). The expression levels of miR-484 were normalized to expression levels of U6 small nuclear RNA (RNU6; Quanta Bio Sciences), as an endogenous control.

For HOXA5 mRNA quantification, first we reverse transcribed total RNA to cDNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's instructions. Then HOXA5 gene expression was measured with the iQ SYBR Green Supermix qPCR Kit (Bio-Rad). GAPDH expression levels were determined as endogenous control. The sequences of the forward and reverse primers for HOXA5 and GAPDH are listed in the appendix in Table 4. Relative expression levels were analyzed by the comparative threshold cycle (2-ΔΔCt) method.

HOXA5 gene overexpression

MDA-MB-231 cells were transfected with lentiviral plasmids containing the specified lentiviral vector for HOXA5 (NM_019102.3) with the CMV promoter (LPP-F0180-Lv105; GeneCopoeia, Rockville, MD) or the mock vector (LPP-NEG-Lv103; GeneCopoeia) according to the manufacturer's instructions. HOXA5 protein expression was then verified by Western blotting.

Orthotopic xenograft TNBC tumor models

For our animal study we obtained female nude athymic mice from M.D. Anderson Cancer Center. We performed our animal study according to an experimental protocol approved by the M.D. Anderson Institutional Animal Care and Use Committee. TNBC cells (MDA-MB-231 and MDA-MB-436) were injected into the mammary fat pad of each mouse at a density of 2×10^6 in 20% matrigel. Approximately two weeks after TNBC cell injection, as tumor volume was in a range of 3-5 mm, we initiated our liposomal-miRNA treatment. Mice were treated with either miR-484 inhibitor or control miRNA inhibitor (0.15 mg/kg≈4μg/mouse) delivered intravenously through the tail vein, once every 4 days for 4

weeks (total of eight i.v. injections). We monitored tumor growth, by weekly measurements of tumor volumes using an electronic caliper. At the end of the experimental protocol, we euthanized the mice with CO_2 and determined their weight to measure tumor growth. Tumor tissues were dissected for further analysis by immunohistochemistry, TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling), western blot, and qRT-PCR.

Preparation of miRNA nanoparticles

For *in vivo* targeting of miR-484, we incorporated anti-miR484 oligonucleotides into liposomal nanoparticles which were composed of dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and pegylated distearoylphosphatidylethanolamine (DSPE-PEG-2000) (AvantiLipids) according to our previously described protocol (161).

Immunohistochemistry

The effect of miR-484 inhibition on TNBC cell proliferation and angiogenesis *in vivo* was determined by immunostaining tumor sections for Ki-67 and CD31 respectively according to the manufacturer's protocol. Formalin-fixed paraffin-embedded tumor tissues sections were deparaffinized and dehydrated, then incubated in Dako (for antigen retrieval) at 95°C for 40 minutes. Slides were then blocked with endogenous peroxidases with methanol supplemented with hydrogen peroxide (3%) for 15 min, and then incubated with primary antibodies for Ki-67 or CD31 overnight at a temperature of 4°C. The following day, slides were incubated with secondary antibodies for 1 hour at room temperature. Tumor sections were then counterstained with hematoxylin for approximately 30 seconds and analyzed by light microscope (Nikon Eclipse TE-200-U).

Evaluation of *in vivo* apoptosis (TUNEL assay)

We evaluated the effect of miR-484 inhibition on TNBC apoptosis *in vivo*, by measuring the nuclear DNA fragmentation using the TUNEL assay kit (Promega) according to the manufacturer's recommended protocol. Tumor sections from mice treated with either control inhibitor or miR-484 inhibitor were incubated with biotin-dUTP and terminal deoxynucleotidyl transferase for 1h. Next, we incubated tissue sections with fluorescein conjugated avidin in the dark for half an hour, and then counterstained with Hoechst 33342 dye (Thermo Scientific) DNA. TUNEL positive cells were then determined in five separate fields for each slide using an inverted fluorescence microscope.

Statistical analyses

Unless otherwise stated, data is expressed as means ± standard deviations (SDs) of three independent experiments. We analyzed our data by the two tailed Student t-test to compare significant differences between means of data sets, and p-values indicate the probability of the means being significantly different, where *p≤0.05, **p≤0.01, ***p≤0.001, ****p ≤0.001. Data analysis was performed by Graph Pad Prism software (version 6.02) for student t-Test and analysis of variance (ANOVA).

CHAPTER III:

RESULTS

Aim 1:

miR-484 inhibition decreases cell viability & proliferation in TNBC cells

Given the observed upregulation of miR-484 in TNBC patients and cells lines, we sought to determine the role of miR-484 in TNBC cells *in vitro* by various functional assays. First, we verified successful transfection efficiency and found that miR-484 inhibitor transfected cells (MDA-MB-231) showed significant downregulation of miR-484 levels compared to control inhibitor transfected cells, while cells treated with miR-484 mimic had significant upregulation of miR-484 levels compared to control mimic treated cells (Fig. 8). Next, we examined the short-term effects of miR-484 on cell proliferation, by the MTS assay on three different TNBC cells (MDA-MB-231, MDA-MB-436, BT-20) and normal mammary epithelial cells (MCF-10A), treated with either miR-484 inhibitor or control inhibitor for 24, 48, and 72h. Our results showed that miR-484 inhibition significantly decreased cell viability in TNBC cells at the indicated time points (Fig. 9, $p \leq 0.0001$), while no significant decrease in cell viability was observed in normal mammary epithelial cells MCF-10A (Fig. 9).

Furthermore, we determined the long-term effect of miR-484 on cell proliferation by the colony formation assay in MDA-MB-231, MDA-MB-436, and BT-20 cells. Inhibition of miR-484 (25nM) significantly decreased colony formation in all TNBC cell lines (MDA-MB-231: 41.94% ±10.07 p= 0.0099; MDA-MB-436: 44.19% ±10.66; p= 0.0119; BT-20: 50 % ± 6.193 p=0.0051) compared to cells treated with control inhibitor (Fig. 10). Moreover, treatments of TNBC cells (MDA-MB-231 and MDA-MB-436) with miR-484 mimic significantly increased cell viability and proliferation by the MTS assays and colony formation assays respectively (Fig. 11 & 12), suggesting miR-484 induces cell proliferation in TNBC cells.

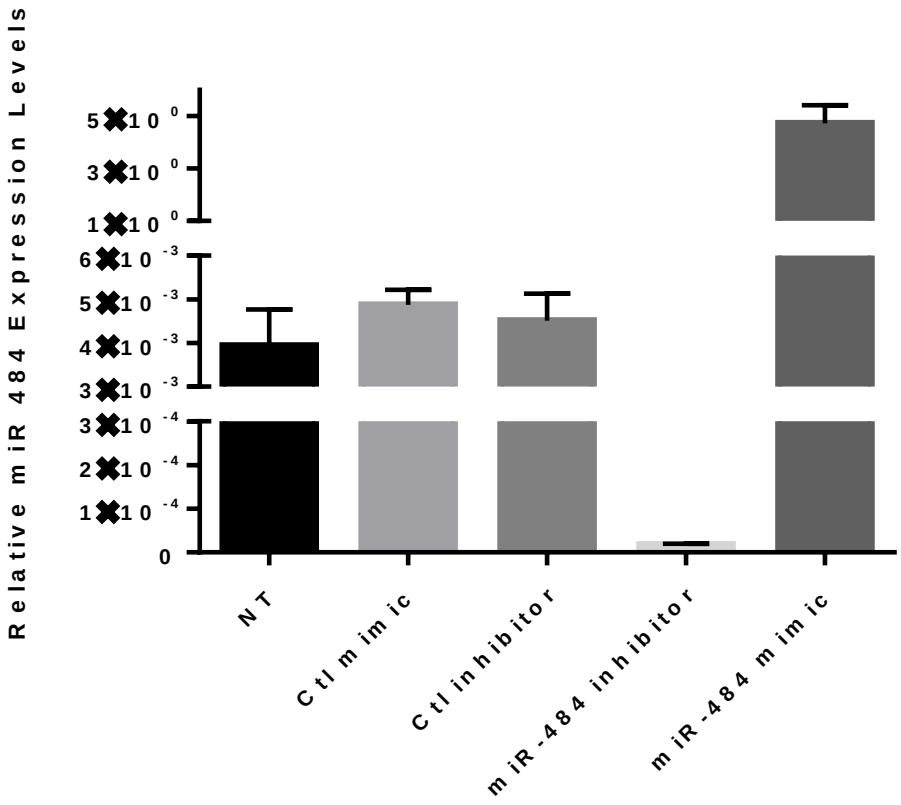


Figure 8: miR-484 inhibitor decreases the expression of miR-484 and miR-484 mimic leads to increased miR-484 expression. MDA-MB-231 cells were treated with either miR-484 inhibitor or miR-484 mimic or control miRs (inhibitor or mimic) at 100nM for 48h. miR-484 expression levels were analyzed by qRT-PCR and normalized to U6.

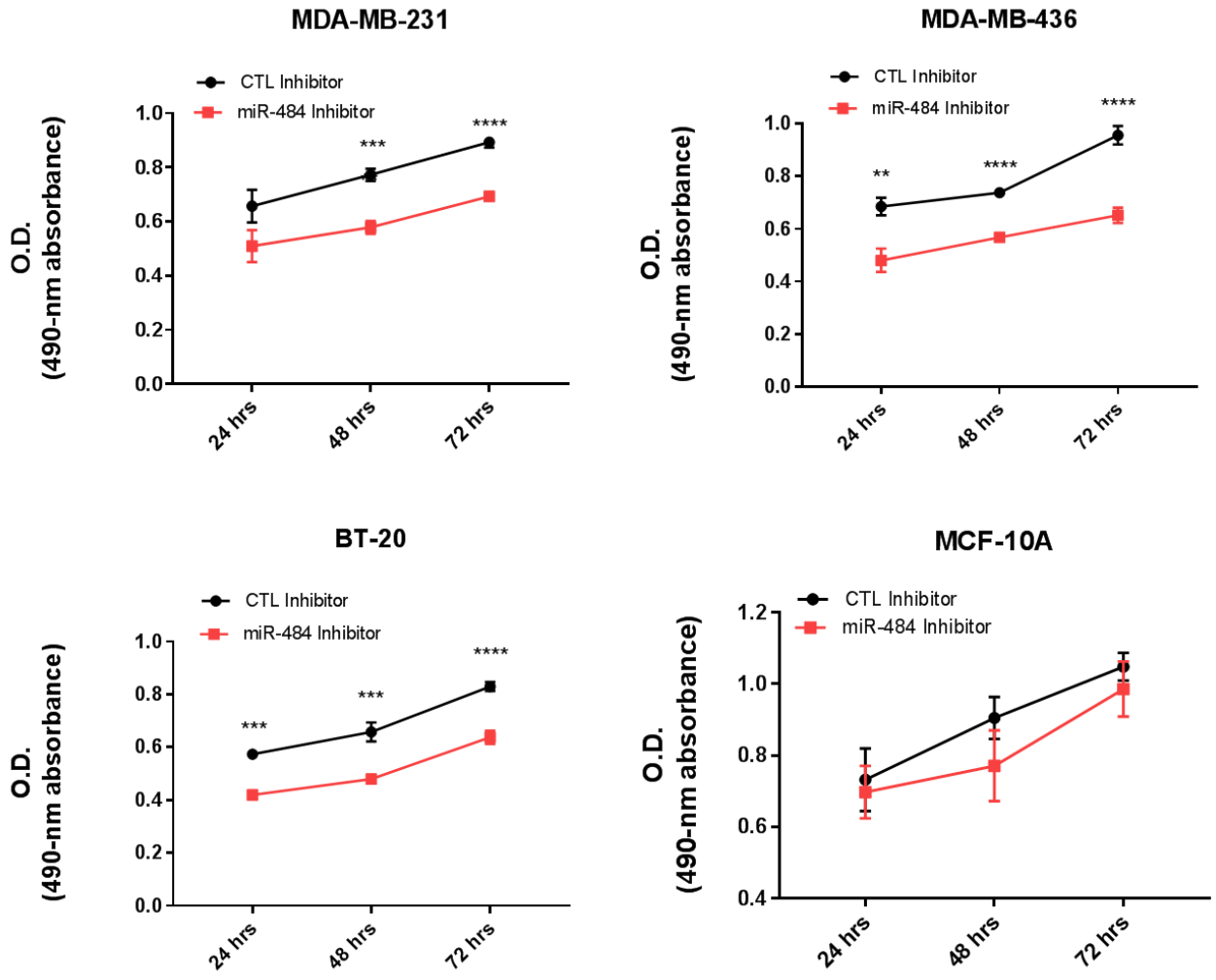


Figure 9: Inhibition of miR-484 decreases cell viability in TNBC cells. Effects of miR-484 inhibition on cell viability was assessed in MDA-MB-231, MDA-MB-436, BT-20, and MCF-10A cells treated with 50nM miR-484 inhibitor or control inhibitor for 24, 48, and 72h and examined by the MTS assay. The data are means ± SDs. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

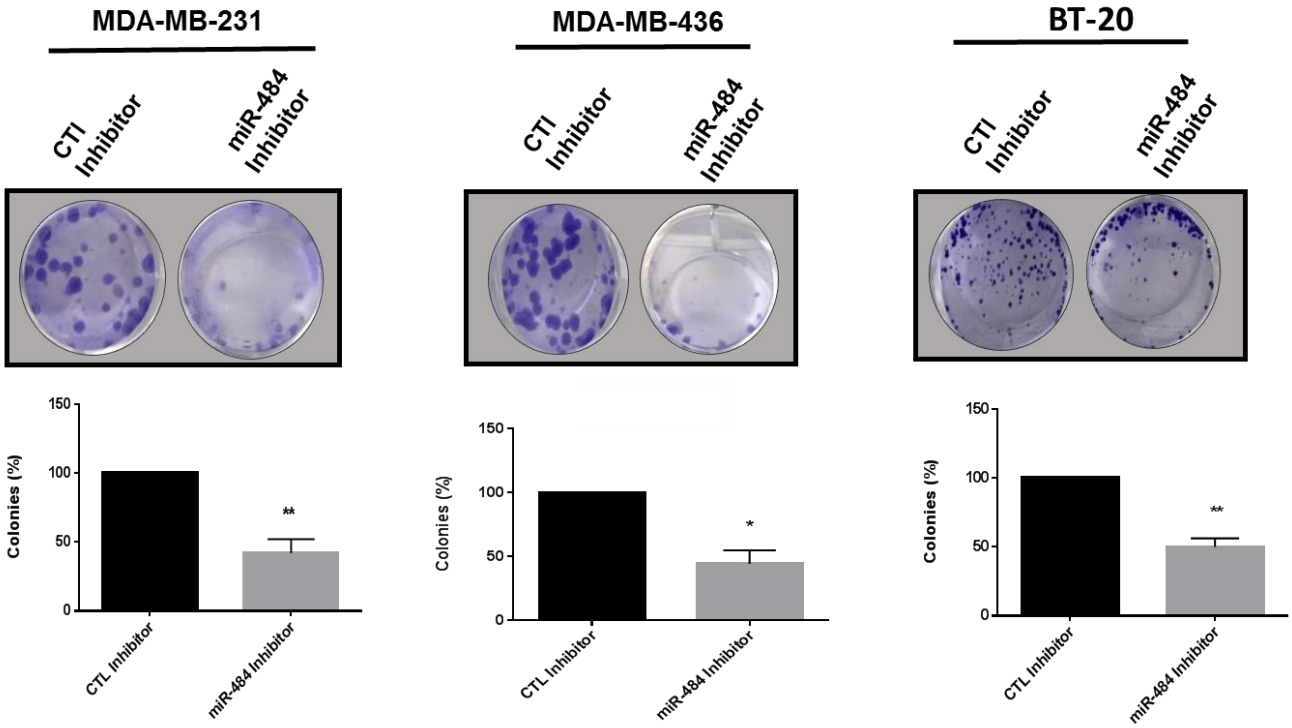


Figure 10: Inhibition of miR-484 decreases colony formation in TNBC cells. Effects of miR-484 inhibition on colony formation of MDA-MB-231, MDA-MB-436, and BT-20 cells. Colony percentage was normalized to the number of colonies formed by cells transfected with negative control miRNA. Data is expressed as means ± SDs. *p≤0.05, **p≤0.01.

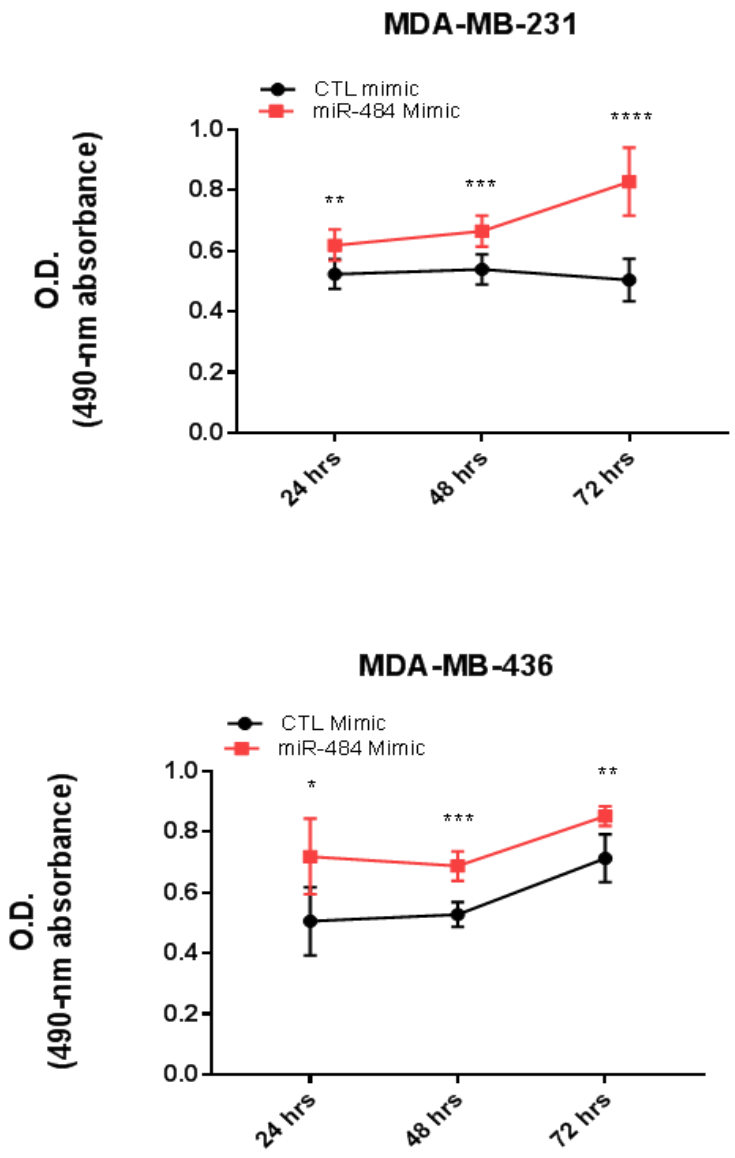


Figure 11: Ectopic overexpression of miR-484 increases cell viability in TNBC cells. MDA-MB-231 and MDA-MB-436 cells were treated with either miR-484 mimic or control mimic (50nM) for 24, 48, or 72 hrs. miR-484 treatment significantly increased cell viability in TNBC cells. Data = means ± SDs *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

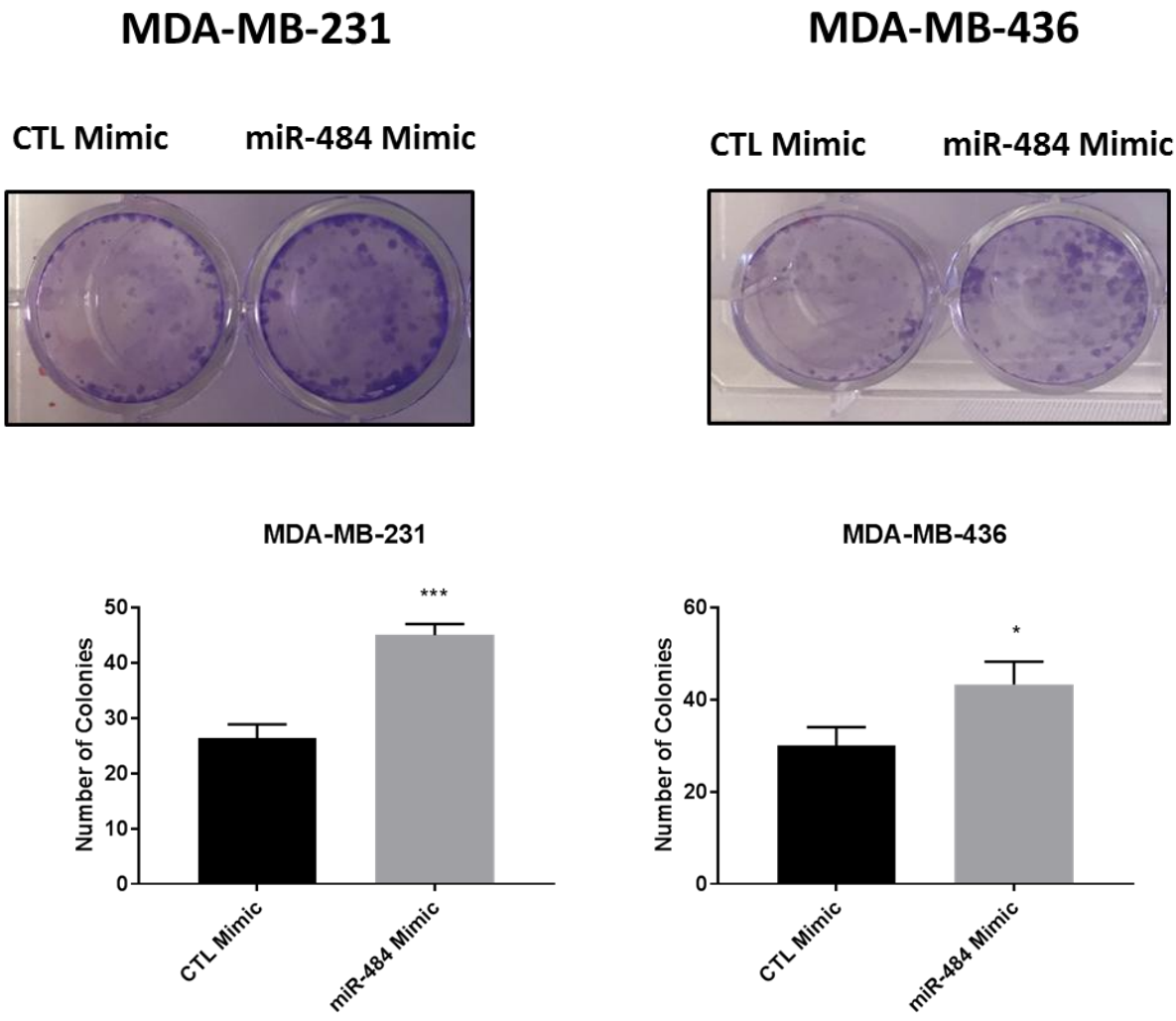


Figure 12: Ectopic overexpression of miR-484 increases proliferation in TNBC cells. miR-484 increases cell proliferation in TNBC cells. Cells were treated with miR-484 mimic or control mimic and the number of colonies were counted after 10 days using image J software. The data are means ± SDs. *p≤0.05, **p≤0.01, ***p≤0.001.

miR-484 promotes cell cycle progression in TNBC cells

Deregulation of the cell cycle is often observed in many tumors, which can result in uncontrolled cell proliferation, further promoting the process of tumorigenesis (240). The cell cycle is composed of sequential, tightly regulated events, that drive DNA replication and cell division (241). Briefly, the cell cycle is divided into 4 main phases: S phase, for DNA synthesis, M phase, in which mitosis occurs, and two gap phases G1 and G2. Some differentiated cells may also enter a period of prolonged quiescence called G0 before entering G1 (242). Transitions between different phases of the cell cycle is regulated by changes in the activity of specific cyclins and cyclin dependent kinases (CDKs) (240). In particular, the G1/S transition is a critical cell-cycle event that may be dyregulated in BC (243). This phase is predominantly under the control of cyclin D–CDK4/6 and cyclin E–CDK2, and can be negatively regulated by CDK inhibitors such as p21 and p27 (244).

Considering the effect of miR-484 on TNBC cell proliferation, we determined the role of miR-484 on cell cycle progression. TNBC cells (MDA-MB-231, MDA-MB-436, and BT-20) were treated with miR-484 inhibitor or control inhibitor and subjected to flow cytometry for cell cycle analysis. Treatment of MDA-MB-231, MDA-MB-436, and BT-20 cells with miR-484 inhibitor (100nM) significantly increased the percentage of cells in G0/G1 phases of the cell cycle (MDA-MB-231 by 6.83%, MDA-MB-436 by 9.43%, and BT-20 by 9.61%) and significantly decreased the percentage of cells in S phase (MDA-MB-231 by 13.71%, MDA-MB-436 by 9.65%, and BT-20 by 18.32%) compared to cells treated with control inhibitor miRNA (Fig. 13). Moreover, we determined the mechanism by which miR-484 regulates the cell cycle by determining the expression of G1/S phase checkpoint regulators by Western blot analysis. Our results indicated that miR-484 inhibition reduced

the expression Cyclin D1, Cyclin E, CDK 2, CDK4, CDK6, and induced cyclin dependent kinase inhibitors p21 and p27 in TNBC cells (Fig. 14), further suggesting that miR-484 increases cell proliferation and cell cycle progression in TNBC.

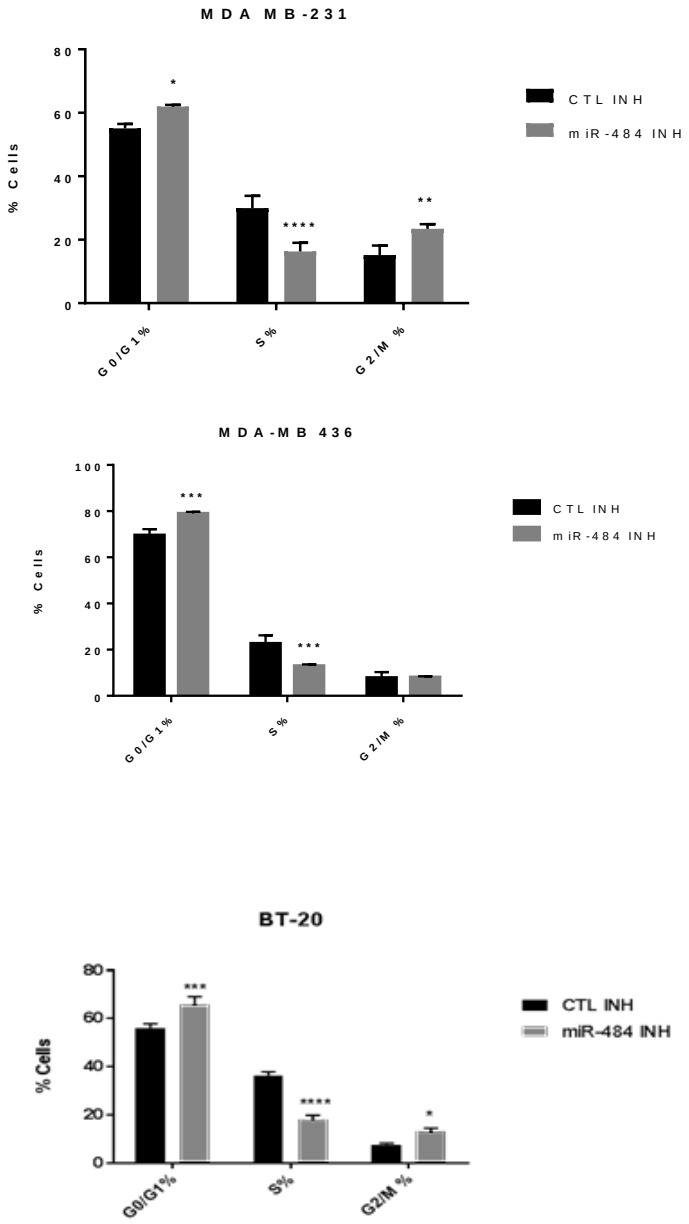


Figure 13: miR-484 inhibition induces G1/S phase cell cycle arrest in TNBC. Cell cycle analysis after treatment with miR-484 inhibitor or negative control miRNA shows that miR-484 inhibition increased the percentage of TNBC cells in G1 phase and decreased the percentage of cells in S phase. Data are represented as mean ± SD of three independent experiments. *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001.

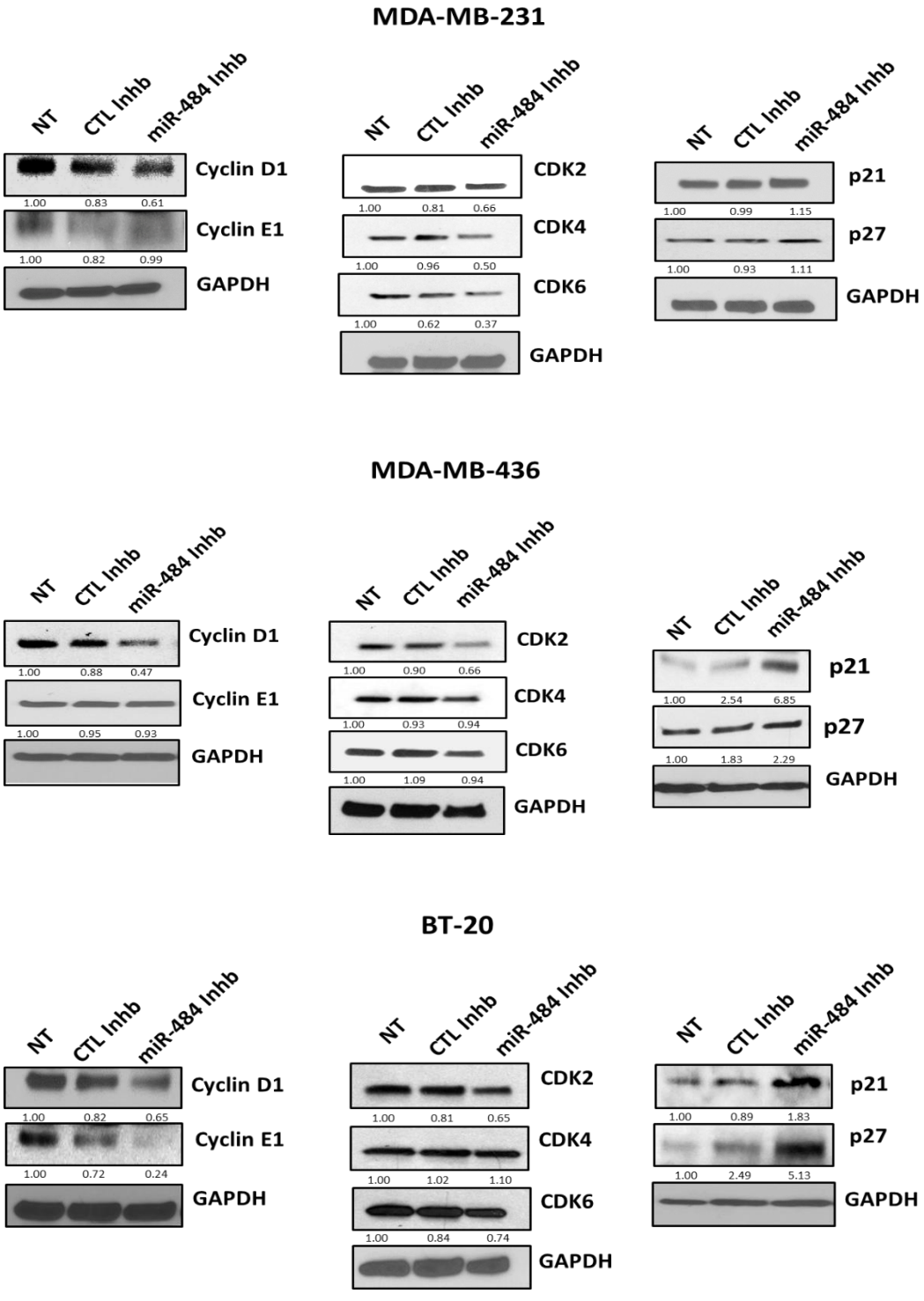


Figure 14: miR-484 regulates G1/S phase checkpoint mediators in TNBC. Expression levels of CDK2, CDK4, CDK6, cyclin D1, cyclin E1, p21, and p27 were determined by Western blot in TNBC cells (MDA-MB-231, MDA-MB-436, and BT-20) transfected with miR-484 inhibitor or negative control miRNA. GAPDH was used as a loading control.

Inhibition of miR-484 reduces cell motility and invasion in TNBC cells

Metastasis is the *primary* cause of cancer related mortality and involves dissemination of the primary tumor to the surrounding tissues and distant organs (245). Each step of the metastatic cascade is dependent on the motility and invasive capacity of tumor cells including their ability to penetrate the basement membrane, escape from the primary tumor site, migrate through the lymphatic and blood vessels, and finally intravasate or extravasate to the distant organs (246).

Considering the significant association of miR-484 with poor OS in BC patients, we determined the role of miR-484 on cell motility and invasion in TNBC cells by performing *in vitro* wound healing and invasion assays. MDA-MB-231, MDA-MB-436, and BT-20 cells were transfected with either control inhibitor or miR-484 inhibitor (100 nM) for 48h and subsequently wound healing assay was performed as previously described (237). We observed that TNBC cells treated with miR-484 inhibitor showed decreased wound healing percentage compared to control inhibitor transfected cells (MDA-MB-231 p=0.0109; MDA-MB-436 p= 0.0118, and BT-20 p=0.009) (Fig.15), suggesting that miR-484 increases TNBC cell motility. Furthermore, we determined the role of miR-484 on TNBC cell invasion using transwell invasion assay. Our results also showed that miR-484 inhibition decreased the number of invading cells compared to control miRNA-inhibitor treatment in MDA-MB-231 (p=0.0006), MDA-MB-436 (p=0.0002), and BT-20 cells (p=0.0019 (Fig. 16), suggesting that miR-484 expression increases the invasiveness of TNBC cells. Moreover, overexpression of miR-484 in MDA-MB-231 and MDA-MB-436 cells with miR-484 mimic significantly increased cell motility and invasion compared to

control cells, providing further evidence that miR-484 promotes cell motility and invasion (Figures 17 and 18).

Regulation of cell migration and invasion in cancer cells is mediated by signaling pathways, including SRC and focal adhesion kinase pathway (FAK) (247). The SRC family of non-receptor protein tyrosine kinases are known to play critical roles in cell proliferation, migration/invasion, and metastasis in many cancers including BC (248). Src functions by mediating multiple downstream effects of receptor tyrosine kinases, such as the EGFR family (249, 250), and its expression is reported to be elevated in many solid tumors, including BC (251) (249). Increased Src activity can be attributed to an increase in its transcription or to overexpression of its upstream regulators such as EGFR, HER2, platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), as well as integrins, and focal adhesion kinase (FAK) (252-254). Focal adhesion kinase (FAK), is another critical mediator of cell adhesion and migration, which can be recruited by intergrins to form a dual complex with Src that promotes cell motility and survival (255). Thus, both Src and FAK may be important therapeutics targets in tumorigenesis (256). Figure 19 shows that miR-484 inhibition in MDA-MB-231, MDA-MB-436, and BT-20 cells results in a significant reduction in both p-SRC (Tyr-416) and p-FAK (Tyr-397) levels which is consistent with the previously described interaction between Src and FAK in tumor cells (256).

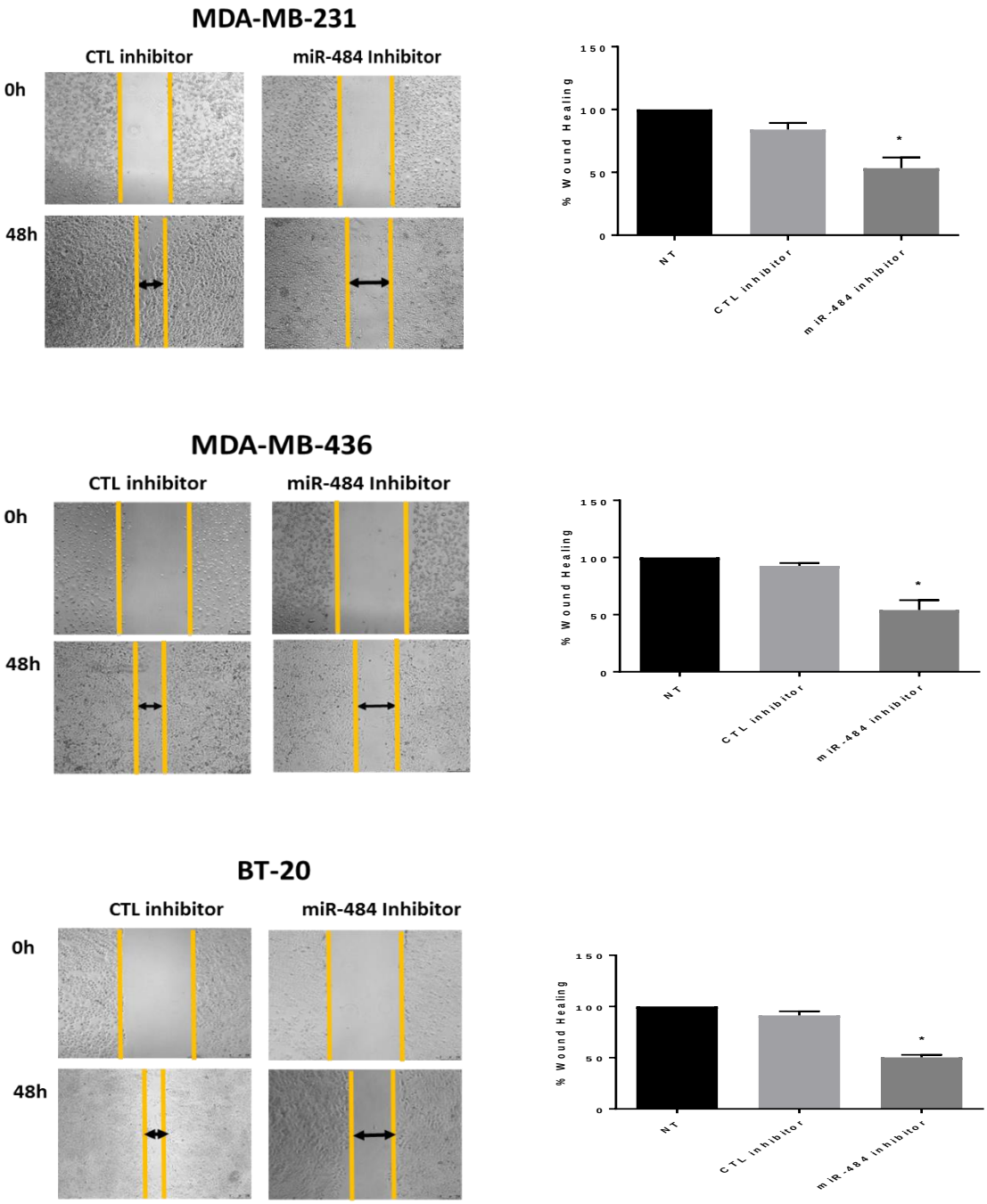


Figure 15: miR-484 inhibition reduces cell motility in TNBC. MDA-MB-231, MDA-MB-436, and BT-20 cells were treated with miR-484 inhibitor, or negative control inhibitor (100nM), or did not undergo transfection (NT), and cell motility was assessed by the wound healing assay. Images are shown at 0 and 48h time points. Wound closure percentage was normalized to untreated cells. Data is shown as means ± SDs.

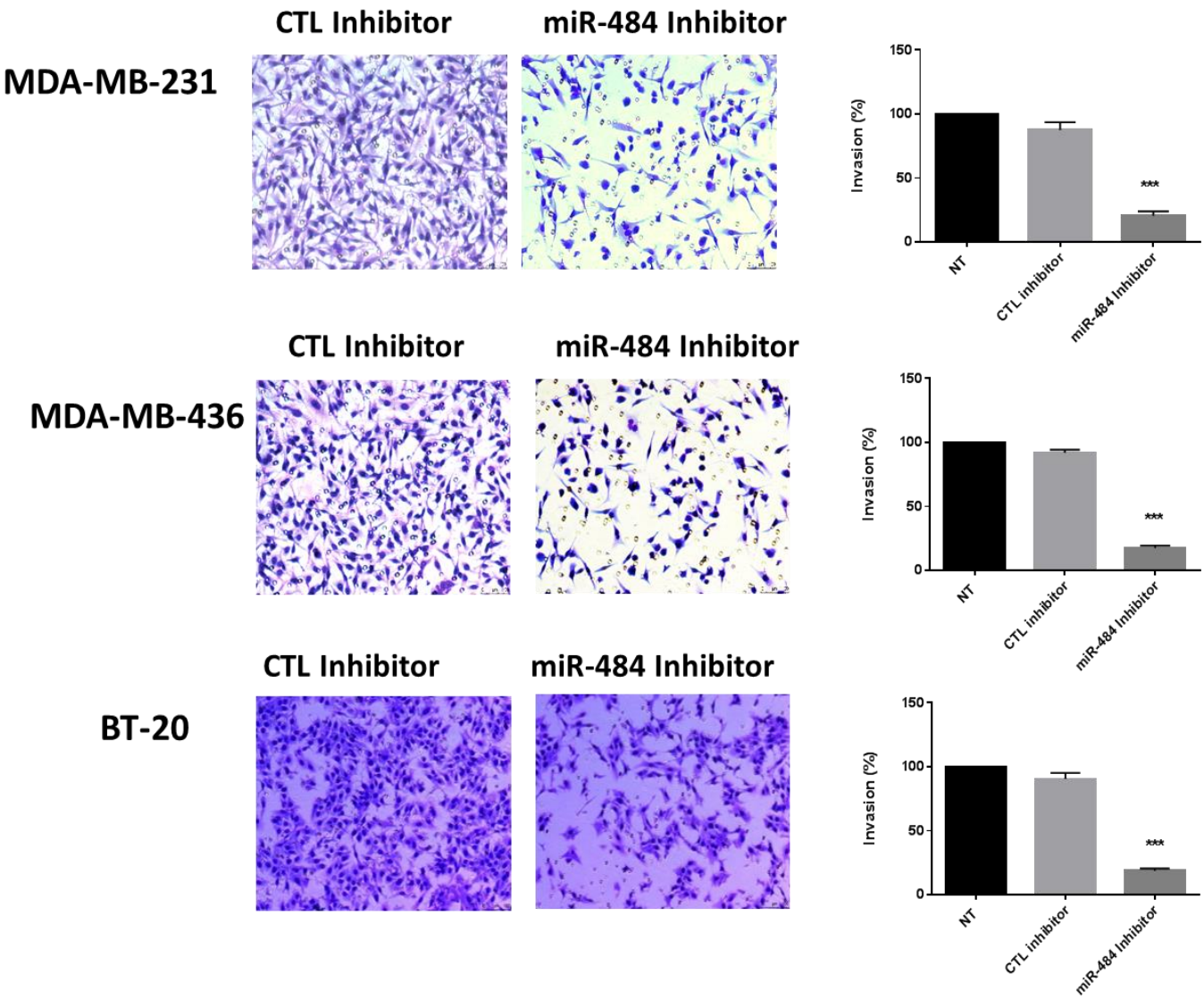


Figure 16: miR-484 inhibition reduces TNBC cell invasion. MDA-MB-231, MDA-MB-436, and BT-20 cells were treated with miR-484 inhibitor or negative control inhibitor miRNA (100nM) or not treated (NT). After 48h of transfection, cells were transferred to matrigel-coated transwell inserts. 24h later the invaded cells were quantified and normalized to the number of invaded cells from the NT group. Data represents means ± SDs from triplicate experiments (***p≤0.001).

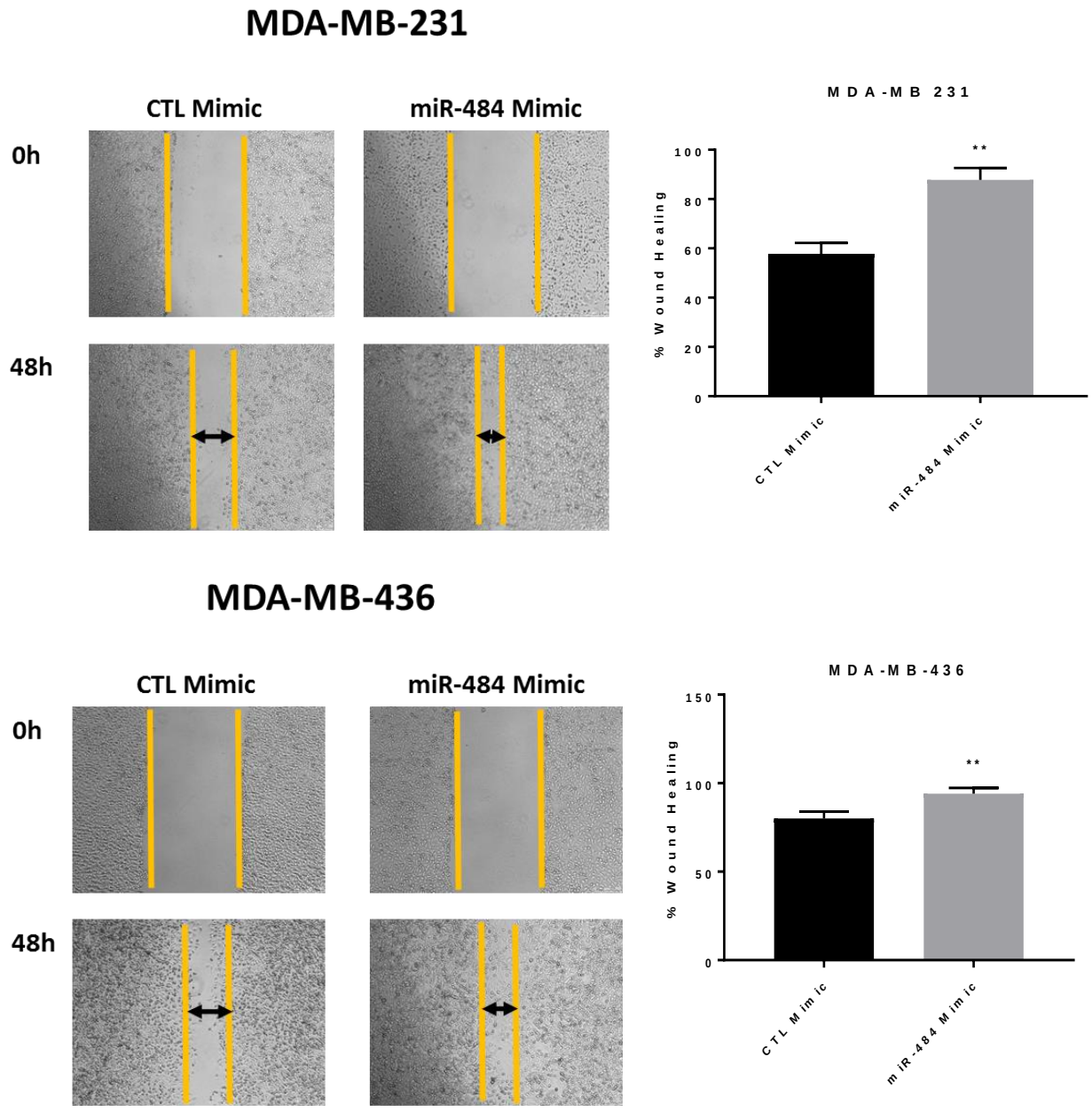


Figure 17: miR-484 overexpression increases cell motility and invasion in TNBC. MDA-MB-231, MDA-MB-436 cells were treated with miR-484 mimic, or negative control mimic, and cell motility was assessed by the wound healing assay. Images were taken at 0 and 48h. The percentage wound healing was quantified and shown on the right panel as means ± SDs (**p≤0.01).

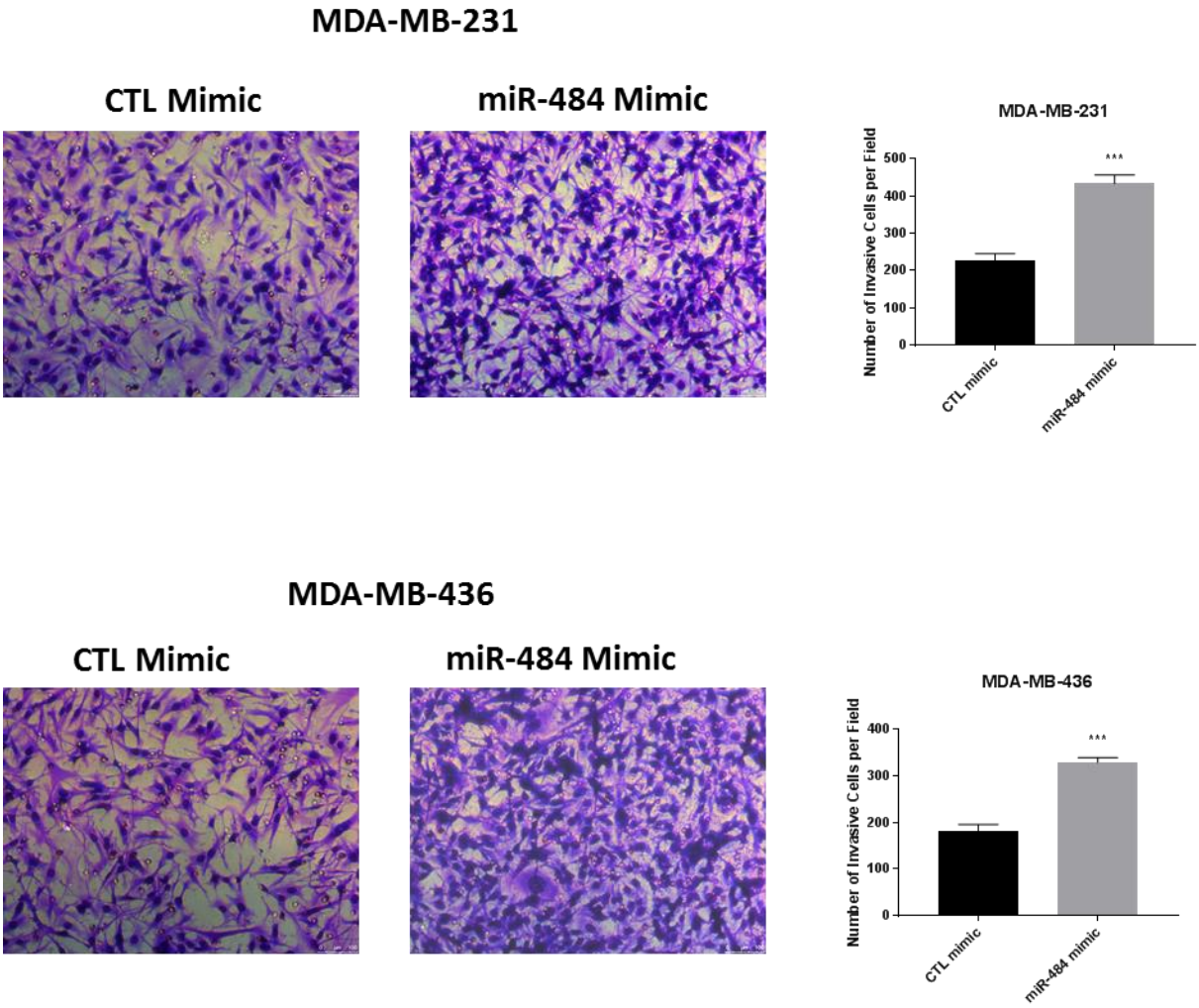


Figure 18: Ectopic over-expression of miR-484 increases invasion in TNBC cells. MDA-MB-231, MDA-MB-436 cells were transfected with miR-484 mimic or control mimic for 48h and transferred to matrigel-coated transwell inserts and incubated for an additional 24h. The number of invaded cells per field was quantified and shown as mean ± SDs from triplicate experiments (***p≤0.001).

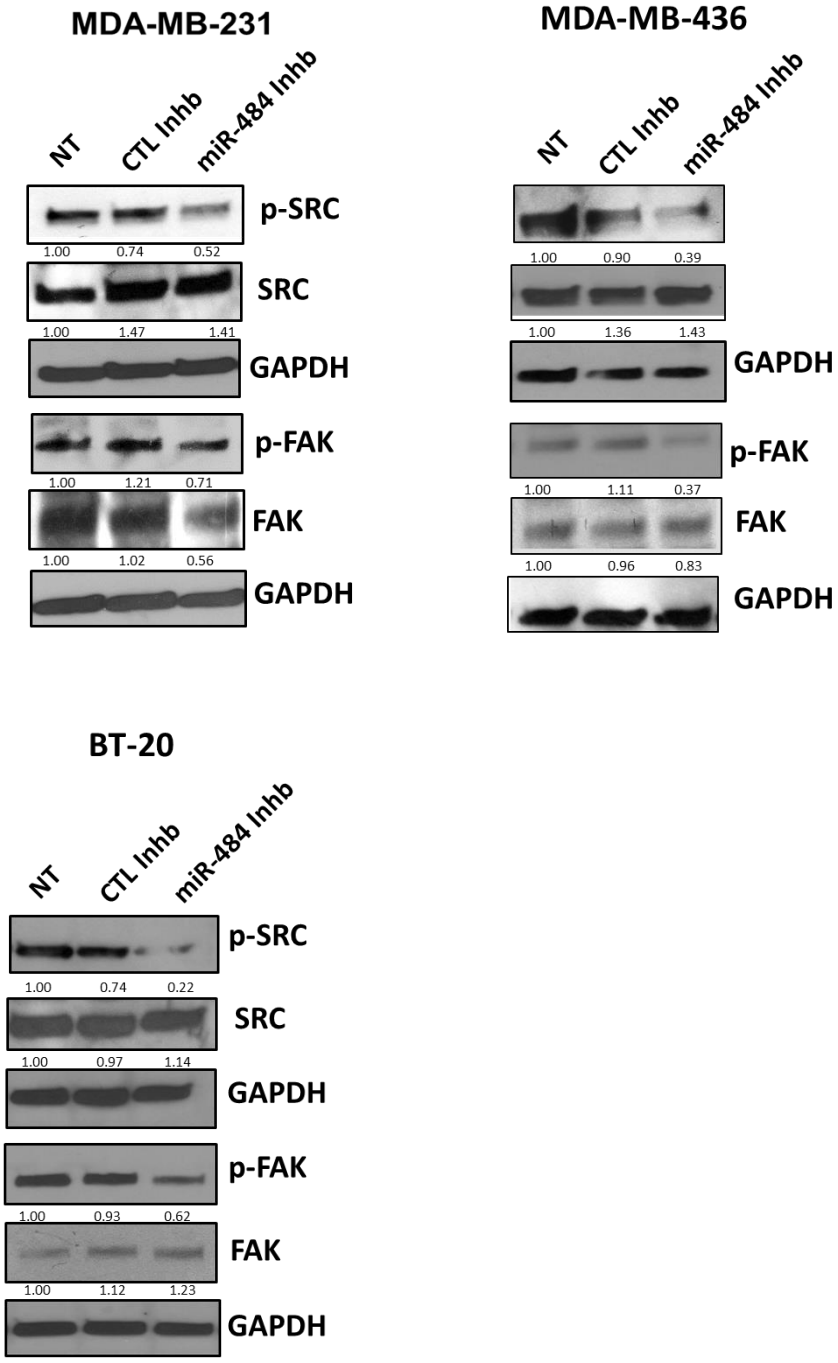


Figure 19: miR-484 inhibition reduces p-SRC and p-FAK expression in TNBC cells. Expression levels of p-SRC, SRC, p-FAK, FAK were determined by Western blot in TNBC cells treated with miR-484 inhibitor (100nM) for 48h or negative control miRNA. GAPDH was used as a loading control.

miR-484 Inhibition induces apoptosis in TNBC cells

Given the observed effect of miR-484 inhibition on reducing cell growth in TNBC, we subsequently investigated its role in programmed cell death. Programmed cell death I, or apoptosis, is mainly induced by two main mechanisms: intrinsic or mitochondrial apoptosis; and extrinsic or death receptor mediated apoptosis (257). Both pathways lead to the activation of the caspase family of cysteine proteases, which eventually leads to specific morphological features, typical of apoptosis, such as chromatin condensation, DNA fragmentation, membrane blebbing, and finally complete cell lysis (258). Many studies have shown that aberrantly expressed miRNAs are related to apoptosis evasion in tumor progression and tumorigenesis and drug resistance (259).

MDA-MB-231, MDA-MB-436 and BT-20 cells were treated with either miR-484 inhibitor or control inhibitor for 48h. Apoptosis following miR-484 inhibition was determined by Annexin V/ Propidium Iodide (PI) staining, followed by flow cytometry (FACS) to determine the percentage of apoptotic cells. The percentage of both early and late apoptotic cells was significantly higher in TNBC cells treated with miR-484 inhibitor compared to control cells (MDA-MB-231 $p=0.0002$, MDA-MB-436 $p=0.0014$, BT-20 $p=0.0036$), suggesting that miR-484 inhibition induces cell death (Fig. 20). Furthermore, we confirmed apoptosis induction by determining the expression of apoptosis-related proteins such as PARP, caspase-3, caspase-2, caspase-8 by Western blot (Fig. 21).

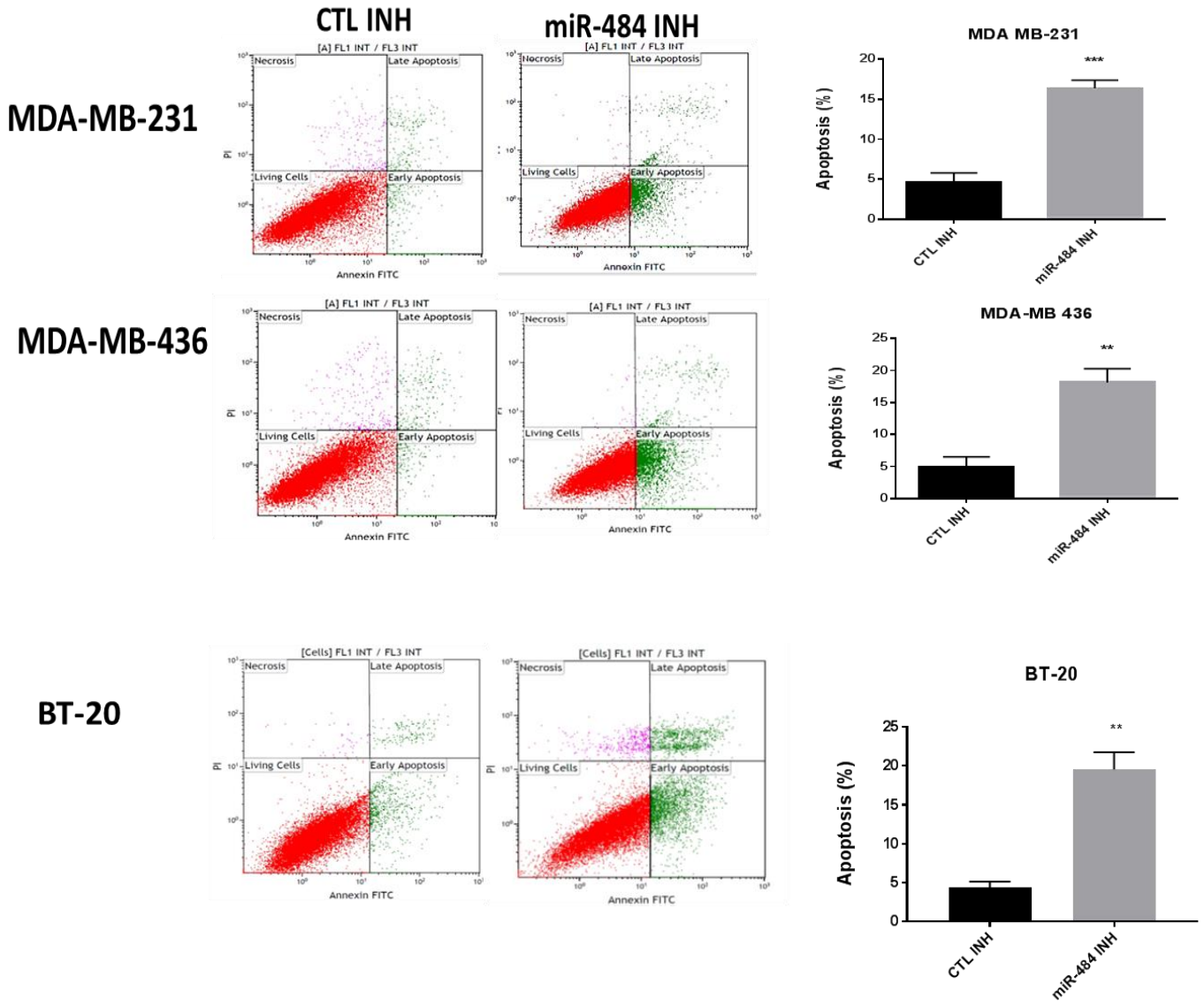


Figure 20: Inhibition of miR-484 induces apoptosis in TNBC cells. TNBC cells were treated with either miR-484 inhibitor or control inhibitor (100nM) for 48h, and stained by Annexin V/PI followed by flow cytometry to determine the number of apoptotic positive cells. Representative percentages are the sum of both early and late apoptosis. Data are represented as means ± SD. *p≤0.05. All experiments were independently triplicated.

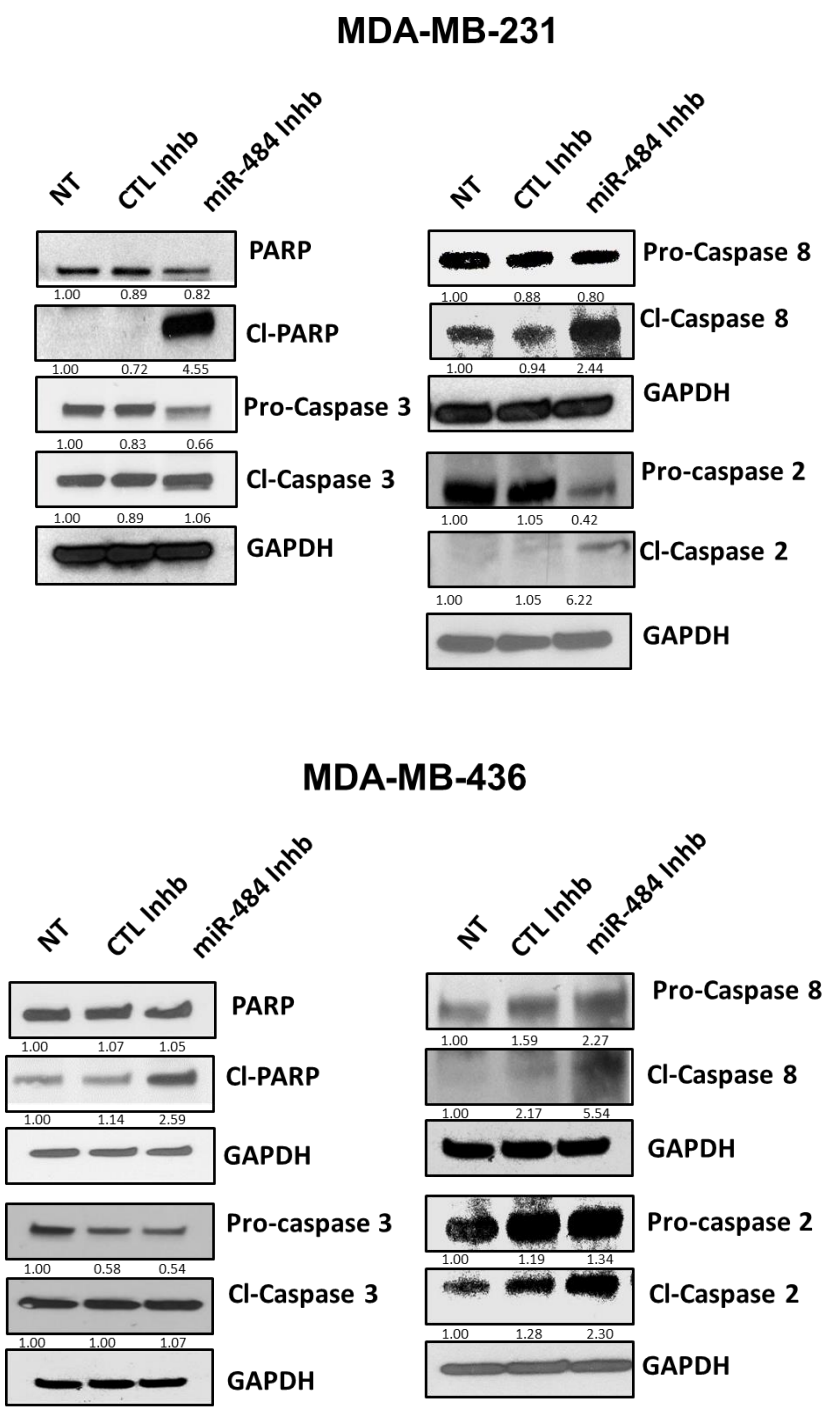


Figure 21: miR-484 inhibition regulates the expression of apoptotic markers in TNBC. Expression levels of apoptotic markers (PARP, Caspase-3, Caspase-2, Caspase-8) in MDA-MB-231 and MDA-MB-436 cells were detected by WB after 48h transfection with miR-484 inhibitor or negative control inhibitor miRNA (100nM). GAPDH was used as a loading control.

Aim 2:

HOXA5 is a predicted target for miR-484

We retrieved miRNA-target interaction predictions for miR-484 from miRWalk2.0 (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) that integrates results from twelve different predictive algorithms (DIANA-microTv4.0, DIANA-microT-CDS, miRanda-rel2010, mirBridge, miRDB4.0, miRmap, miRNAMap, PicTar2, PITA, RNA22v2, RNAhybrid2.1, and Targetscan6.2). We selected the 147 targets which were predicted by at least nine algorithms (3/4 of the total number of programs checked). Among them we chose the 16 experimentally validated targets (listed in Appendix Table 5) as retrieved from miRWalk2.0. Based on a literature search, we focused on *HOXA5* as it was previously shown to have a tumor suppressive role in BC (260, 261). The predicted binding site for miR-484 on HOXA5 3'-UTR is shown in Figure 22, and this binding site was also found to be highly conserved among many species (Fig. 23).

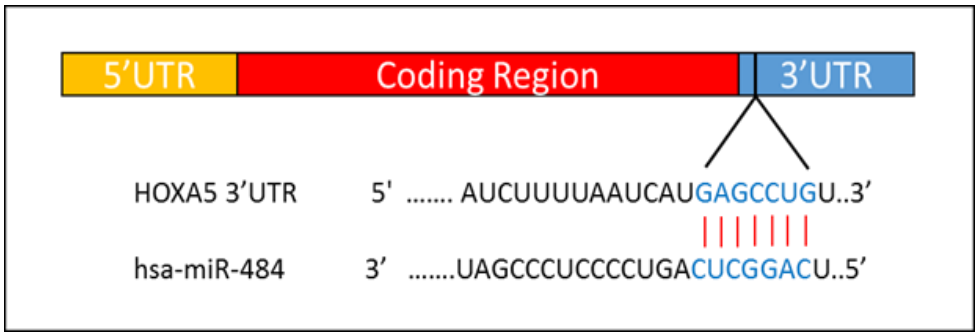
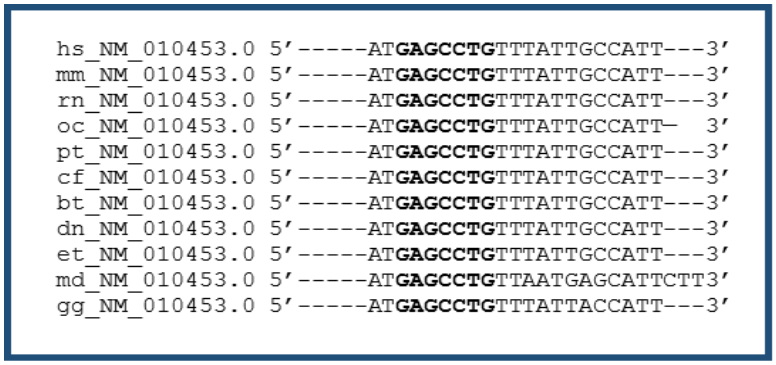
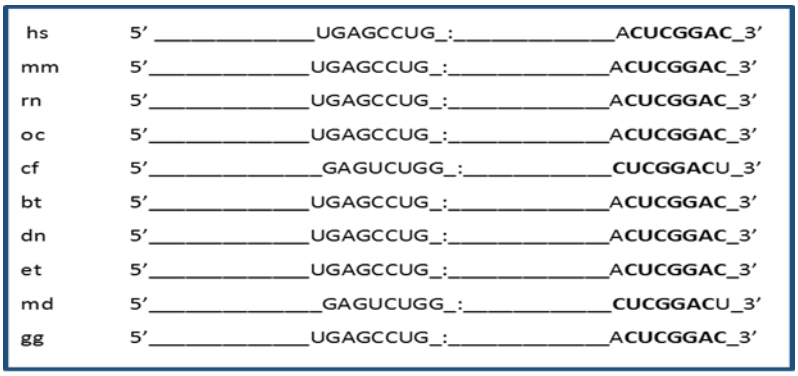


Figure 22: Predicted binding site of miR-484 and HOXA5 3'-UTR.



HOXA5



miR-484

Figure 23: miR-484 and HOXA5 binding sites are highly conserved across many species.

High miR-484 expression is correlated with low HOXA5 expression in BC patients & cell lines

The homeobox genes (HOX genes) are composed of 39 members, organized in four clusters (A, B, C, and D), located on chromosomes 7, 17, 2, and 12, respectively (262). *HOXA5* belongs to the cluster A family of HOX regulatory genes. The homeobox sequence (183 nt) of HOX genes encode homeoproteins that can act as transcription factors, to either activate or repress the expression of downstream effector target genes (263, 264). Numerous studies during the last several decades, have highlighted the importance of HOX genes in normal tissues, as well as in many clinical diseases and carcinomas (265). The HOX family genes play fundamental roles in the anterior-posterior patterning during embryonic development (266, 267). They have also been shown to be aberrantly expressed and/or mutated in many cancers, including leukemia, colon, prostate, breast, and ovarian cancers (268). In particular, homeobox A5 (HOXA5) has been shown to be a key regulator of cell differentiation and organogenesis. HOXA5 has been implicated in the development of the axial skeleton, as wells are respiratory system, mammary glands, and digestive tracts (269). In the context of BC, HOXA5 expression was found be reduced in more than 60% of BC cell lines, partially due to hypermethylation of its promoter region (261). Additionally, HOXA5 has been shown to induce apoptosis, both in a p-53 dependent or caspase 2 and 8 dependent manner in BC cells (260, 261). Furthermore, the loss of HOXA5 expression was shown to lead to the functional activation of Twist, a negative regulator of p53 (270), resulting dysregulation of the cell cycle and promotion of breast carcinogenesis (271). Collectively, these studies indicate that HOXA5 may serve as a tumor suppressor gene in BC.

To evaluate the potential interaction between miR-484 and HOXA5, we analyzed the TCGA database of BC patients (n=833) and performed a spearman rank correlation ($p \leq 0.00001$, $R=0.31$) and found that miR-484 was inversely correlated with *HOXA5* expression in patients' tumors (Fig. 24). Furthermore, HOXA5 protein expression was found to be reduced in all BC cell lines (MDA-MB-231, MDA-MB-436, MDA-MB-453, BT-20, MDA-MB-453, MDA-MB-361, MCF-7, and T-47) compared with normal immortalized breast epithelial cell lines (MCF-10A and HMEC) by Western blot analysis (Fig. 25), suggesting an inverse relationship between *HOXA5* and miR-484 expression, and the possibility that miR-484 regulates *HOXA5* mRNA expression.

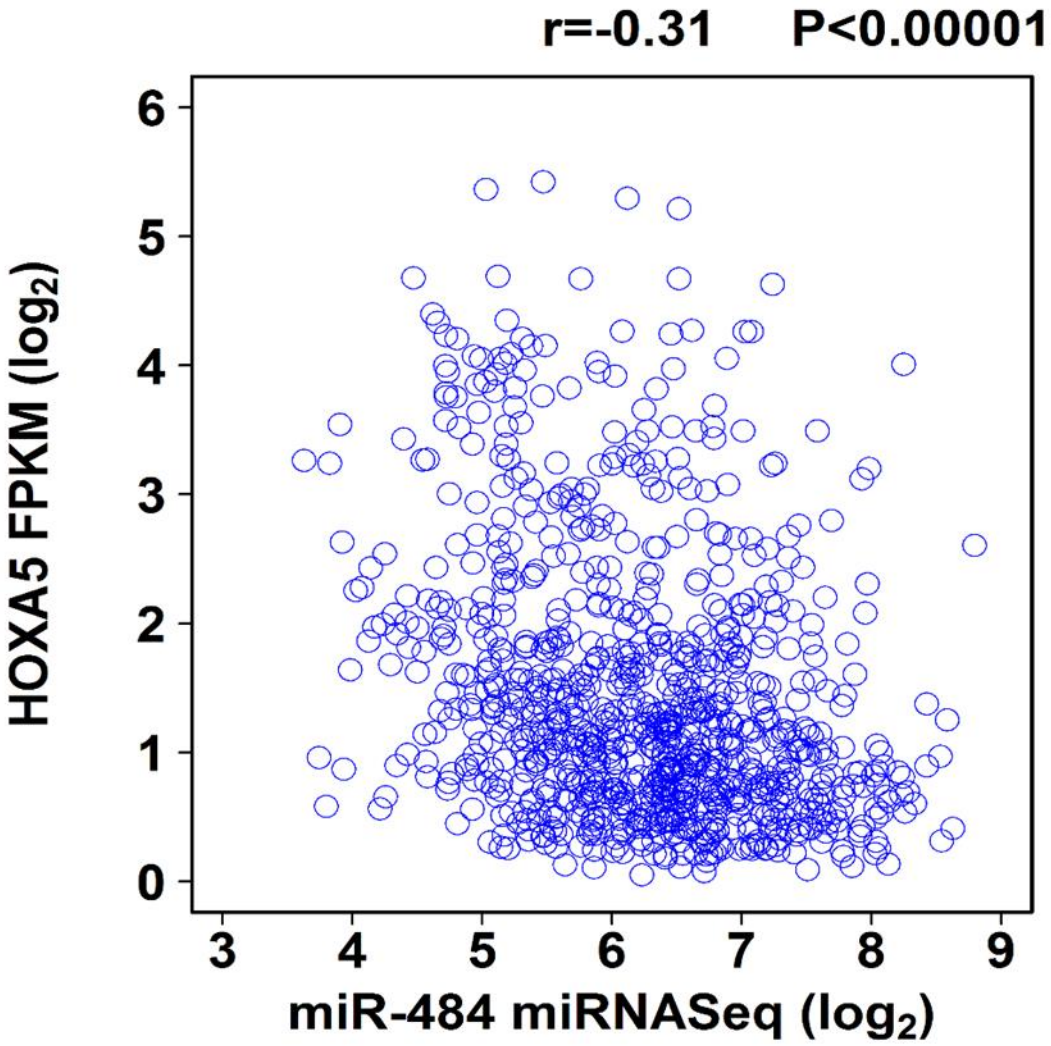


Figure 24: miR-484 and HOXA5 expression levels are inversely correlated in BC patients. Spearman correlation analysis showed a negative and significant correlation between miR-484 and its target gene HOXA5 in BC patients (n=833). R=0.31, p≤0.0001.

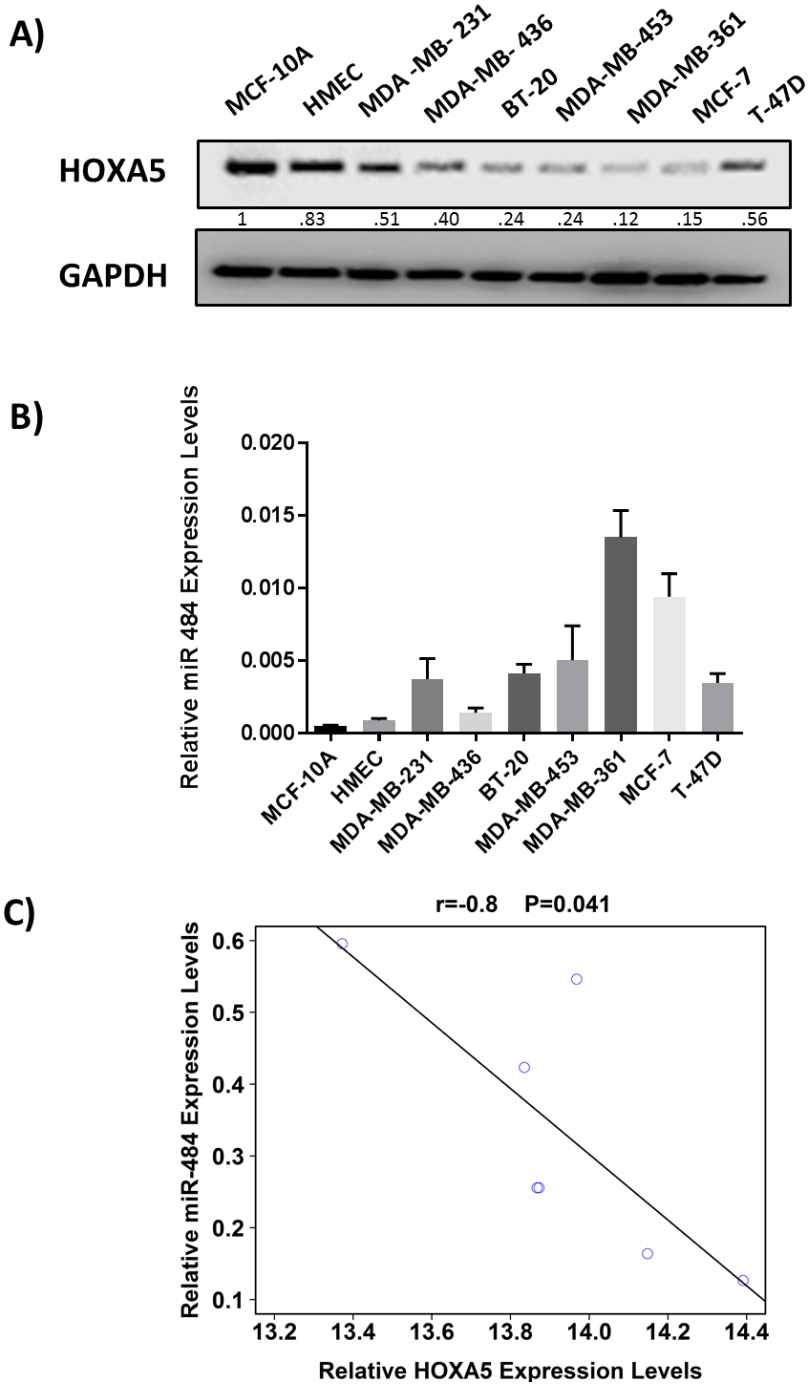


Figure 25: miR-484 and HOXA5 expression levels are inversely correlated in BC cell lines. A) HOXA5 expression levels are lower in BC cell lines compared to normal breast epithelial cells MCF-10A and HMEC. Basal HOXA5 expression levels were analyzed by WB and GAPDH was used as loading control. B) miR-484 basal expression levels were assessed by qRT-PCR. U6 was used as internal control. C) Pearson correlation analysis showing a negative and significant correlation between miR-484 and HOXA5 in BC cells. R=0.8, p=0.041.

miR-484 directly binds to the 3'-UTR of HOXA5 to regulate its expression

miRNAs are involved in post-transcriptional regulation of gene expression mainly by directly binding of the 3'-UTR of their target mRNAs to negatively regulate their expression (72). To evaluate the effect of miR-484 on HOXA5 gene and protein expression in TNBC cells, we transfected MDA-MB-231, MDA-MB-436, and BT-20 cells with miR-484 inhibitor (100nM) or negative control inhibitor for 48h. Inhibition of miR-484 resulted in significant reduction in HOXA5 protein and mRNA expression in the cell lines detected by Western blot and qRT-PCR analysis, respectively (Figures 26 and 27). Collectively, these results suggests that miR-484 suppresses HOXA5 protein and mRNA expression levels in TNBC cells.

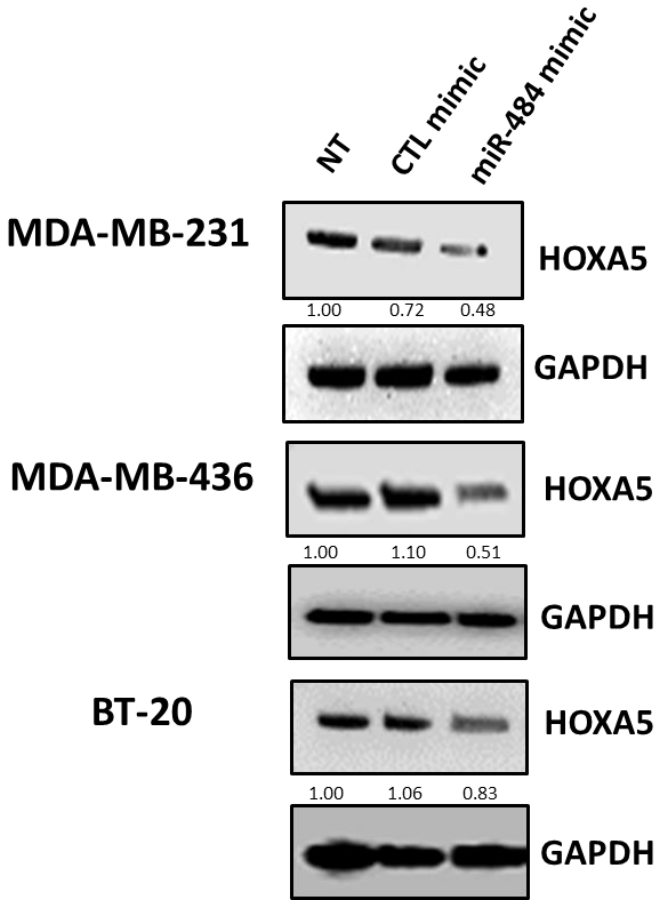


Figure 26: miR-484 reduces HOXA5 protein expression levels in TNBC cells.
MDA-MB-231, MDA-MB-436, and BT-20 cells were treated with miR-484 mimic or control mimic (100nM) for 48h and cell lysates were analyzed for HOXA5 expression by WB analysis. GAPDH was used as loading control.

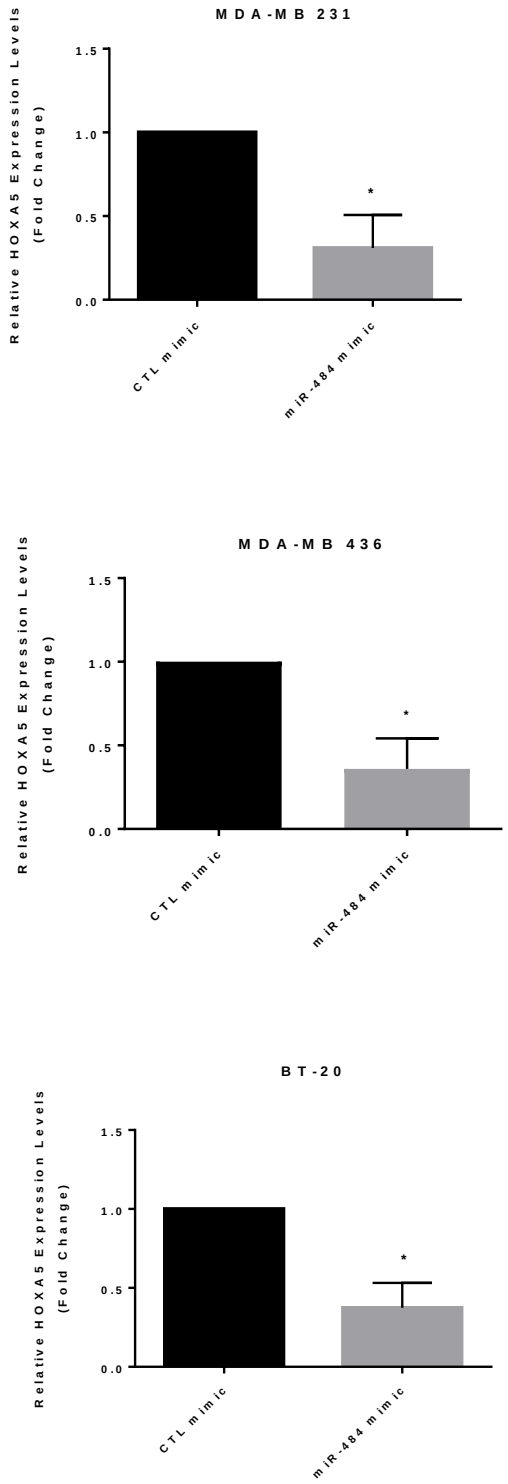


Figure 27: miR-484 reduces HOXA5 mRNA expression levels in TNBC cells. Cell lines were analyzed for HOXA5 mRNA levels by qRT-PCR 48h after miR-484 transfection. Data is represented as fold change normalized to GAPDH expression levels. *p≤0.05.

miR-484 directly binds to HOXA5 3'-UTR to negatively regulate its expression

To further prove the direct role of miR-484 on *HOXA5* mRNA regulation we identified the consensus sequences on the 3′-UTR region of the *HOXA5* gene for binding to miR-484 and performed a luciferase gene reporter assay. The human wild type (WT) HOXA5 3'-UTR was cloned upstream of a firefly luciferase gene in a reporter vector (pEZX-MT06) plasmid. A similar vector containing the mutated sequence (GAGCCTG> GCTACAG) in the miR-484 binding site of the HOXA5 3'-UTR-mut (pMSCV–HOXA5-3'-UTR-mut) was used as a negative control. The resulting plasmids were separately transfected into MDA-MB-231 and MDA-MB-436 cells along with miR-484 mimic or negative control miRNA (100nM). Firefly luciferase activity was measured and normalized to Renilla Luciferase activity. As shown in Figure 28, cells treated with miR-484 and expressing the WT 3'-UTR of *HOXA5* had significant reduction in luciferase activity compared to cells treated with control miRNA (MDA-MB-231 $p=0.0021$ and MDA-MB-436 $p\leq0.0001$). Moreover, cells expressing the pEZX-MT06 miRNA reporter vector containing the mutated miR-484 binding site (pMSCV–HOXA5-3'-UTR-mut) showed no significant difference in luciferase activity between miR-484 and control miRNA transfections. Thus, our findings suggest that miR-484 binds specifically to the WT *HOXA5* 3'-UTR to negatively regulate its mRNA expression.

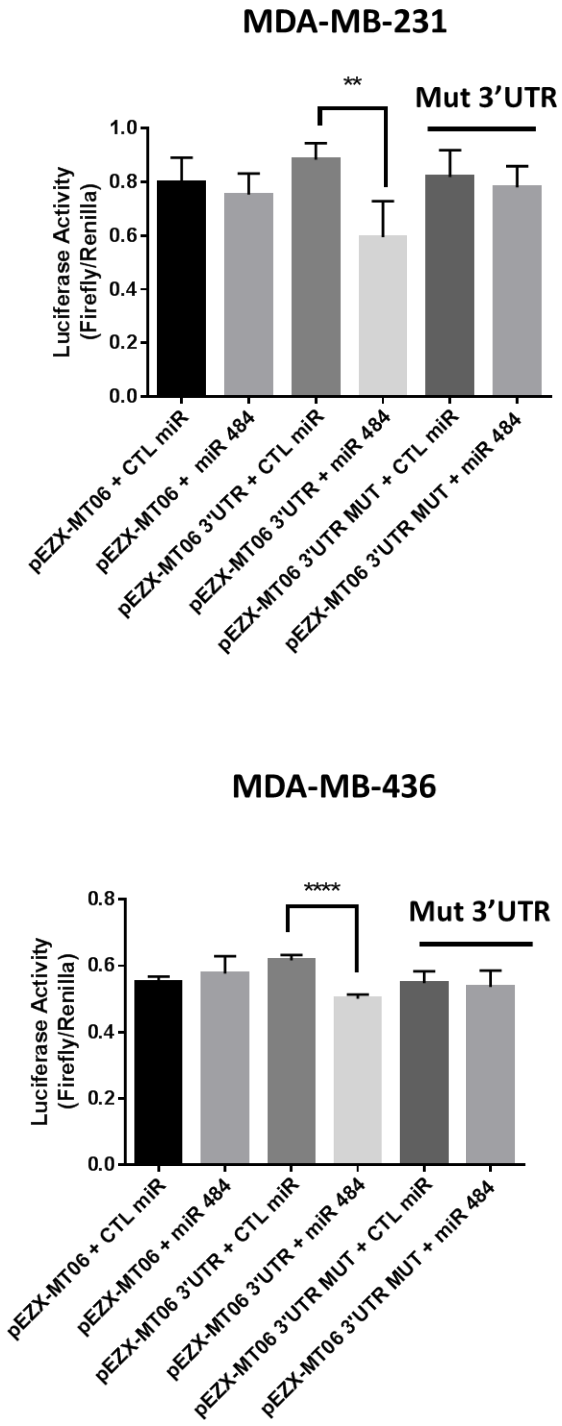


Figure 28: miR-484 directly binds to HOXA5 3'-UTR in TNBC cells. Luciferase reporter assay showing that miR-484 directly binds to the 3'-UTR of HOXA5 luciferase reporter in MDA-MB-231 and MDA-MB-436 cells. Firefly luciferase activity was normalized to endogenous Renilla luciferase activity. Data are represented means ± SDs for three independent experiments. *p≤0.05

miR-484 mediates its effects through inhibition of HOXA5 tumor suppressor in TNBC cells

Reduced cell proliferation in cancer is often associated with concomitant activation of cell death pathways and inhibition of cell cycle progression (272). We have shown that miR-484 inhibition significantly induces apoptosis and promotes G1/S cell cycle arrest in TNBC cells. Furthermore, HOXA5 expression has been shown to lead to activation of cell death pathways (260, 261) and aberrant cell cycle regulation (271). Therefore, we examined the role of HOXA5 in mediating apoptosis in response to miR-484 inhibition. First, we transduced MDA-MB-231 cells with *HOXA5*-expressing lenti-based vector and control empty-vector that lack *HOXA5* gene. Light microscopy revealed that *HOXA5* overexpressing cells displayed typical apoptotic morphology such as cell shrinkage and appeared denser compared to controls (273) (Fig. 29). Furthermore, *HOXA5* overexpressing cells showed increased apoptosis by FACS, which was reversed by expression of miR-484 mimic (Fig. 30). This finding was also associated with a reduction in HOXA5 expression levels (Fig. 31). Moreover, *HOXA5* overexpression recapitulated the effects of miR-484 inhibition on apoptotic markers such as PARP and capase-3 and G1/S cell cycle regulators such as Cyclin D1, Cyclin E1, CDK4, and induced the expression of CDK inhibitors p21 and p27 (Fig. 32). Overall, our findings suggest that miR-484 could promote TNBC cell survival through downregulation of *HOXA5* tumor suppressor gene.

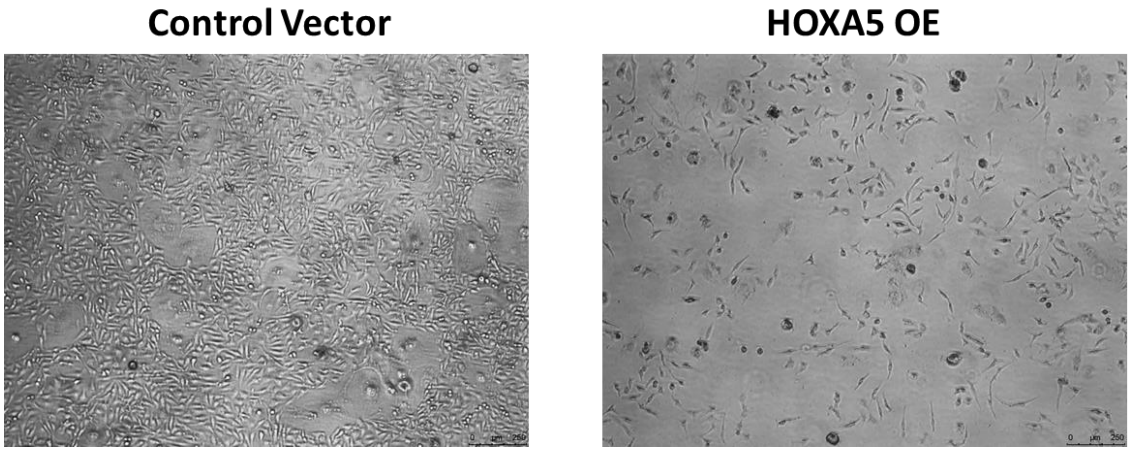


Figure 29: HOXA5 overexpressing cells display typical apoptosis morphological features. MDA-MB-231 cells were transduced with lentiviral expression vector incorporating WT-HOXA5 (NM_019102.3) (HOXA5-OE) for overexpression of HOXA5 or the mock empty vector (EV) (LPP-NEG-Lv103) and examined under the light microscope 48h after transduction. HOXA5 overexpressing cells appeared smaller and denser compared to cells expressing control empty vector. Magnification 4X.

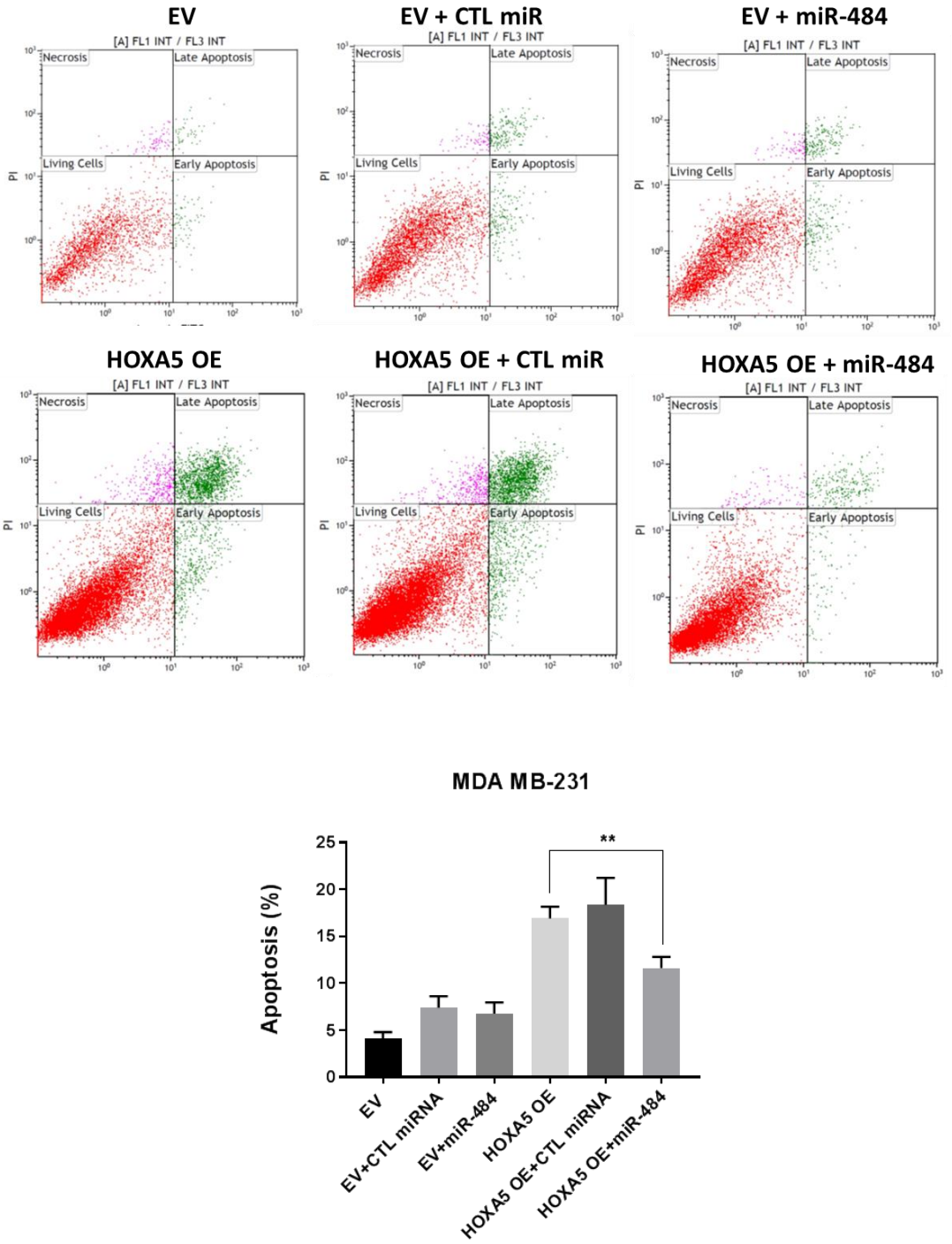


Figure 30: miR-484 reverses HOXA5 induced apoptosis. MDA-MB-231 cells over-expressing either HOXA5 or mock empty vector were co-transfected with either miR-484 mimic or control mimic (100nM) and collected after 48h. Cells were stained with Annexin/PI for FACS analysis of the apoptotic positive cells. Data are represented as mean ± SD. **p≤0.01. All experiments were independently triplicated.

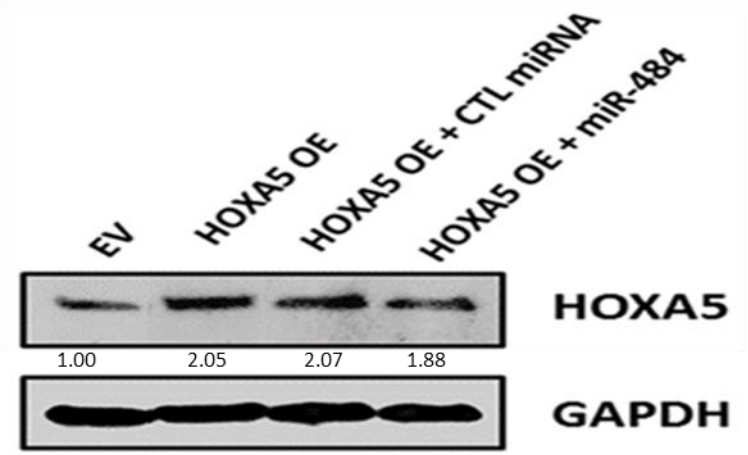


Figure 31: miR-484 partially reduces HOXA5 expression in HOXA5 overexpressing cells. MDA-MB-231 cells overexpressing HOXA5 (HOXA5-OE) or expressing control empty vector (EV) were treated with either miR-484 mimic or control mimic (100nM) and collected after 48h. Cell lysates were analyzed for HOXA5 expression levels by Western blot. GAPDH was used an internal control.

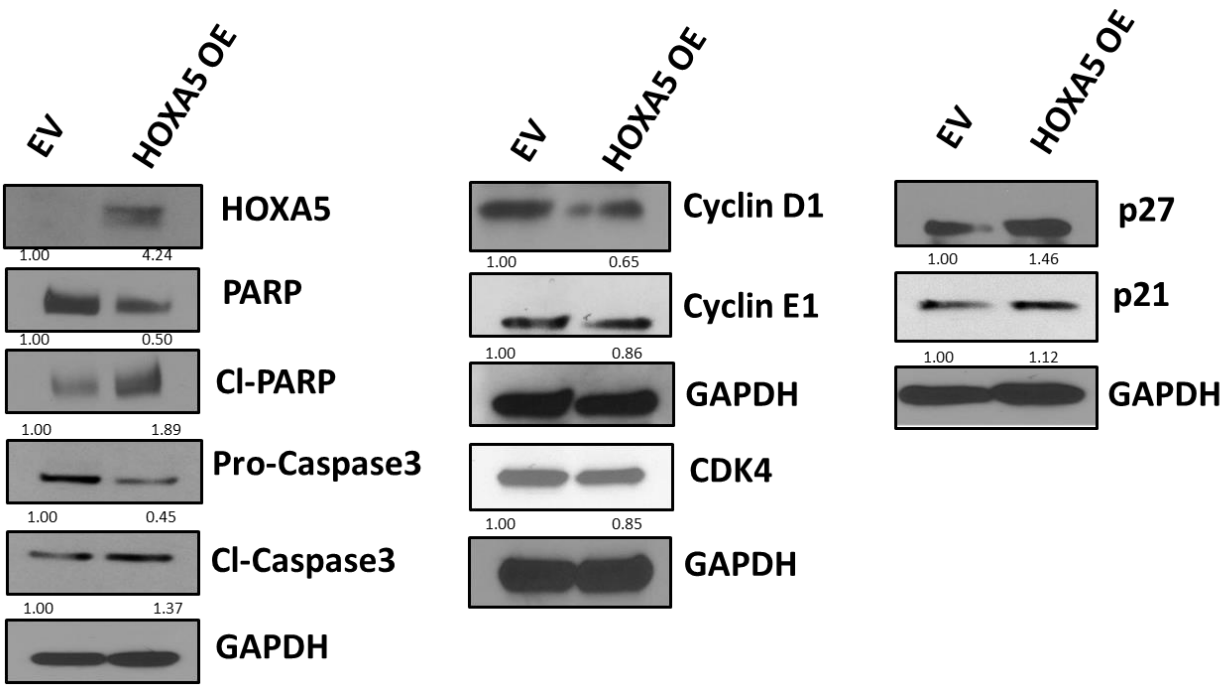


Figure 32: HOXA5 overexpression recapitulates miR-484 inhibition on apoptosis and cell cycle markers. MDA-MB-231 cells overexpressing HOXA5 lentiviral vector (HOXA5-OE) or expressing control empty vector (EV) were treated with either miR-484 mimic or control mimic (100nM) and collected after 48h. The levels of HOXA5, PARP, caspase-3, Cyclin D1, Cyclin E1, p27, and p21 were determined by WB. GAPDH was used as loading control.

miR-484 alters multiple proteins/cancer signaling pathways in TNBC:

To determine the potential signaling pathways that are regulated by miR-484 in TNBC, we performed a reverse phase protein array (RPPA) analysis of MDA-MB-231 cells treated with either miR-484 mimic or control miRNA. Samples were probed with 304 proteins, including total and phospho-proteins. Among the proteins that were probed for, we found a total of 55 proteins that were significantly upregulated with miR-484 overexpression compared to controls, and a total of 61 proteins that were significantly downregulated with miR-484 treatment compared to controls. Significantly altered proteins after miR-484 transfection are shown in the heat map in Figure 33. Of particular interest to us, we observed significant downregulation of caspase-8 (FCH=-1.09 p-value=0.11), p53 (FCH=-1.074 p-value=0.014), Bax (FCH=-1.04 p-value=0.031) with miR-484 overexpression, and significant upregulation of cyclin D1 (FCH=+1.18 p-value=0.012) with miR-484 treatment. Furthermore, our ingenuity pathway analysis showed that miR-484 overexpression in MDA-MB-231 cells resulted in alteration in many signaling pathways related to cell proliferation, apoptosis, and cell cycle regulation (Fig. 34), which is consistent with our previously mentioned findings. Figure 35 summarizes the findings from our IPA analysis and illustrates the interaction between miR-484 and HOXA5 in regulating certain apoptosis related proteins such as PARP and caspases as well as cell cycle regulators such as cyclins and CDKs.

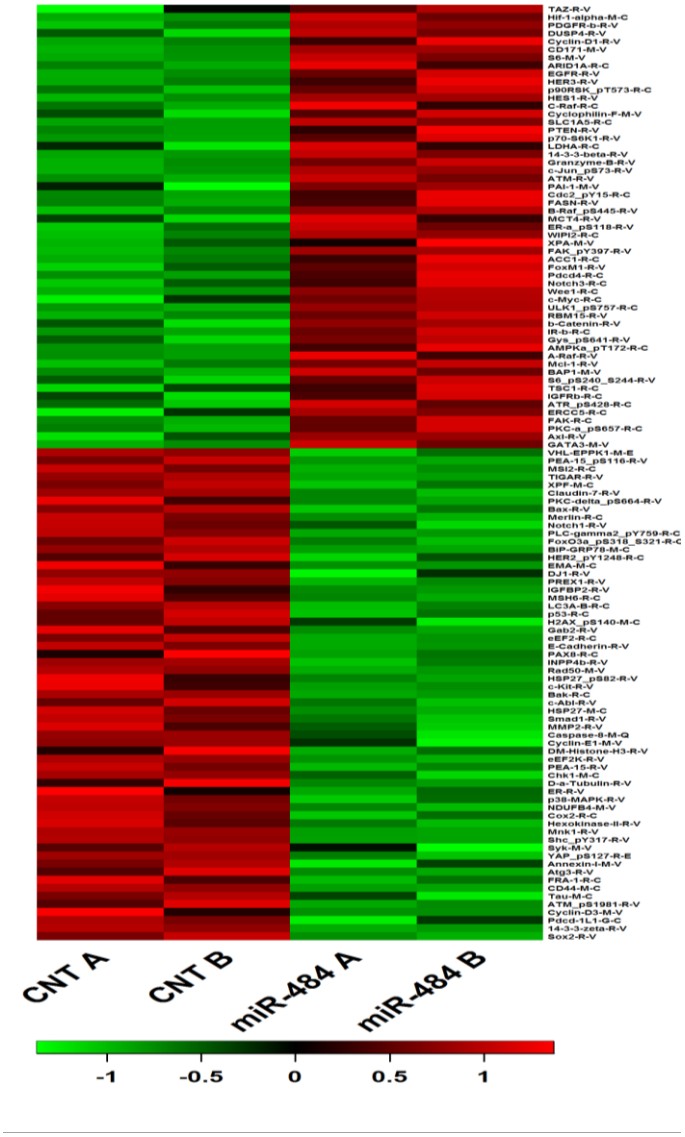


Figure 33: Overexpression of miR-484 significantly alters multiple proteins involved in cancer signaling in TNBC. Heat map of RPPA analysis showing significantly altered proteins after miR-484 transfection in MDA-MB-231 cells. Green color indicates that expression levels were reduced with miR-484 treatment compared to control miRNA treatment, while red color indicates that the expression levels were increased with miR-484 transfection compared to controls.

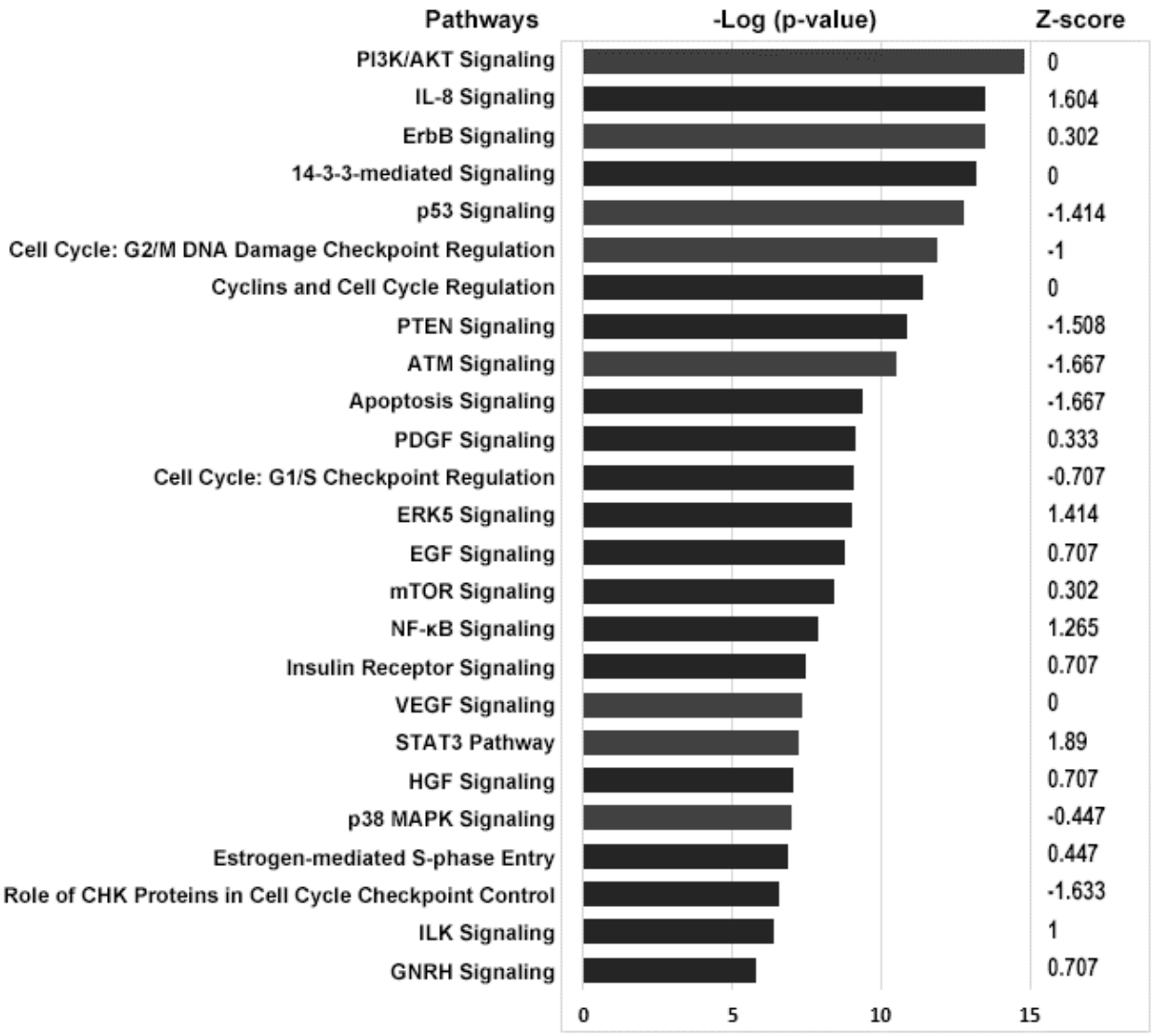


Figure 34: miR-484 overexpression significantly alters multiple cancer signaling pathways in TNBC. The pathway annotations obtained by Ingenuity Pathway Analysis (IPA) show that ectopic overexpression of miR-484 in MDA-MB-231 cells led to alteration in multiple canonical pathways related to cancer.

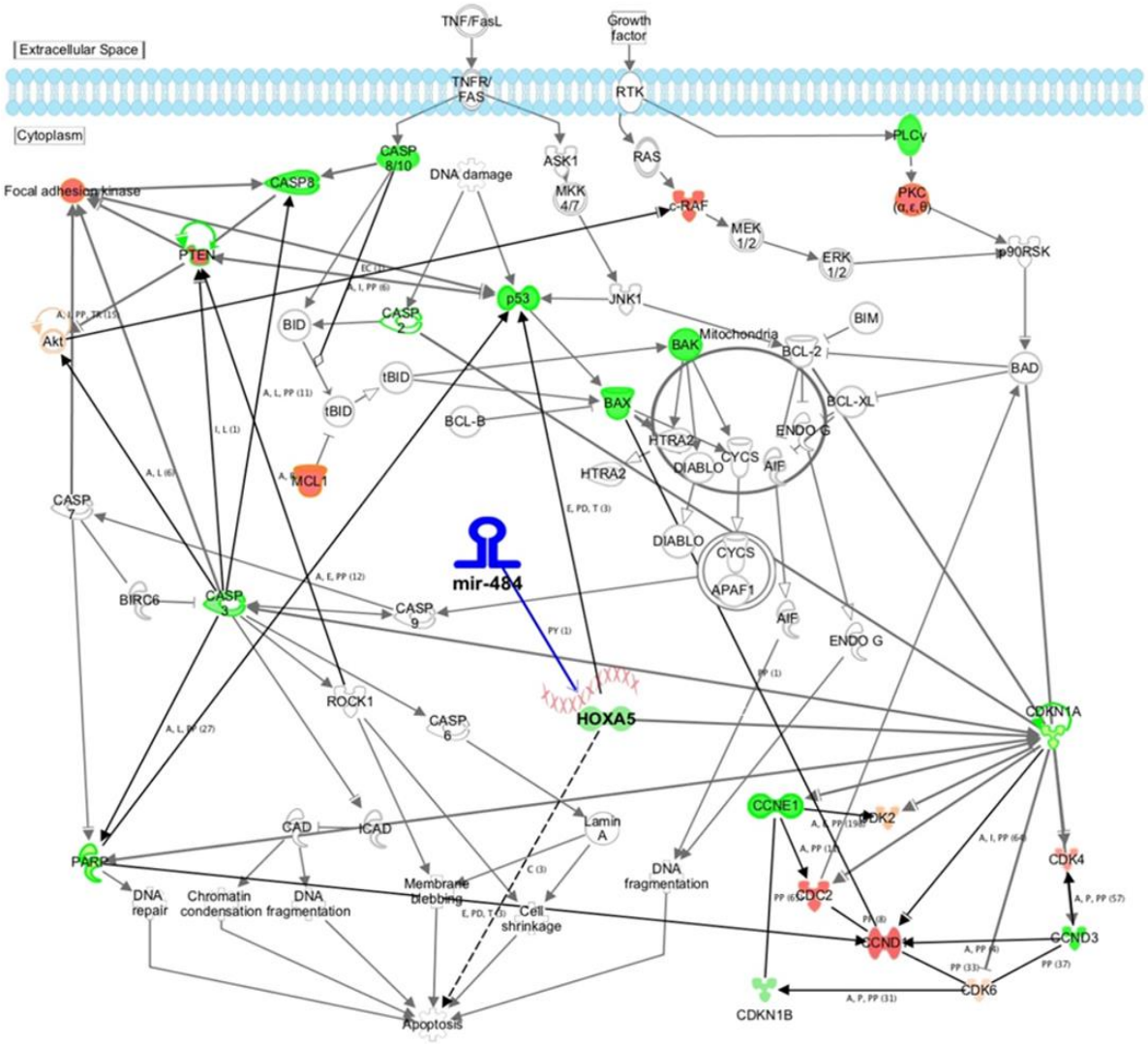


Figure 35: Ectopic overexpression of miR-484 regulates HOXA5 and multiple downstream targets in TNBC. Ingenuity Pathway Analysis (IPA) showing the canonical pathways/proteins that were significantly downregulated (green) or upregulated (red) by miR-484 in TNBC cells. Graphs produced by RPPA analysis of MDA-MB-231 treated with miR-484 or control mimic for 72h.

Aim 3:

In vivo therapeutic targeting of miR-484 suppresses growth of orthotopic TNBC xenograft tumors and induces HOXA5 expression

We have shown that miR-484 is upregulated in TNBC cell lines and is associated with poor patient survival and prognosis. Therefore, to demonstrate the *in vivo* effects of miR-484 in promoting TNBC tumorigenesis and progression as well as the therapeutic potential of targeting this oncogenic miRNA, we inhibited miR-484 in orthotopic MDA-MB-231 and MDA-MB-436 TNBC mouse models. Tumor cells (2 X 10^6 cells/mouse) were orthotopicaly injected in into the mammary fat pad of female nude athymic mice (n=5). After approximately one week, we injected dimyristoyl-sn-glycero-3-phosphocholine-based liposomal nanoparticles (237) incorporating anti-miR-484 (0.15 mg/kg, i.v.) once a week, for 4 weeks. At the end of the treatment we evaluated the *in vivo* effects of miR-484 downregulation on tumor growth and analyzed for proliferation, angiogenesis, and apoptosis by IHC. Mice treated with miR-484 inhibitor showed decreased expression of miR-484 levels in tumors (Fig. 36) compared to control inhibitor, and had a significant decrease in tumor volume compared to control mice (Fig. 37) ($p \leq 0.05$).

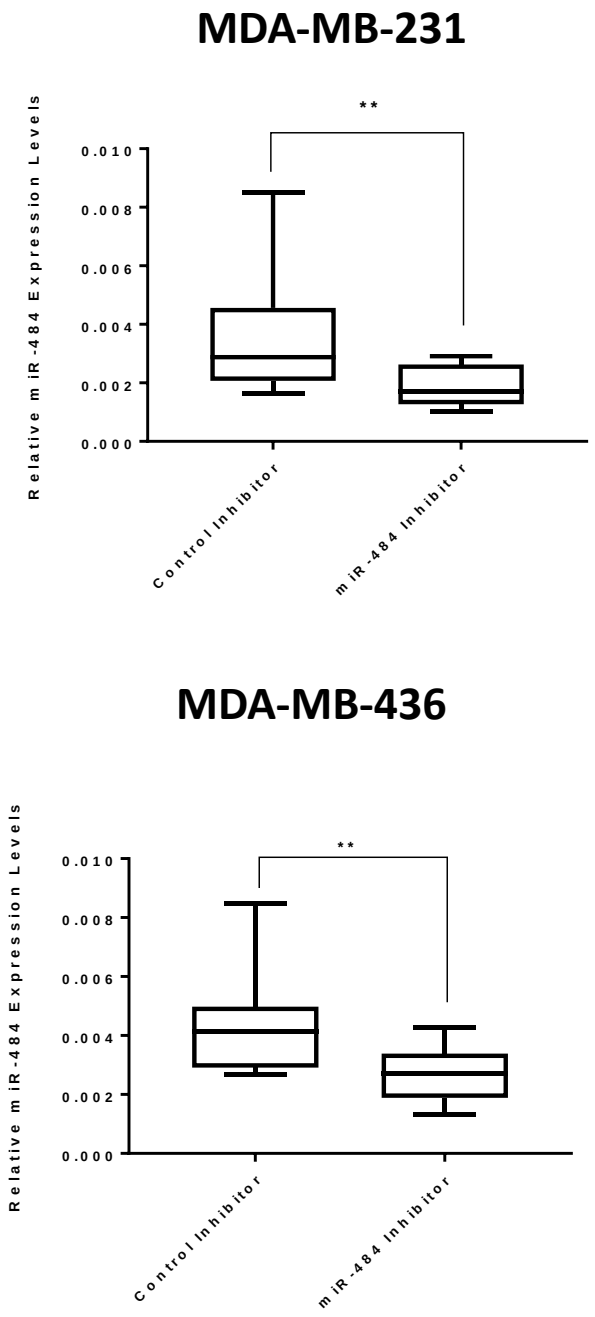


Figure 36: Systemic delivery of Anti-miR-484 reduces miR-484 expression levels in orthotopic xenograft TNBC mouse models. MDA-MB-231 and MDA-MB-436 cells were orthotopically injected in female nude athymic mice (n=5). Mice were then treated with either control inhibitor or miR-484 inhibitor liposomal nano-particles delivered I.V. once every 4 days, for 4 weeks. miR-484 expression levels were analyzed from tumor samples by qRT-PCR. U6 was used as internal control.

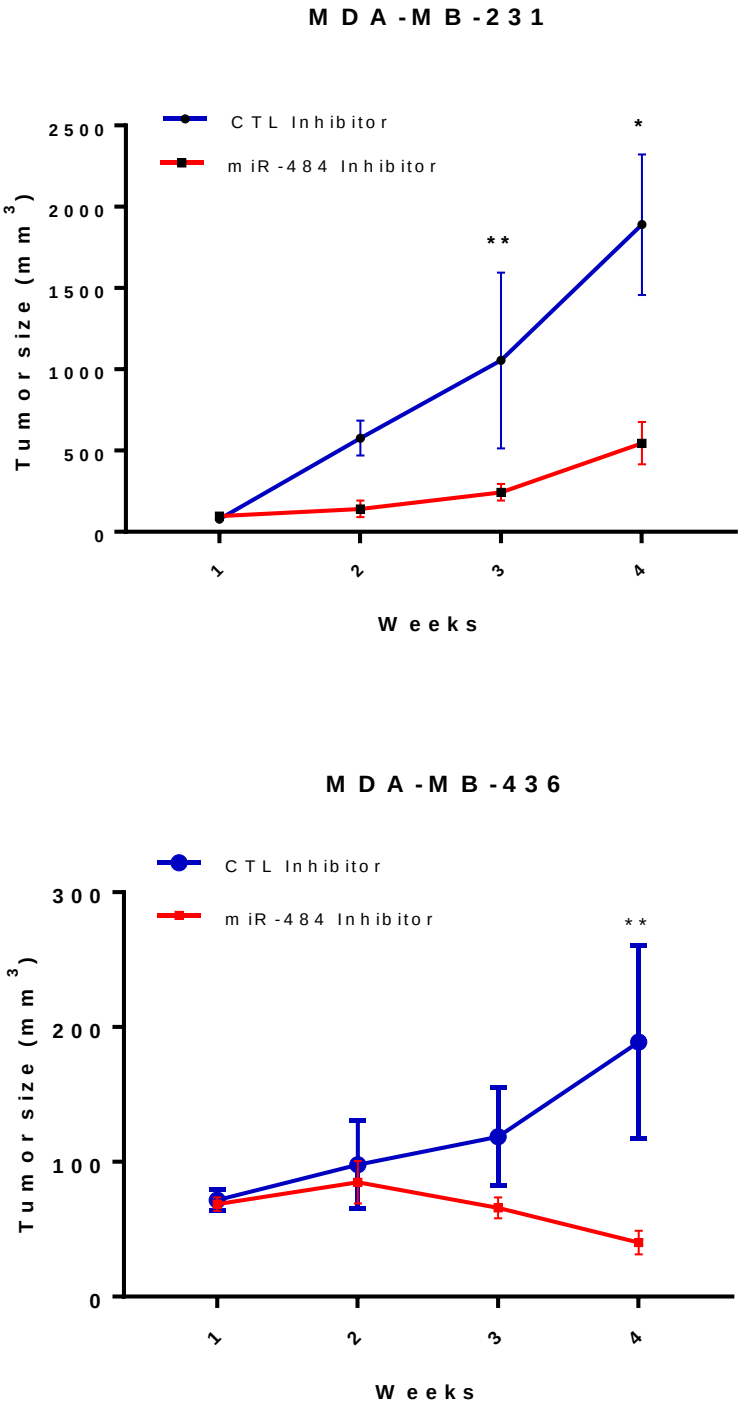


Figure 37: *In vivo* systemic delivery of Anti-miR-484 nanoparticles decreases tumor volume in TNBC mouse xenografts. Tumor volumes were determined once a week for 4 weeks and data is represented as means ± SD. *p=≤0.05.

To assess the effects of miR-484 inhibition on cell proliferation, angiogenesis, and apoptosis, tissue sections were stained with hematoxylin and eosin followed by immunohistochemical analysis for Ki-67 expression as a proliferation marker, and CD31 as a marker for angiogenesis. Additionally, we analyzed the effects of miR-484 inhibition on apoptosis by the Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay which detects nuclear DNA fragmentation in apoptotic cells. Our results showed that mice treated with miR-484 inhibitor had a greater reduction of Ki-67–positive tumor cells compared to mice treated with control inhibitor (MDA-MB-231 p=0.0011, MDA-MB-436 p=0.0093) (Fig. 38). Furthermore, miR-484 inhibition dramatically decreased micro-vessel density, as represented by CD31-positive cells, compared to control inhibitor miRNA (Fig. 39) in both orthotopic tumor models (MDA-MB-231 p=0.024, MDA-MB-436 p=0.0192), suggesting that inhibition of miR-484 has an antiangiogenic effect in TNBC mouse models. Additionally, miR-484 inhibition significantly increased the number of TUNEL-positive cells compared to control inhibitor miRNA (Fig. 40) (MDA-MB-231 p=0.0019, MDA-MB-436 p=0.0049), suggesting that miR-484 inhibition has a pro-apoptotic effect *in vivo*. Overall, our findings indicate that miR-484 inhibition decreased tumor growth in orthotopic TNBC mouse models through significant suppression of cell proliferation and angiogenesis and induction of apoptosis. Furthermore, we assessed the HOXA5 expression levels by both WB and PCR and observed that miR-484 inhibition significantly increased HOXA5 protein and mRNA expression levels by Western blot (Fig. 41) and qRT-PCR analysis (Fig. 42) respectively in both tumor models, providing further evidence that miR-484 pro-tumorigenic effect in TNBC mouse models is mediated by suppression of HOXA5 in TNBC.

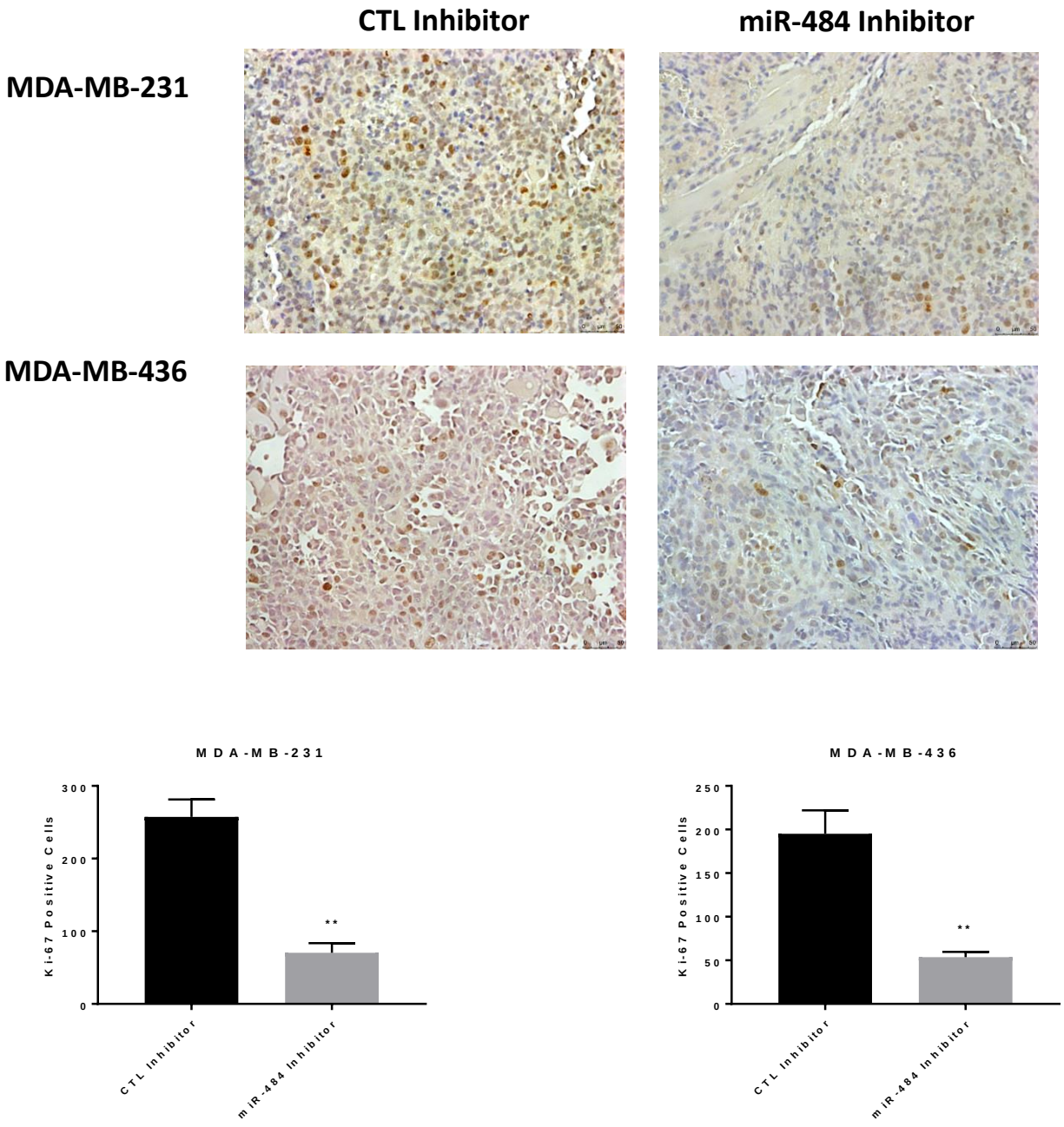


Figure 38: miR-484 inhibition decreases Ki-67 expression. Tumor cell proliferation was analyzed by determining Ki-67 expression in tumor tissues by immunohistochemistry. Magnification X20.

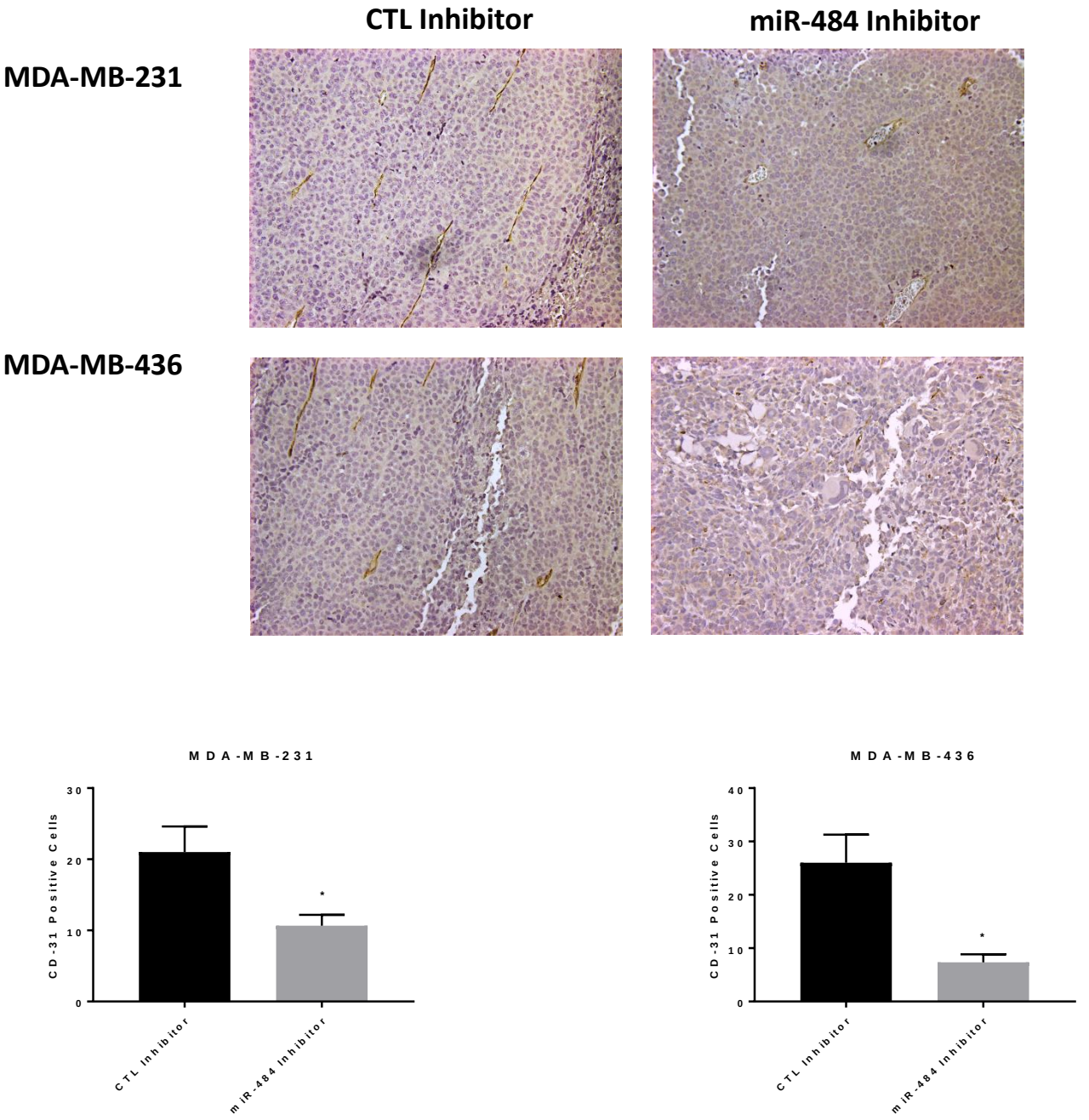


Figure 39: miR-484 inhibition decreases CD31 expression. Tumor tissue sections were analyzed for CD31 expression as a micro-vessel density marker by immunohistochemistry. Magnification X20.

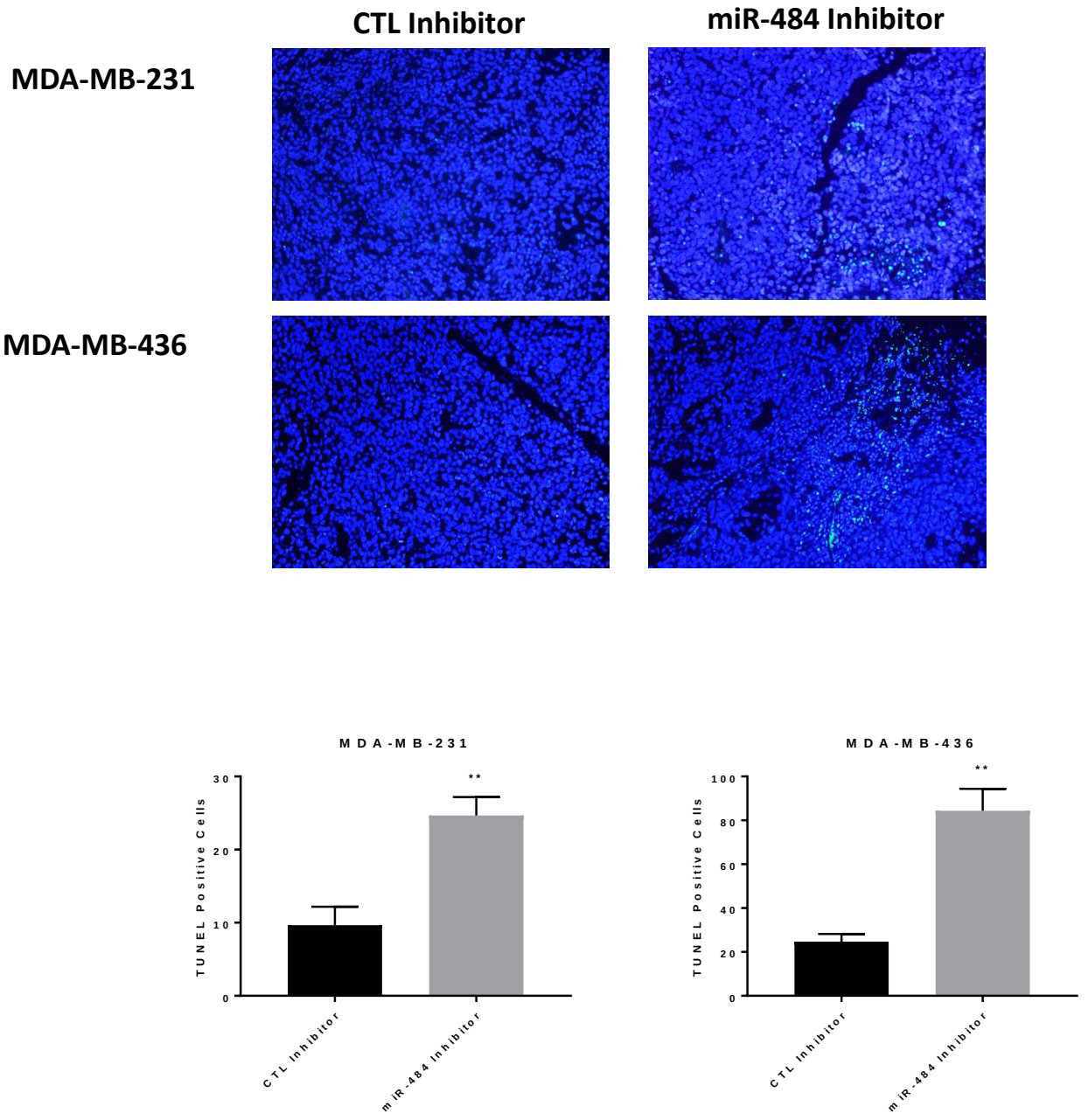


Figure 40: miR-484 inhibition induces TUNEL positive cells in TNBC mouse models. Analysis of *in vivo* apoptosis induction was performed by the TUNEL assay in TNBC tumor xenografts. Magnification×20.

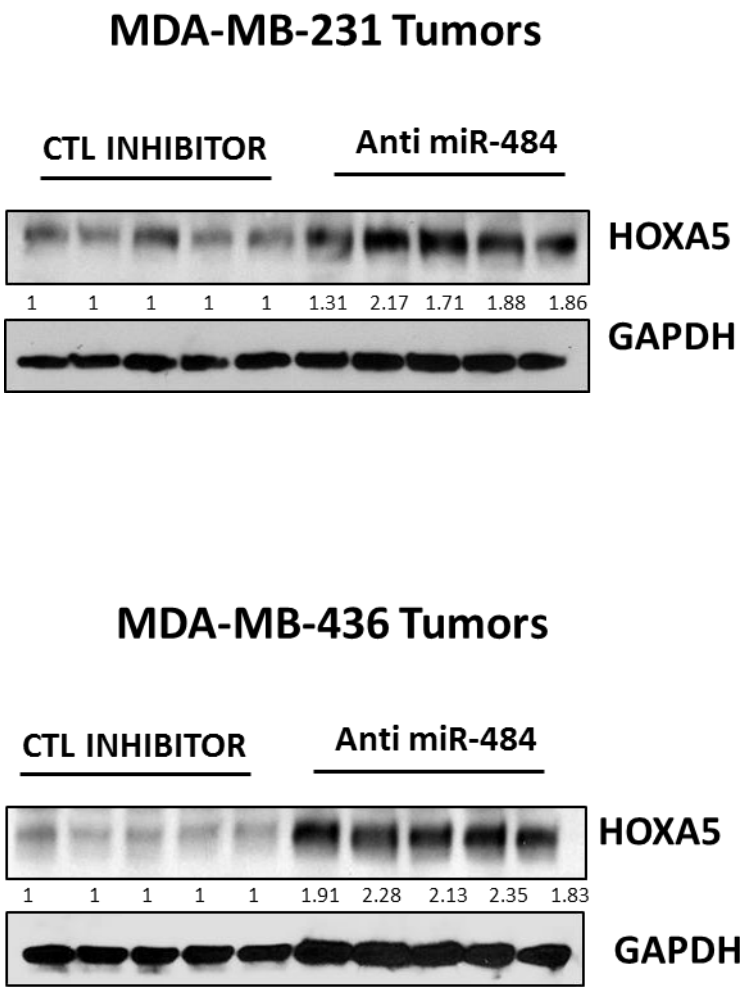


Figure 41: miR-484 inhibition increases HOXA5 protein expression levels in TNBC orthotopic xenografts. TNBC tumor cell lysates were analyzed for HOXA5 expression levels by Western blot analysis. GAPDH was used as a loading control.

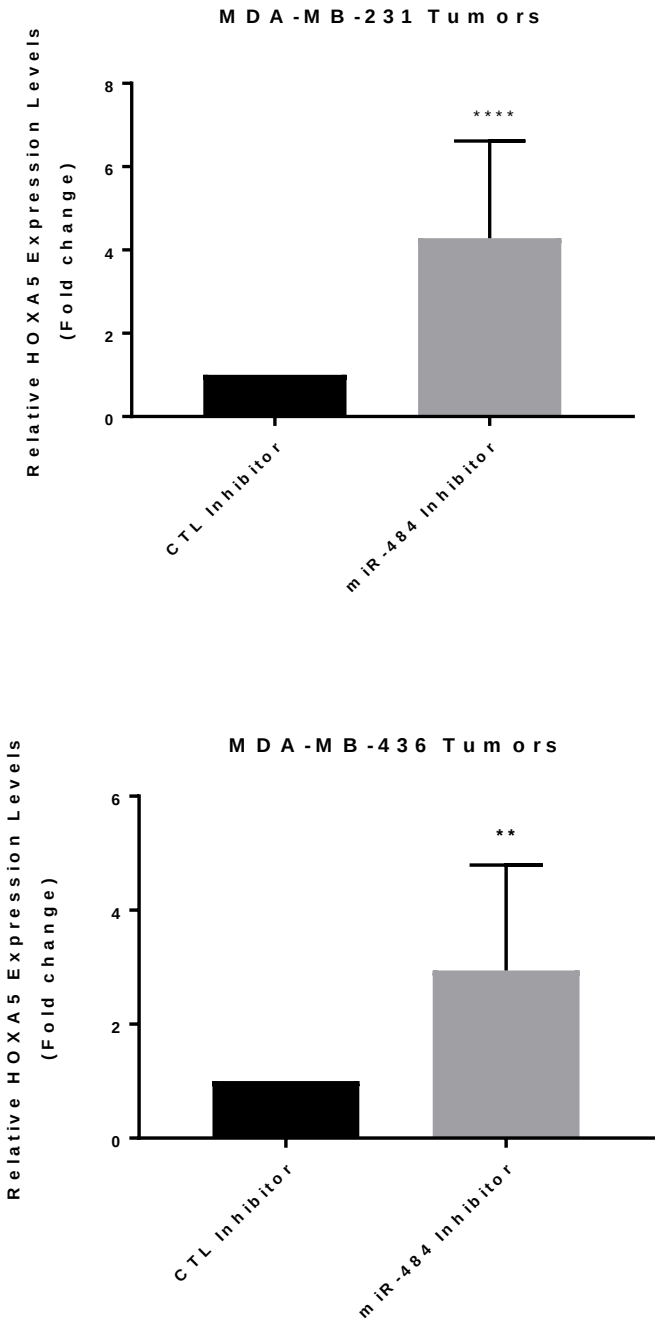


Figure 42: miR-484 inhibition increases HOXA5 mRNA expression levels in TNBC orthotopic xenografts. RNA was isolated from TNBC tumor samples and analyzed for miR-484 expression by qRT-PCR. miR-484 inhibition increase HOXA5 mRNA expression levels in TNBC xenograft tumors. Data is represented as fold change normalized to GAPDH as endogenous control.

CHAPTER IV:
DISCUSSION

DISCUSSION:

Triple negative breast cancer is a very heterogenous and aggressive BC subtype, that lacks specific markers (i.e. ER, PR and HER2) for effective targeted therapy (eg. anti-estrogens, anti-HER2 therapies) (6, 49). Currently, TNBC has six different genetically defined subtypes, making it highly difficult to identify common molecular targets for development of targeted therapies (7). Several gene expression and miRNA profiling studies have been carried out in order to identify particular miRNA signatures in TNBC patients (274). Moreover, several miRNAs have been identified to play a crucial role in TNBC carcinogenesis, providing a basis for their possible therapeutic application with promising results (274). Thus, the application of miRNA based therapy represents an innovative approach, especially for TNBC patients with limited therapeutic options.

The key findings in our study is that miR-484 is a clinically significant oncogenic miRNA that is highly expressed in TNBC patients and is associated with poor OS and prognosis. Additionally, we found that miR-484 acts as an onco-miR by directly binding and regulating the expression of the tumor suppressor gene *HOXA5* in TNBC. Our study also provides the first evidence that *in vivo* therapeutic targeting of miR-484 by systemically injected anti-miR-484 nanoparticles significantly inhibits tumor growth and induces *HOXA5* expression in TNBC tumor models.

Significance of miR-484 in Cancer

Certain miRNAs have been shown to behave either as oncogenic or as tumor suppressor miRNAs depending on the cellular context (103). Such is the case for miR-484, as it has been reported to either act as a tumor suppressor or oncogenic miRNA depending on the cancer type.

The potential oncogenic role of miR-484 has been previously reported in renal cell carcinoma (RCC), where miR-484 was shown to correlate with drug resistance to Sunitinib (tyrosine kinase inhibitor) (275, 276). Patients expressing high miR-484 levels had a median time to progression (TTP) of 5.8 months, whereas patients with low miR-484 expression had a median TPP of 8.9 months. Although the exact mechanism of action of miR-484 in RCC is not yet elucidated, this study suggests that miR-484 may be utilized as a potential predictive biomarker in RCC patients treated with Sunitinib. Additionally, Wang and colleagues showed that miR-484 targets mitochondrial fission protein Fis1, which is induced by anoxia; thereby inhibiting mitochondrial fission and apoptosis in cardiomyocytes and in adrenocortical cancer cells (277). Furthermore, they showed that the transcription factor Foxo3a activated miR-484 expression by binding to its promoter region, and that this binding was attenuated by anoxia (277). Other studies have also suggested the clinical significance of miR-484 as a diagnostic biomarker in cancer. For example, miR-484 was found to be a predictive biomarker for prostate cancer recurrence (278). Another study reported that miR-484 is a predictive biomarker that is highly expressed in metastatic CRC patients treated with combination 5-flurouracil/oxaliplatin (279). However, to our knowledge the mechanism of action of miR-484 in prostate or colon cancer has not been defined as of yet.

On the other hand, miR-484 was reported to act as a tumor suppressor miRNA in cervical cancer, leading to suppression of proliferation, migration/invasion, and induction of apoptosis *in vitro*. Mechanistically, ZEB1 and SMAD2 were identified as miR-484 targets using predictive algorithms and miR-484 was shown to reduce their expression levels, while overexpression of ZEB1 and SMAD2 reversed the events mediated by miR-484 in cervical cancer cells (280). miR-484 was also reported to be among three miRNAs implicated in classifying ovarian cancer patient response to chemotherapy (281). Moreover, miR-484 was found to modulate the tumor vasuclature by targeting VEGF-B in tumor cells and VEGF-R2 in adjacent endothelial cells (281).

Clinical and Functional Significance of miR-484 in Breast Cancer

Here we report for the first time that miR-484 is highly expressed in TNBC subtype of BC patients compared to non-TNBC and normal subtypes. Moreover, we found that high miR-484 expression is correlated with worse OS and prognosis in BC patients. In support of our findings, a previous study found that miRNA-484 is differentially expressed in different clinical and molecular subclasses of invasive BC (234). Utilizing genome-wide data for miRNA/mRNA expression and DNA methylation, an integrated survival analysis was performed on 466 BC patients. This analysis revealed a distinct prognostic signature, composed of seven miRNAs, including miR-484, and 30 mRNA genes, and was successfully validated on eight other BC cohorts (234). Furthermore, Zearo and colleagues reported that miR-484 is significantly upregulated in the serum of early BC patients, suggesting its potential as an early diagnostic biomarker in BC (235). Thus, our data, complemented by the previous findings highlight the clinical significance of miR-484

as a biomarker in BC, and further demonstrated the significance of miR-484 expression in TNBC.

To our knowledge, we are the first to report the functional role of miR-484 in TNBC. Our *in vitro* functional assays showed that miR-484 inhibition significantly reduced TNBC cell viability, cell proliferation, motility/migration, while inducing G1/S cell cycle arrest, and apoptosis. Moreover, opposite effects were observed by the treatment of cells with miR-484 mimic, providing further evidence for the oncogenic role of miR-484 in TNBC. In support of our findings, Ye and colleagues recently showed that miR-484 is implicated in cell proliferation and cell cycle regulation. In their study, miR-484 overexpression promoted cell proliferation and cell cycle progression by targeting cytidine deaminase enzyme in gemcitabine resistant BC cells (282). We also utilized RPPA as an unbiased platform to provide us with the proteomic analysis in order to understand the potential role of miR-484 *in vitro* after its overexpression in TNBC cells (239). The comprehensive analysis of the RPPA data unraveled the link between miR-484 and signaling pathways involved in apoptosis and cell cycle progression, which we further confirmed by western blot analysis.

Deregulated Expression of Tumor Suppressor *HOXA5* by Onco-miR-484 in Breast Cancer

HOX genes are defined by a DNA-binding domain called the homeodomain which encodes for transcription factors that can function to either upregulate or repress the transcription of downstream targets. Numerous studies over the past several decades have demonstrated that HOX genes play a crucial role in the normal temporo-spatial limb

(283) and organ (284-286) development along the anterior-posterior (A-P) axis (287). Additionally, several studies have also revealed that HOX genes can be aberrantly expressed or mutated in many cancers, acting to either promote or suppress tumor development (288, 289), by regulating processes such as cell proliferation, angiogenesis, apoptosis, and tumor metastasis (290-293). This aberration could be mainly attributed to three main mechanisms: 1) temporospatial deregulation, where HOX gene expression in tumors is temporospatially different than in normal tissues; 2) gene dominance, where HOX genes are expressed at higher levels in cancer tissues versus normal; and 3) epigenetic deregulation in which HOX genes are either downregulated or silenced in tumors (288).

HOXA5 is a member of the cluster A family of HOX genes located on chromosome. 7p15.2 (289). HOXA5 has been shown to be a key regulator of cell differentiation and organogenesis particularly in the axial skeleton, respiratory system, mammary glands, and digestive tracts (269). HOXA5 has also been shown to regulate many processes in carcinogenesis namely in breast, lung, colon, ovarian, and hematological malignancies (269).

Previous literature suggest that HOXA5 may function as a tumor suppressor in BC. HOXA5 expression has been shown to be decreased in almost 60% of BC cell lines, which is partially attributed to hypermethylation of the HOXA5 promoter region (261). Moreover, reduced HOXA5 expression was found to be correlated with progression to higher-grade BC stages (261, 294), further supporting our findings of the association of

low HOXA5 with poor OS in BC patients. In the context of BC, HOXA5 has been shown to have a growth suppressive effect by promoting apoptosis in a p53-dependant or independent manner. Raman and colleagues showed that HOXA5 interacts with the p53 promoter to activate it expression and thus induce p53-mediated apoptosis in MCF-7 ER+ BC cells (261). Additionally, HOXA5 was shown to bind with TWIST (a negative regulator of p53), thereby reducing its suppressive effect on p53 in BC cells (271). Alternatively, HOXA5 was also shown to induce apoptosis in a p53-independent independent way via caspases 2 and 8 (260).

Other studies have also shown that HOXA5 is involved in retinoic acid (RA) induced apoptosis in BC cells, where RA was shown to induce HOXA5 expression to mediate its growth suppressive effects. Furthermore, a follow up study revealed a post-transcriptional modulation of RA-induced HOXA5 expression, where miR-130a and the RNA binding protein-human antigen R were found to be involved in HOXA5 upregulation following RA treatment (295).

Furthermore, Teo and colleagues defined the role of HOXA5 in maintaining certain molecular features such as cell-cell adhesion and markers of differentiation in mammary epithelial cells. In their study, reduced HOXA5 expression was shown to increases the self-renewal capacity and the acquisition of a more aggressive phenotype in mammary epithelial cells, via a reduction in E-cadherin and CD24 levels, whereas HOXA5 overexpression promoted the differentiation of the progenitor population to a more differentiated state (296).

Utilizing our miRNA target prediction strategy, we identified *HOXA5* as a target for miR-484, and demonstrated that miR-484 directly binds to the *HOXA5* 3'-UTR to negatively regulate its expression. Furthermore, we showed that miR-484 is inversely correlated with HOXA5 expression in BC patients and cell lines, suggesting that high expression of miR-484 in BC patients, particularly in TNBC patients, may be one of the major causes that contribute to the suppression of *HOXA5* tumor suppressor gene. In agreement with previously published data (260, 261), we found that HOXA5 over expression promoted cell death through apoptosis, which was associated with an increase in the active forms of caspase-2 and capsase-8. Furthermore, the growth inhibiting effects of *HOXA5* were reversed with ectopic overexpression of miR-484 in TNBC cells. Moreover, we also showed that *HOXA5* overexpression recapitulated the effects of miR-484 inhibition on cell cycle progression, whereby we observed the inhibition of cell cycle proteins including cyclin D1, cyclin E1, as well as CDK4 which is being targeted by novel inhibitors in the clinical trials (297). Since p53 is mutated in almost 80% of TNBC patients (298) and the TNBC cell lines used in our study (MDA-MB-231, MDA-MB-436, and BT-20) harbor p53 mutations (299), it is possible that HOX5 mediated effects may mostly be mediated through p53 independent mechanisms.

Aberrant HOXA5 and miR-484 levels were also reported in other cancer types besides BC, suggesting the existence of a possible regulatory pathway in other tumors. For instance, HOXA5 expression levels were found to be reduced in non-small cell lung cancer (NSCLC) patients, where HOXA5 was shown to induce cell proliferation by upregulating Cdkn1a, encoding the cyclin-dependent kinase inhibitor p21 (300-302).

Interestingly, miR-484 was shown to promote NSCLC oncogenesis through inhibiting apoptotic protease activating factor (Apaf-1) associated with the suppression of apoptosis (303). However, whether miR-484 function in NSCLC is also via targeting HOXA5 levels would be a point of further investigation.

miR-484 was also reported to be highly expressed in serum of colorectal cancer (CRC) patients, with its highest expression in the later stages (III-IV) (279, 304), suggesting that it may function as an oncogenic miRNA in CRC. On the contrary, HOXA5 levels were shown to be downregulated in CRC tumors, which was associated with upregulation of the Wnt/β-catenin pathway. Moreover, HOXA5 overexpression in CRC lead to reduction of their self-renewal capacity via inhibition of Wnt signaling, along with reduction in tumor size and metastasis (305).

HOXA5 expression was also found to be lost in angiogenic endothelial cells of the tumor vasculature, suggesting the role of HOXA5 in suppressing tumor angiogenesis. Previous studies reported that HOXA5 overexpression was found to inhibit the expression of pro-angiogenic factors such as VEGFR2, while inducing the anti-angiogenic factor Thrombospondin-2 (TSP-2) (306). Additionally, restoring the expression of HOXA5 also inhibited angiogenesis in brain hemangiomas in mice, which was associated with increased TSP-2 and reduced hypoxia inducible factor 1 (HIF-1α) expression levels (307). In our study, our RPPA analysis revealed that miR-484 induces HIF-1α expression in TNBC, one of the major drivers of oncogenesis. Additionally, we found that *in vivo* inhibition of miR-484 reduced angiogenesis in TNBC tumor xenografts, which was

associated with an increase in HOXA5 expression levels. Collectively, these data suggest that miR-484 may promote angiogenesis in TNBC, and the possible existence of a regulatory pathway between miR-484 and HOXA5/HIF-1α in modulating angiogenesis in TNBC, could be a point of further exploration.

miR-484 as a Novel Molecular Target in TNBC

Since numerous studies have demonstrated that miRNAs are aberrantly expressed in many cancers, and have the ability to regulate multiple cancer-related genes and pathways simultaneously, the use of miRNA based therapies represents a promising therapeutic approach against cancer (308). Indeed several miRNAs are currently in clinical development or are being evaluated in clinical trials as a therapeutic modality against cancer (203).

One of the key findings in our study is that *in vivo* therapeutic targeting of miR-484 by systemically injected anti-miR-484 nanoparticles significantly inhibits tumor growth in TNBC tumor models, with no sign of toxicity during 4 weeks of treatment. Considering the clinical significance and broad expression of miR-484 in TNBC cell lines and BC patients (non-TNBC and TNBC tumors), miR-484 represents an excellent molecular target in BC especially in the TNBC subtype.

A major obstacle in the field of miRNA-based cancer therapy is developing a safe and effective systemic delivery of therapeutic miRNAs *in vivo*. Some obstacles that hinder successful miRNA delivery *in vivo* include degradation by enzymatic nucleases, as well as poor cellular uptake, and poor stability (309).

Thus, the ideal delivery system for miRNAs or miRNA antagomirs should provide sufficient target binding that is tumor tissue specific, and be packaged in a carrier that is biodegradable and non-immunogenic (225). One such strategy that has been extensively investigated in the field of RNA interference is the use of nanocarriers. Nanoparticles are submicron in size, usually made up of natural or synthetic lipids or polymers, that can be utilized to deliver various cargos such as drugs and oligonucleotides *in vivo* (230). Nanoparticles also offer the advantage that they can be coated with high-affinity ligands for tumor-specific receptors to achieve controlled and/or sustained delivery (308).

Liposomal nanoparticles are among the favorable options for systemic miRNA delivery *in vivo*. (230). Advantages of these nanoliposomes include their biocompatible and biodegradable characteristics, and lack of any apparent toxicity (230). Several studies have shown that incorporation of miRNA mimics/inhibitors in neutral nanoliposomes achieved significant reduction in tumor volume and altered the expression of target genes in many cancer models including subcutaneous xenografts and orthotopic tumor models (161, 237, 310). Moreover, neutral nanoliposomes did not cause any detectable distress or toxicity and were found to be safe in mice (161).

In our study, we provide the first evidence that *in vivo* therapeutic targeting of miR-484 by nanoliposomes made of DMPC successfully delivered anti-sense miR-484 and reduced miR-484 expression in orthotopic TNBC mouse models as detected by qRT-PCR. Furthermore, mice treated with miR-484 inhibitor showed an increased expression of HOXA5, as well as reduced intra-tumoral proliferation and angiogenesis, and induction of apoptosis. Additionally, we observed a significant inhibition in tumor growth in the miR-

484 targeted group compared to controls, with no observed side effects, suggesting that miR-484 could be a potential therapeutic target in TNBC. Mechanistically, given the previously described role of HOXA5 in inducing apoptosis in BC cells *in vitro* (260, 261), as well as it anti-angiogenic effect on endothelial cells (306, 311), the observed miR-484 effects *in vivo* may be in part via induction of HOXA5 expression.

In conclusion, our study provides new insight into the role and mechanism of action of miR-484 in TNBC as a potential molecular target, which can further be utilized to develop safe and effective miRNA-based therapies for TNBC patients with limited therapeutic options. Collectively, our *in vitro* and *in vivo* data, as well as the protein array results suggest that miR-484 promotes tumor growth, invasion, metastasis, and progression in TNBC cells by regulating multiple oncogenic pathways. The key findings for our three specific aims are summarized in Figure 43. Thus, our data suggest that miR-484 may function as an “onco-miR” in TNBC and may therefore serve a potential therapeutic target.

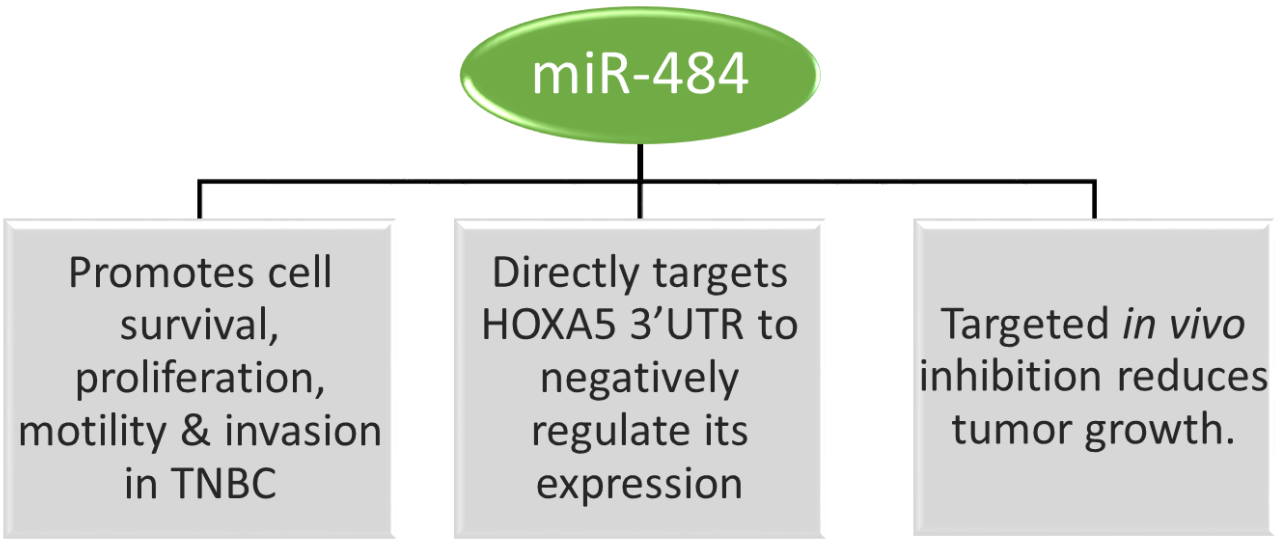


Figure 43: Summary of the key findings depicting the role and mechanism of action of miR-484 in TNBC.

CHAPTER V:
FUTURE DIRECTIONS

Evaluating the role of miR-484 and HOXA5 in other breast cancer subtypes:

One of the critical findings in our study is that miR-484 is significantly associated with poor OS in BC patients. According to our TCGA analysis we also found that miR-484 is upregulated in all BC subtypes compared to matched normal tissues, with the highest expression in the TNBC subtype. Furthermore, we showed that HOXA5 and miR-484 expression levels are inversely correlated in BC patients and cell lines, suggesting that miR-484 may also promote tumor growth and progression in other BC subtypes by targeting HOXA5. Therefore, further evaluation of role of miR-484 in other BC tumors and whether it functions by targeting HOXA5 may be investigated.

Determining the mechanism of aberrant miR-484 expression in Breast Cancer:

Aberrant miRNA expression could be due to genetic, epigenetic factors, or factors that affect miRNA biogenesis/processing (312). However, the causes for dysregulation of miR-484 expression in TNBC are currently not known. miRNA transcription can be activated by transcription factors that bind to its promoter region. In search for possible transcription factors on the miR-484 promoter region (biobase.mdanderson.edu), we found that Nuclear Factor Kappa-B (NF-kB) (Rel A p65 subunit) has multiple predicted binding sites on the miR-484 promoter region (data not shown). NF-kB is a transcription factor that is involved in almost all aspects of human cancer (313, 314), and represents a key regulator of TNBC (315, 316). Moreover, NF-kB has been implicated in the dysregulated expression of many miRNAs (317). Thus, further confirmation by CHIP assay of whether NF-kB directly binds to the miR-484 promoter to regulate its expression could be investigated.

Further confirmation that miR-484 mediated events in TNBC are through down regulation of HOXA5:

In this study we showed that inhibition of miR-484 in TNBC cells significantly reduced cell proliferation, motility/invasion, and induced cell cycle arrest and apoptosis. On the other hand, miR-484 overexpression resulted in increased cell proliferation, survival, motility and invasion, suggesting that it functions as an oncogenic miRNA in TNBC. Furthermore, we identified HOXA5 as a direct target of miR-484 and found that miR-484 directly binds to the 3'-UTR of HOXA5 to negatively regulate its expression. Additionally, HOXA5 over-expression recapitulated the effects of miR-484 inhibition on apoptosis induction, while miR-484 overexpression reversed this effect, suggesting that miR-484 mediates its effects through HOXA5 suppression. However, further examination of whether miR-484 effects on cell proliferation, motility, and invasion are through HOXA5 downregulation should be considered. Thus, determining whether siRNA mediated knockdown of HOXA5 can recapitulate miR-484 effects in TNBC could be examined.

Analysis for *in vivo* toxicity of Anti-miR-484 treatment in TNBC mouse models:

In our study, no significant changes in mouse body weights, nor changes in behavioral or eating habits were detected during the 4 weeks of the treatment of mice, suggesting that anti-miR-484 therapy exerted no or limited side effects. However, further confirmation by clinical biochemistry analyses for mice treated with either miR-484 inhibitor or control inhibitor nanoliposomes should be compared. This can include biochemical parameters for kidney, liver, and blood toxicity such as, blood urea nitrogen, glucose, aspartate

aminotransferase, alanine aminotransferase, creatinine, total bilirubin, and lactic dehydrogenase.

Further confirmation of miR-484 oncogenic effects in TNBC mouse models:

We showed that treatment with miR-484 inhibitor reduced tumor growth, proliferation, and angiogenesis, and induced apoptosis in TNBC mouse models. Moreover, we observed that these effects were associated with increased HOXA5 expression levels. However, further confirmation of the oncogenic effects of miR-484 can be explored by injecting mice with miR-484 mimic to determine its effects on proliferation, angiogenesis, and apoptosis would be warranted. Additionally, tumor samples from mice treated with either miR-484 inhibitor or mimic can be evaluated for the proliferation, angiogenesis, and apoptosis markers by western blot.

Determining the effect of miR-484 inhibition in combination with standard chemotherapy in TNBC:

According to our *in vitro* and *in vivo* results, miR-484 promotes tumor growth and progression in TNBC and therefore represents a potentially novel therapeutic target. However, further evaluation of combining miR-484 inhibitors with standard chemotherapeutics could be evaluated in order to determine a possible synergistic effect and maximize treatment efficacy. Our preliminary experiments have shown that combination of miR-484 inhibitor with standard chemotherapeutics such as paclitaxel or doxorubicin significantly reduced TNBC cell proliferation than either mono therapies (data not shown). Therefore, further investigation of whether miR-484 increases doxorubicin or paclitaxel sensitivity in TNBC cells may be explored.

APPENDIX

Table 3: Antibodies used in Western Blot analysis

Target Protein	Source	Catalog Number	Application
GAPDH	Cell Signaling Technology	5174	WB, IHC, IF
HOXA5	Santa Cruz	365784	WB, IP, IF
Caspase-2	Cell Signaling Technology	2224	WB
Caspase-8	Cell Signaling Technology	9746	WB, IP
Cleaved Caspase-8	Cell Signaling Technology	9496	WB, IHC, IF
Caspase-3	Cell Signaling Technology	9662	WB, IHC, IP
PARP	Cell Signaling Technology	9532	WB, IP, IF
p-FAK (pY397)	B&D Biosciences	611722	WB
FAK	B&D Biosciences	610087	WB
p-SRC (Tyr416)	Cell Signaling Technology	2101	WB
SRC	Cell Signaling Technology	2109	WB, IHC, IF, IP
CDK2	Cell Signaling Technology	2546	WB, IP
CDK4	Cell Signaling Technology	12790	WB, IHC, IF
CDK6	Cell Signaling Technology	13331	WB, IP
Cyclin D1	Cell Signaling Technology	2978	WB, IHC
Cyclin E1	Cell Signaling Technology	20808	WB
p21	Cell Signaling Technology	2947	WB, IHC, IP, IF
p27	Cell Signaling Technology	3686	WB, IP, IF

Table 4: Oligonucleotide sequences for quantitative reverse transcription polymerase chain reaction

Target Gene	**Forward Sequence**	**Reverse Sequence**
HOXA5	5’-AGTCATGACAACATAGGCGGC-3’	5’-CGGGTCAGGTAACGGTTGAA-3’
GAPDH	5’-CAAGGTCATCCATGACAACTTTG- 3’	5’-GTCCACCACCCTGTTGCTGTAG-3’

Table 5: Experimentally verified targets for miR-484 by miRWalk2.0

RefseqID	Gene
NM_000702	ATP1A2
NM_152272	CHMP7
NM_020699	GATAD2B
NM_005523	HOXA11
NM_019102	HOXA5
NM_001004317	LIN28B
NM_000254	MTR
NM_003204	NFE2L1
NM_002616	PER1
NM_024297	PHF23
NM_138300	PYGO2
NM_031459	SESN2
NM_144582	TEX261
NM_013390	TMEM2
NM_005781	TNK2
NM_017590	ZC3H7B

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Vita

Nashwa Kabil was born in Cairo, Egypt and moved to the U.S.A. with her family in 1988. After completing high school, she attended Ain Shams University in Cairo, Egypt, where she earned her M.D. degree. She later obtained her Master's degree in Physiology from Cairo University, Egypt in 2010. She joined the University of Texas Graduate School of Biomedical Sciences in 2014 and the lab of Dr. Lopez-Berestein in 2015 at the Experimental Therapeutics Department, M.D. Anderson Cancer Center. She worked under the supervision of Dr. Bulent Ozpolat to complete her Ph.D dissertation project.