



# miR-221 Promotes Tumorigenesis in Human Triple Negative Breast Cancer Cells

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## Abstract

Patients with triple-negative breast cancers (TNBCs) typically have a poor prognosis. TNBCs are characterized by their resistance to apoptosis, aggressive cellular proliferation, migration and invasion, and currently lack molecular markers and effective targeted therapy. Recently, miR-221/miR-222 have been shown to regulate ER $\alpha$  expression and ER $\alpha$ -mediated signaling in luminal breast cancer cells, and also to promote EMT in TNBCs. In this study, we characterized the role of miR-221 in a panel of TNBCs as compared to other breast cancer types. miR-221 knockdown not only blocked cell cycle progression, induced cell apoptosis, and inhibited cell proliferation *in-vitro* but it also inhibited *in-vivo* tumor growth by targeting p27<sup>kip1</sup>. Furthermore, miR-221 knockdown inhibited cell migration and invasion by altering E-cadherin expression, and its regulatory transcription factors Snail and Slug in human TNBC cell lines. Therefore, miR-221 functions as an oncogene and is essential in regulating tumorigenesis in TNBCs both *in vitro* as well as *in vivo*.

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## Introduction

microRNAs (miRNAs) are non-coding, single-stranded ~22 nucleotides long small RNAs that act as agents of the RNA interference pathway and negatively regulate the translation by either cleaving or degrading their targeted transcripts [1]. Because miRNAs usually bind to their targets with incomplete complementarity, a single miRNA can potentially regulate the translation of multiple target genes involved in various cellular processes [2,3]. In fact, miRNAs have been implicated in the regulation of a variety of biological functions, including cellular proliferation, differentiation, and apoptosis [4,5]. Growing evidence indicates that miRNAs can function as tumor suppressors or oncogenes [4], and miRNA expression profiling analyses have revealed characteristic miRNA signatures in a variety of human cancers [6,7]. Furthermore, miRNAs are frequently located at fragile genomic regions susceptible to amplification, deletion, or translocation during tumor development [8]. Since miRNAs are believed to be pivotal players in tumor development, investigations of differential expression of miRNAs and their corresponding targets might prove to be instrumental for the diagnosis and treatment of various cancers.

Molecular profiling has allowed us to classify breast cancers to five subtypes based on their distinctive gene expression signatures [9]. The five subtypes are luminal A, luminal B, Human Epidermal Growth Factor Receptor 2 (HER2) positive, basal-like, and normal-like breast cancers. Basal-like tumors are characterized by the expression of genes specific for basal epithelial cell proliferation, inhibition of apoptotic pathways, and aggressive migration and invasion [9–11]. Basal-like breast cancers (BLBCs) are often stained negative by immunohistochemistry

for estrogen receptor (ER), progesterone receptor (PR), and HER2 and thus are called triple negative breast cancers (TNBCs). Although BLBC and TNBC share numerous clinical and pathological features, they are not identical [12,13]. In the majority of cases, however, these two categories share similar clinical characteristics, prognosis and treatment options and thus, the term "TNBC" will be used in this study to collectively describe BLBC and TNBC cell lines and patient populations. Clinical studies have shown that TNBCs are the most aggressive breast cancer type and TNBC patients are frequently faced with poor prognosis and high mortality [14,15]. Thus, the development of targeted therapies for TNBCs is urgently and critically needed for this patient population.

miR-221 and miR-222 are encoded in tandem from a gene cluster located on chromosome X and have been shown to be up-regulated in a panoply of cancer types. Due to their seed sequence similarity, both miRNAs have been shown to directly target p27<sup>kip1</sup>, Bmf, PTEN, Mdm2, PUMA, and TRPS1 [16–22]. In breast cancer, miR-221/miR-222 have been shown to be involved in regulation of ER $\alpha$  expression, suppression of ER $\alpha$ -mediated signaling, as well as drug resistance mechanisms [23–27]. More recently miR-221/miR-222 have been shown to be over-expressed in triple-negative primary breast cancers or cell lines [28–30]. In this study, we investigated the molecular mechanisms of cellular transformation regulated by miR-221/222 specifically in a panel of human TNBC cell lines compared to other breast cancer types and validated our findings *in vivo*. We show that miR-221 is an oncogene and modulates cell proliferation and tumor progression via targeting p27<sup>kip1</sup> and EMT transition in TNBCs both *in vitro* as well as *in vivo*.

## Materials and Methods

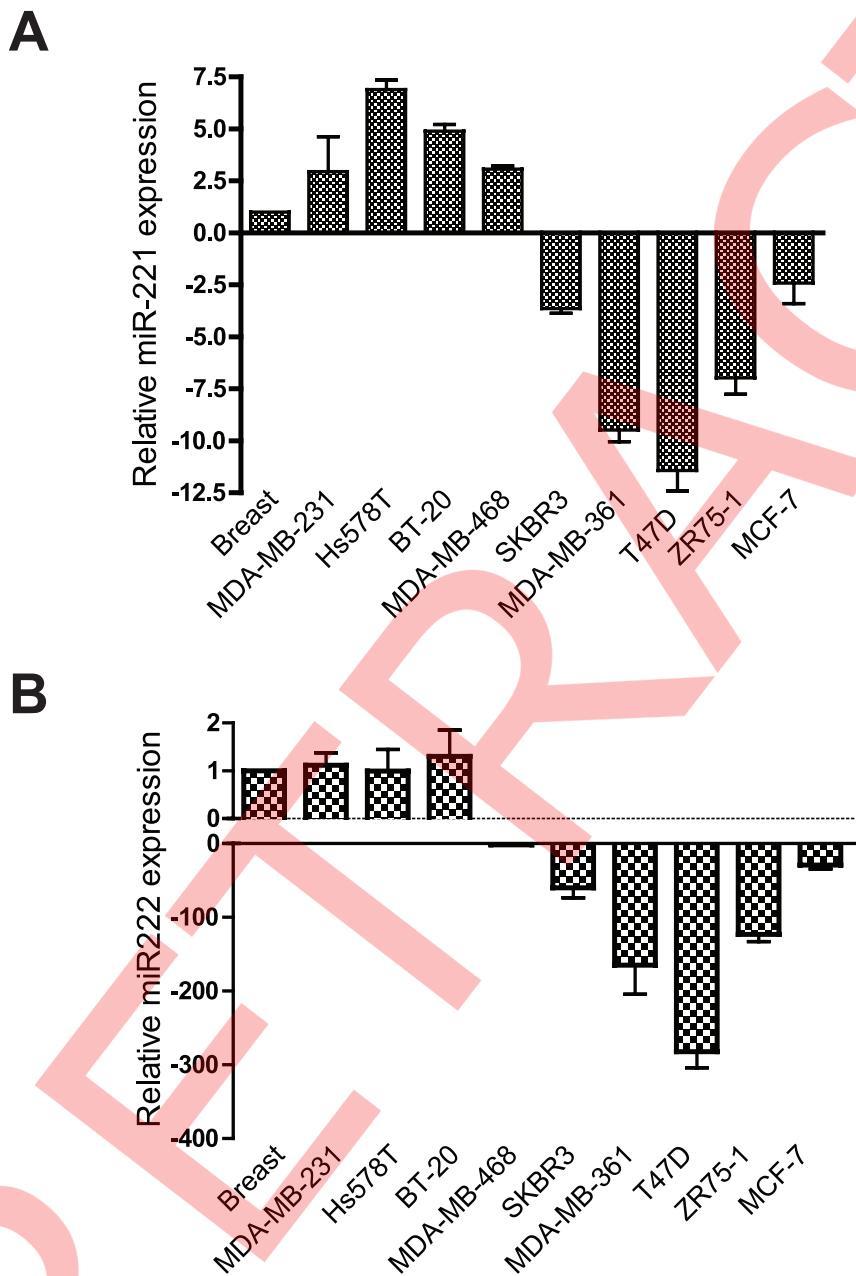
### Cell Culture

All cell lines were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with L-glutamine and grown in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C.

### Stable Cell Line Generation

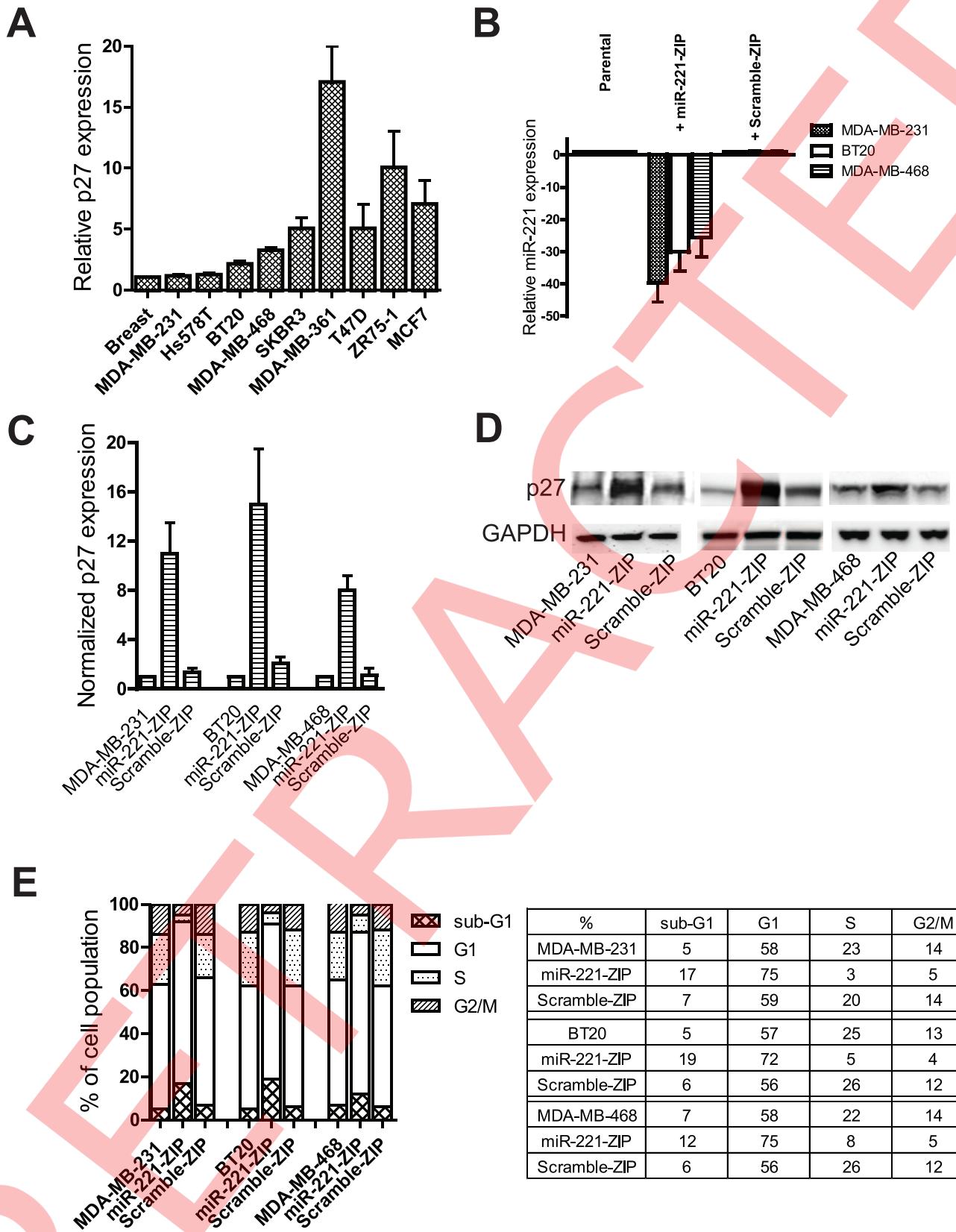
System Bioscience's miRZip™ anti-sense miRNAs are stably expressed RNAi hairpins that inhibit miRNA activity. The miRZip shRNAs produce short, single-stranded anti-miRNAs

that competitively bind their endogenous miRNA target and inhibit its function. The miRZip short hairpin RNAs are cloned into SBI's pGreenPuro™ shRNA expression vector, an improved third generation HIV-based expression lentivector. The lentiviral vector contains the genetic elements responsible for packaging, transduction, and stable integration of the viral construct into genomic DNA, inducing expression of the anti-miRNA effector sequence. For production of a high titer of viral particles, we used the ViraPower™ Lentiviral Support Kit (Invitrogen) employing Lipofectamine™ 2000 (Invitrogen) for transfecting the miRzip vectors into HEK-293T cells. Because infected cells stably express cop-GFP and puromycin, as well as the anti-miRNA cloned into



**Figure 1. miR-221 is over expressed in TNBCs.** qRT-PCR was performed to quantitatively measure RNA expression levels of miR-221 (A) and miR-222 (B) in a panel of breast cancer cell lines. All expression levels are displayed as fold changes normalized to the expression level in normal breast tissue.

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**Figure 2. miR-221 modulates cell cycle progression by targeting p27<sup>kip1</sup>.** (A) mRNA expression level of p27<sup>kip1</sup> was measured in a panel of breast cancer cell lines. Data are displayed as fold changes normalized to the expression level in normal breast tissue. (B) TNBC lines, MDA-MB-231, BT-20, and MDA-MB-468 cells were established to stably express anti-miR-221 (miR-221-ZIP) or a control scramble miRNA (Scramble-ZIP). miR-221 expression level was measured and is displayed as fold changes normalized to the expression level of parental cell lines. (C) Transcript expression level of p27<sup>kip1</sup> was measured and is displayed as fold changes normalized to the expression level of parental cell lines. (D) Western blot analysis of

%	sub-G1	G1	S	G2/M
MDA-MB-231	5	58	23	14
miR-221-ZIP	17	75	3	5
Scramble-ZIP	7	59	20	14
BT20	5	57	25	13
miR-221-ZIP	19	72	5	4
Scramble-ZIP	6	56	26	12
MDA-MB-468	7	58	22	14
miR-221-ZIP	12	75	8	5
Scramble-ZIP	6	56	26	12

MDA-MB-231, BT-20, and MDA-MB-468 cells stably expressing anti-miR-221 or scramble miR-ZIP depicting changes in p27 protein level. GAPDH was used as loading control. (E) MDA-MB-231, BT-20, and MDA-MB-468 cells stably expressing anti-miR-221 or scramble miR-ZIP were cultured for 72 hours to reach 80–90% confluence before harvesting and cell cycle analysis was performed and displayed as percentages of each cell cycle stage. These experiments were repeated at least three times, and representative data is shown.

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the miRZip<sup>TM</sup> vector, we successfully used puromycin to select for the infected cells harboring the miRzip.

#### RT-PCR

TaqMan miRNA assays (Life Technologies, CA) were used to quantify the expression levels of mature miR-221 and miR-222 as well mRNAs p27, Snail, Slug, vimentin and E-Cadherin. Total RNA extracted by mirVana (Life Technologies) was reverse transcribed in a reaction mixture containing miR-specific stem-loop RT primers. Quantitative polymerase chain reaction (qPCR) was performed in triplicate with reactions containing amplified cDNA and TaqMan primers in Universal Master Mix without AmpErase UNG (Applied Biosystems). The qPCR was conducted at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds in 7900 HT Real Time PCR system (Applied Biosystems) and threshold cycles ( $C_T$ ) were calculated using Sequence Detection Software (SDS v2.2.1, Applied Biosystems). All mRNA quantification data were normalized to GAPDH. All miRNA data are expressed relative to a RNU48 small nuclear (sn) RNA TaqMan PCR performed on the same samples, unless otherwise specified. Fold expression was calculated from the triplicate of  $C_T$  values following the  $2^{-\Delta\Delta C_T}$  method.

#### Immunoblotting

Cells were lysed in buffer composed of 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 50 mM HEPES, 10% glycerol, 1 mM EGTA, 1% Triton X-100, 0.5% NP-40 supplemented with 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF, 1 mM NaF, 1 mM β-glycerophosphate, protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktail (Roche) added prior to use. Protein concentration was determined using the BCA Protein Assay (Pierce/Thermo Fisher Scientific) per manufacturer's instructions. Protein (30–50 µg) was resolved by SDS-PAGE and transferred onto nitrocellulose membrane. Blots were probed with primary antibodies to detect proteins of interest. After incubation with secondary antibodies, membranes were visualized by chemiluminescence (Pierce/Thermo Fisher Scientific). All antibodies were purchased from Cell Signaling Technology, with the exception of GAPDH (Santa Cruz Biotechnology).

#### Cell Proliferation, Apoptosis Assay, Migration, and Invasion Assays

Resazurin Fluorescent Assay was used for the proliferation studies. Briefly, Cells were seeded at 3000–5,000 cells/100ul/well in DMEM +10% FBS in a 96 well plate, and incubated overnight at 37°C in 5% CO<sub>2</sub>. Resazurin (Sigma) fluorescent dye was added (1:100) to each well. The cells were incubated at 37°C in 5% CO<sub>2</sub> for 4 hours at which point the plate was read for fluorescence at 530/590 nm on the HTS 7000 plate reader. Cell Signaling Technologies PathScan® Apoptosis Multi-Target Sandwich ELISA Kits were used in the apoptosis assays. Briefly, antibodies for cleaved caspase 3 and phosphorylated BAD had been coated onto microwells. After incubation with the cell lysates, the target protein was captured by the coated antibody. Following extensive washing, a detection antibody was added to detect the captured target protein. An HRP-linked secondary antibody was then used to recognize the bound detection antibody. HRP substrate, TMB, was added for color development which was measured to quantity

the level of bound target protein. Cell Biolab's CytoSelect<sup>TM</sup> Cell Migration Assay Kit containing polycarbonate membrane inserts (8 µm pore size) in a 24-well plate was used in our migration assays. Migratory cells were able to extend protrusions towards FBS (used as the chemo-attractant), and pass through the pores of the polycarbonate membrane. The non-migratory cells were removed from the top of the membrane and the migratory cells were stained and quantified. Similarly, Cell Biolabs CytoSelect<sup>TM</sup> Cell Invasion Assay Kit containing basement membrane-coated inserts were used to assay the invasive properties of the cells. The upper surface of the insert membrane is coated with a uniform layer of dried basement membrane matrix solution. This basement membrane layer serves as a barrier to discriminate invasive cells from non-invasive cells. The non-invasive cells were removed from the top of the membrane and the invading cells were stained and quantified.

#### Cell Cycle Analysis

MDA-MB-231, BT-20, and MDA-MB-468 parental cells and cells stably expressing miR-221-ZIP, or scramble miRNA-ZIP were cultured to 70–90% confluence and allowed to adhere overnight. Cells were collected, fixed, and permeabilized using the Cell Cycle Phase Determination Kit (Cayman Chemical) following the manufacturer's protocol. Samples were stored at –20°C until DNA stained with propidium iodide and read on a BD FACSCalibur (BD Biosciences). Data analysis was done with FCS Express (De Novo Software). All experiments were repeated at least 3 times and representative data set is shown.

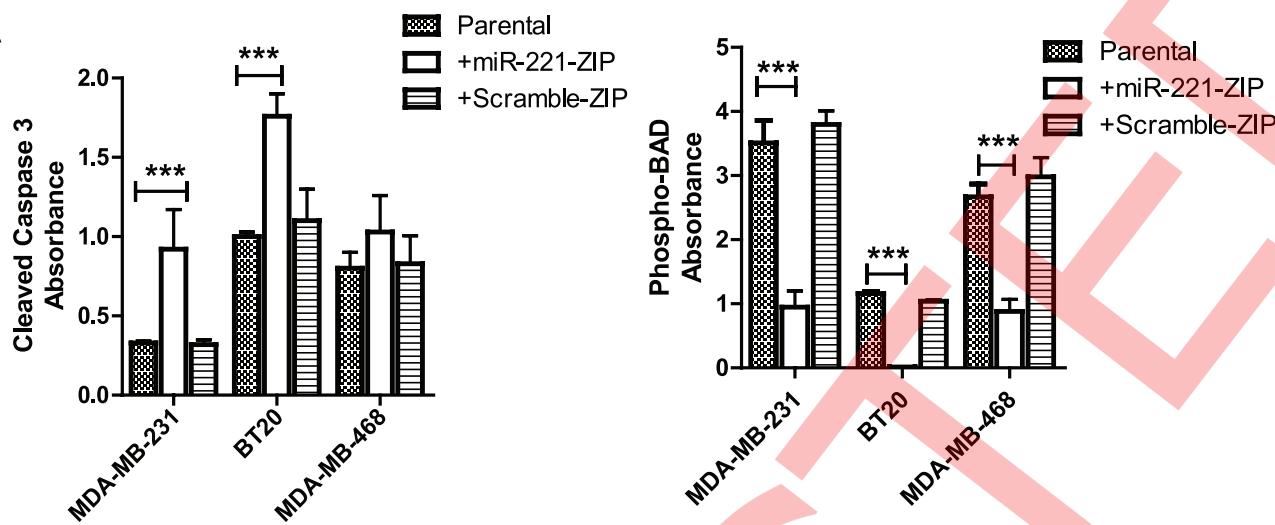
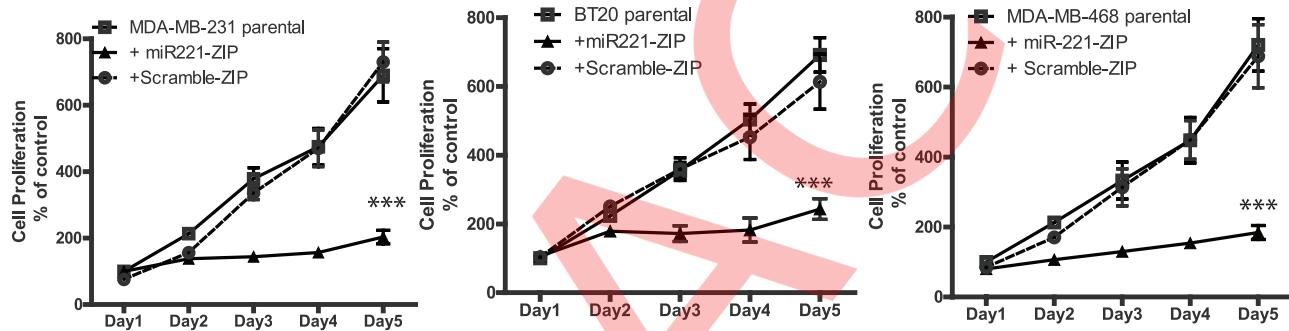
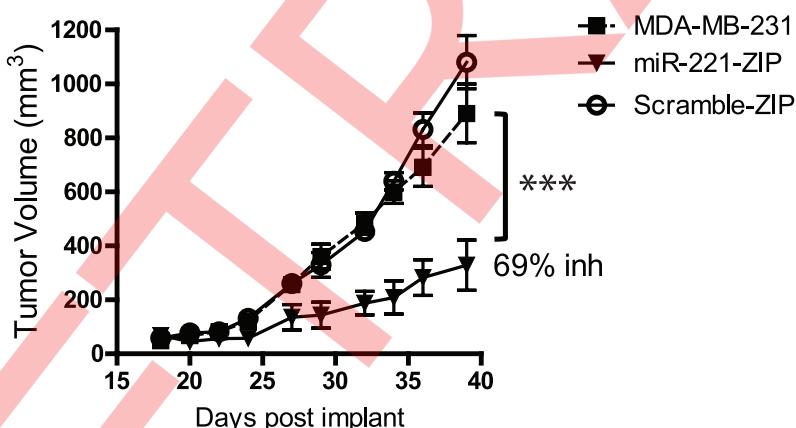
#### Animal Studies

Six- to eight-week-old nu/nu athymic female mice were obtained from Jackson Labs. The mice were maintained in pressurized ventilated caging at the Pfizer La Jolla animal facility. All animal studies were done under the ethical approval by Pfizer Institutional Animal Care and Use Committees. Tumors were established by injecting  $5 \times 10^6$  cells suspended 1:1 (v/v) with reconstituted basement membrane (Matrigel, BD Biosciences). Tumor dimensions were measured with Vernier calipers, and tumor volumes were calculated using this formula:  $\pi/6 \times (\text{larger diameter}) \times (\text{smaller diameter})^2$ . Tumor growth inhibition percentage (TGI %) was calculated as  $100 \times (1 - \Delta T/\Delta C)$ . One way ANOVA Statistical analysis were performed and noted as \*\*\* depicting p-value less than or equal to 0.001.

## Results

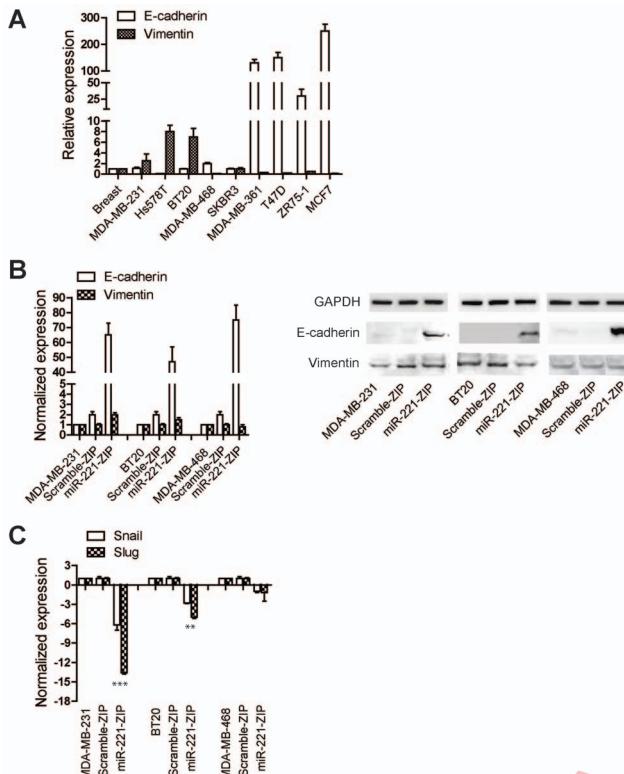
#### miR-221 is Specifically Up-regulated in Human TNBC Cell Lines

The expression level of miR-221 and miR-222 were examined by qRT-PCR in 4 TNBC lines: MDA-MB-231, Hs-578T, BT-20, and MDA-MB-468; 2 HER2 positive lines: SKBR3 and MDA-MB-361; and 3 ER positive lines: T47D, ZR75-1, and MCF-7. In comparison to the expression level in normal breast tissue (RNA acquired from Applied Biosystems), miR-221 is up-regulated in all the TNBC lines while down-regulated in the non-TNBC lines (Figure 1A). Surprisingly, although clustered with miR-221, the expression level of miR-222 is only up-regulated mildly (1–1.5 fold) in Hs-578-T and BT-20 (compared to normal breast tissue), but

**A****B****C**

**Figure 3. miR-221 knockdown induces apoptosis, inhibits cell proliferation and suppresses tumor growth in mice.** MDA-MB-231, BT-20, and MDA-MB-468 cells stably expressing miR-221-ZIP or scramble-ZIP were used to perform apoptosis, cell proliferation, and *in-vivo* tumor growth assays. (A) Cleaved caspase 3 and phosphorylation of BAD were measured as apoptosis markers. All cell lines were seeded at similar density as the parental cell lines and cultured for 72 hours until the parental cell lines reached 80–95% confluencey, before cell lysates were prepared and subject to cleaved caspase 3 assays. (B) Cell proliferation measurements were normalized to the readings in parental cells at day 1. (C) Nude mice were implanted subcutaneously with MDA-MB-231 parental cells, and MDA-MB-231 cells stably expressing miR-221-ZIP or scramble-ZIP. Tumor measurements were recorded and tumor growth inhibition was calculated as described in Materials and Methods. *T*-test was performed in (A) and (B) and one way ANOVA was performed in (C) to compare the differences between parental cells versus miR-221-ZIP cells. \*denotes  $p$ -value  $\leq 0.05$ . \*\*denotes  $p$ -value  $\leq 0.005$ .

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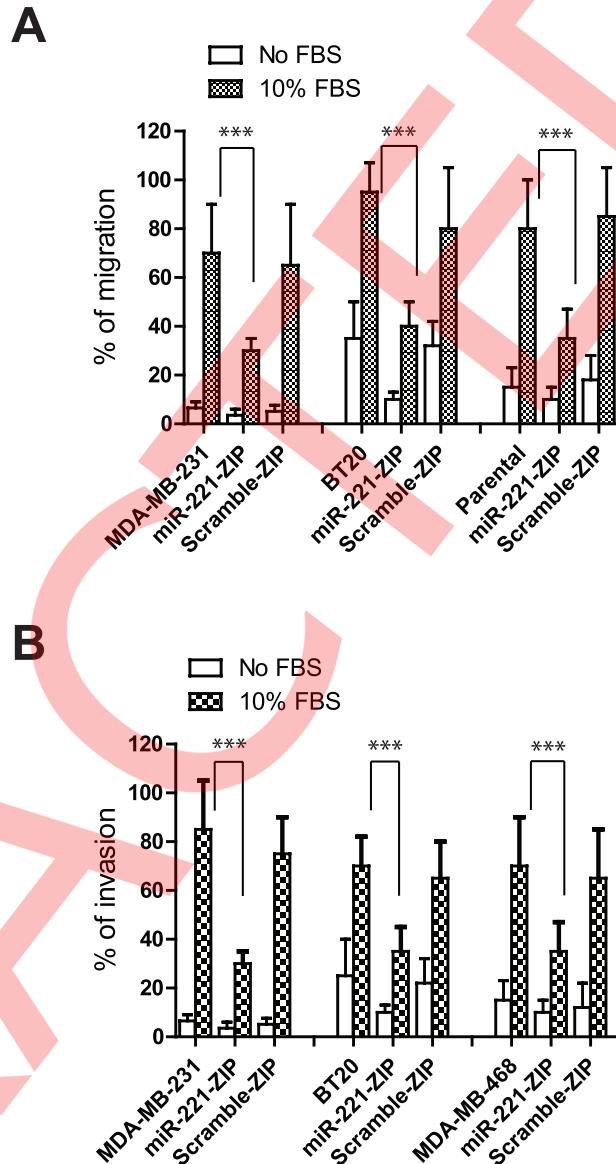
**Figure 4. Down regulation of miR-221 increases E-cadherin levels and decreases the expression levels of Snail and Slug.** (A) The RNA expression level of E-cadherin and vimentin was measured in a panel of breast cancer cell lines. Fold changes are recorded as normalized to normal breast tissue levels. (B) E-cadherin and vimentin expression levels were measured in MDA-MB-231, BT-20, MDA-MB-468 parental cells, as well cells harboring miR-221-ZIP, or scramble-ZIP. Data are normalized to the expression level in parental cells. Western blot analysis was also performed to examine the protein levels of E-cadherin and vimentin. (C) Snail and Slug expression levels were also examined in MDA-MB-231, BT-20, and MDA-MB-468 cells. Data were normalized to the expression level in parental cells and fold changes were plotted. \*\*\*denotes  $p < 0.005$ . \*\*denotes  $p < 0.01$ .

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down regulated in MDA-MB-468 and the other non-TNBC lines tested (Figure 1B). These results indicate that although miR-221/miR-222 are both down-regulated in non-TNBC cells, miR-221 is specifically over-expressed in the TNBC cell lines in comparison to normal breast tissue. Therefore, over-expression of miR-221 may be important in maintaining the characteristics of triple negative breast cancer cells.

#### miR-221 Targets p27<sup>kip1</sup> to Regulate Cell Cycle Progression in TNBCs

p27<sup>kip1</sup>, an inhibitor of cyclin dependent kinase involved in the regulation of the cell cycle, has previously been shown to be a potential target of miR-221 in a variety of cancers [17,24,31,32]. Since highly activated cell proliferation is one of the major characteristics of TNBCs, we investigated whether miR-221 also targets p27<sup>kip1</sup> in this particular breast cancer subtype. If so, we would expect an inverse relationship between the miR-221 and p27 levels. We thus measured the expression level of p27 in a variety of breast cancer cell lines as shown in Figure 2A. As expected, p27 is expressed at much lower levels in TNBCs than in other types of breast cancer cell lines. In fact, p27 expression level



**Figure 5. Down regulation of miR-221 inhibits cell migration and invasion in TNBC lines.** (A) Migration and (B) Invasion assays were performed in the absence or presence of 10% FBS after 72 hours in culture. Data are displayed as the percentage of cells migrated or invaded. T-test was performed to compare the differences between parental cells versus miR-221-ZIP cells, \*\*\*denotes  $p < 0.005$ .

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was inversely correlated to miR-221 expression level in most the breast cancer cell lines tested as shown in Figure 1A and Figure 2A. Since miR-221 and miR-222 are highly homologous and contain identical seed sequences, one might expect them to regulate the same target genes and play similar biological functions in cancer cells [17]. However, the relative expression level of miR-221 versus normal breast tissue is higher in the comparison to miR-222, and since miR-221, but not miR-222, was specifically over expressed in the TNBCs tested, we focused our experiments on miR-221. Next, we successfully and stably knocked down miR-221 using a miR-ZIP lentiviral vector in various breast cancer cells, as shown in Figure 2B. Briefly, miRZip are short, single-stranded anti-miRNAs in a lentivirus backbone that can be stably expressed to specifically target miRNAs of interest and alter translation. Knockdown of

miR-221 in MDA-MB-231, BT-20, and MDA-MB-468 TNBC cell lines induced significant increases of p27<sup>kip1</sup> both in mRNA expression and protein levels as shown in Figure 2C and Figure 2D, confirming that p27<sup>kip1</sup> is a target of this miRNA in TNBCs. Since p27<sup>kip1</sup> is involved in cell cycle regulation by modulating cyclin-dependent kinase (CDK) activity [33], we also investigated the effects of miR-221 inhibition on cell cycle progression in TNBC cell lines. miR-221 knockdown induced a G1 arrest as evidenced by observing a higher number of cells in G1 phase compared to S phase (as shown in Figure 2E). Furthermore, cell cycle profile analysis demonstrated that miR-221 knockdown in MDA-MB-231, BT-20, and MDA-MB-468 cells exhibited higher sub-G1 cell population, again suggesting that miR-221 knockdown restored p27<sup>kip1</sup> levels and subsequently induced apoptosis probably by blocking the aggressive cell cycle progression in these TNBC cell lines.

### Down Regulation of miR-221 in TNBCs Inhibits Cell Proliferation and Tumor Growth in Mice

Since cell cycle analysis demonstrated that miR-221 knockdown was able to block cell cycle progression and induce higher sub-G1 cell population, we next investigated whether miR-221 knockdown also induced apoptosis by direct measurement of apoptosis markers: cleaved caspase 3 and BAD phosphorylation (pBAD). As shown in Figure 3A, down regulation of miR-221 induced significantly higher levels of cleaved caspase 3 in MDA-MB-231 and BT-20 cells, and slightly higher levels of cleaved caspase 3 in MDA-MB-468 cells. Knocking down miR-221, however, did decrease phosphorylation of Bcl-2-associated death promoter (*BAD*), a pro-apoptotic member of the Bcl-2 gene family whose activity is regulated by survival kinases such as AKT, in all three TNBC cell lines (Figure 3A). We next investigated the cell proliferation rates of TNBCs with miR-221 knockdown. Down-regulation of miR-221 in all three TNBC lines (MDA-MB-231, BT-20, MDA-MB-468) decreased cell proliferation rates (Figure 3B), likely due to the cell cycle block and increased apoptosis induced by miR-221 knockdown. Although we did not observe a significant increase of cleaved caspase 3 in MDA-MB-468 cells, miR-221 knockdown indeed was able to decrease pBAD (Figure 3A) and induced G1 arrests (Figure 2E) and subsequently resulted in cell proliferation inhibition probably through mitochondrial apoptosis regulated by BCL2 family [34,35].

To expand on our *in vitro* results, we also investigated whether miR-221 is required for *in vivo* tumor growth. miR-221 stably knocked down MDA-MB-231 cells were implanted in nude mice and tumor growth was measured and plotted to compare with the tumor growth of MDA-MB-231 parental cell line and cells infected with the control ZIP vector alone as shown in Figure 3C. Our results indicated that miR-221 knockdown also inhibited *in vivo* tumor growth in TNBC cell line MDA-MB-231. Therefore, both the *in vitro* assays and *in vivo* studies confirm that miR-221 functions similar to an oncogene and is essential in mediating cell proliferation and tumor progression in TNBC.

### miR-221 Modulates Cell Migration and Invasion by Regulating Epithelial-mesenchymal Transition

Relative to luminal subtypes, TNBCs, having undergone an epithelial to mesenchymal transition (EMT), express higher levels of vimentin and low levels of E-cadherin which allow for their characteristic high migration and invasion capabilities through the basement membrane to promote metastasis [36]. Since miR-221 knockdown can inhibit cell proliferation and tumor growth in mice (Figure 3), we wanted to investigate the molecular mechanism for

the miR-221 mediated cell transformation activity in TNBC human cell lines. Therefore, we next examined the levels of EMT markers and performed cell migration and invasion assays. The levels of E-cadherin and vimentin in a variety of breast cancer cells were quantified relative to the normal breast tissue as shown in Figure 4A. As expected, E-cadherin is highly expressed in luminal and HER2 positive cells but not in TNBC cell lines. Conversely, vimentin is expressed in higher levels in TNBC cell lines compared to non-TNBC cells. E-cadherin and vimentin levels were measured at both the transcript and protein levels in parental, vector control and miR-221 knocked down MDA-MB-231, BT-20, and MDA-MB-468 cells. Results indicate that knocking down miR-221 in these TNBCs significantly increased both the mRNA and protein levels of E-cadherin as shown in Figure 4B. Interestingly, vimentin levels were not altered by knocking down miR-221 in these cell lines. These data suggest that although suppression of E-cadherin is regulated by miR-221, the vimentin level in TNBCs is probably regulated by other mechanisms. Since E-cadherin lacks a miR221 binding site and is likely not a direct target, we next investigated if this regulation is mediated by any of the transcription factors that have previously been reported to directly regulate E-cadherin expression [37]. Figure 4C outlines the effects of miR-221 knockdown on some of the EMT transcription factors known to regulate E-cadherin levels. We observed a robust decrease in the expression levels of mesenchymal markers Snail and Slug by miR-221 knockdown in MDA-MB-231, BT20 and MDA-MB-468 (Figure 4C). As previously reported however, the expression level of Slug in MDA-MB-468 was much lower than the other two TNBC cell lines tested [38].

We next investigated the effects of miR-221 knock down on cell migration and invasion of TNBC cell lines. As expected, MDA-MB-231, BT-20, and MDA-MB-468 showed high migratory and invasive properties in the migration and invasion assays performed upon stimulation with 10% FBS. Knocking down miR-221 decreased the FBS stimulated migration and invasion in all three cell lines as shown in Figure 5A and Figure 5B. Our data thus indicate that miR-221 alters the migration and invasion properties of TNBCs by suppressing E-cadherin expression. miR-221 knockdown in TNBCs restored E-cadherin expression and the increased E-cadherin in these TNBC cells was sufficient to block the activity of cell migration and invasion. Interestingly, although vimentin levels did not change with miR-221 knock down and high vimentin levels were maintained in these transduced TNBCs, it was not sufficient to maintain their migration and invasion capabilities. Therefore, these results suggest that the modulation of E-cadherin by miR-221 plays a critical role in maintaining the triple negative cell phenotype and knockdown of miR-221 can lead to increased E-cadherin and subsequently inhibit cell migration and invasion independent of vimentin levels.

### Discussion

miR-221 has been reported to be dysregulated in a variety of tumor types and has previously been shown to be involved in suppression of ERα expression in luminal breast cancer cells and EMT transition in basal-like breast cancers [23–26,29,30,39]. Here we demonstrate that miR-221 is specifically over expressed in TNBCs and that miR-221 knockdown induces G1 arrest and apoptosis, inhibits cell proliferation and tumor growth (probably by altering expression levels of p27<sup>kip1</sup>), and suppresses the migratory phenotypes of TNBCs by restoring E-cadherin levels. These results suggest that miR-221 is essential in regulating the aggressive characteristics of triple negative or basal like of breast cancer cells, including cell proliferation, suppressed apoptosis, high

migratory and invasive abilities, as well as accelerated *in-vivo* tumor growth. Therefore, our results provide direct evidence that overexpression of miR-221 leads to *in vitro* and *in vivo* tumorigenesis in TNBCs.

Therefore, miR-221 regulates two key mechanisms to promote the aggressive tumorigenic characteristics observed for TNBC: it promotes cell cycle progression by inhibiting p27<sup>Kip1</sup> and it promotes EMT transition by inhibiting the expression of E-cadherin. Both of these mechanisms may account for the aggressive cellular proliferation, suppression of apoptosis, as well as higher cell migration and invasion characteristics associated with BLBCs and TNBCs [9–11]. Although, we could not rule out alternative targets of miR-221 that may explain the cellular phenotypes observed, knockdown of miR-221 alone is sufficient to induce *in vitro* and *in vivo* anti-tumor activities in the TNBC cell lines tested. Hence, the translational suppression of miR-221 targets is crucial in maintaining the aggressive tumor progression of TNBCs. Previously, miR-221 over expression has been shown to alter E-cadherin/vimentin levels in an EMT-induced MCF-7 cell line, which normally does not express endogenous miR-221 [39]. Additionally, EMT transcription factor Slug has previously been shown to decrease both miR-221 and E-cadherin/vimentin levels in MDA-MB-231 cells [30]. In this study, although we did observe changes in Slug and Snail, we did not detect changes in vimentin expression after stable miR-221 knockdown in MDA-MB-231, BT20, and MDA-MB-468 cell lines. Although vimentin levels were not altered, E-cadherin expression changed signifi-

cantly with miR-221 knockdown. Our data suggest that increasing E-cadherin while maintaining vimentin levels in TNBCs seems to be sufficient for inhibiting cell migration and invasion. Therefore, E-cadherin seems to be critical in regulating cell motility, at least in the TNBC cell lines, MDA-MD-231, BT-20, and MDA-MB-468. Although miR-221 was able to regulate E-cadherin expression, we were unable to identify the complementary sequence in the 3'UTR of this protein. Therefore E-cadherin may not be a direct target of miR-221, and its expression is likely affected by alteration in EMT transcription factors Snail and Slug.

In conclusion, we have demonstrated that miR-221 is a potential oncomiR and functions as an oncogene to mediate tumor progression of TNBCs via targeting p27<sup>Kip1</sup> and inhibiting E-cadherin levels to mediate EMT transition. These results may prove useful for therapeutic options for TNBCs when systemic delivery of anti-miR-221 becomes feasible.

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## Author Contributions

Conceived and designed the experiments: RN MJY. Performed the experiments: RN PPM SMB. Analyzed the data: RN MJY. Contributed reagents/materials/analysis tools: RN PPM SMB MJY. Wrote the paper: RN MJY.

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