Published in final edited form as:

Biochem Biophys Res Commun. 2016 August 26; 477(3): 461–466. doi:10.1016/j.bbrc.2016.06.076.

Downregulation of MicroRNA-206 Promotes Invasion and Angiogenesis of Triple Negative Breast Cancer

Zhongxing Liang^{a,b,*}, Xuehai Bian^{a,c}, and Hyunsuk Shim^{a,b}

^aDepartment of Radiology, Emory University, Atlanta, GA 30322, USA

^bWinship Cancer Institute, Emory University, Atlanta, GA 30322, USA

^cDepartment of Thyroid Surgery, China-Japan Union Hospital of Jilin University, Changchun, China

Abstract

Triple negative breast tumors don't respond to Tamoxifen and Herceptin, two of the most effective medications for treating breast cancer. Additionally, triple negative breast cancer (TNBC) intrinsically resists or will eventually acquire resistance to chemotherapy. The purpose of this study is to understand better the molecular basis of TNBC as well as develop new therapeutic strategies against it. Here, we analyzed miRNA-206 expression levels in breast cancer cell lines and tissues. In addition, we investigated whether miR-206 mimics inhibited TNBC tumor invasion and angiogenesis. The results showed that miR-206 was downregulated in TNBC compared to non-TNBC cell lines and tissues. Additionally, the decreased levels of miR-206 were inversely consistent with expression levels of VEGF. Furthermore, the forced expression of miR-206 in the mimic-transfected TNBC cells downregulated VEGF, MAPK3, and SOX9 expression levels. The miR-206 mimics inhibited TNBC breast cell invasion and angiogenesis. These findings demonstrate for the first time the involvement of miRNA-206 in TNBC invasion and angiogenesis and suggest that miR-206 may be an efficient agent for therapy of TNBC.

Keywords

microRNA; triple negative breast cancer; VEGF; invasion; angiogenesis

1. Introduction

Triple negative breast cancer (TNBC) refers to a specific subtype of breast cancer that lacks expression of the estrogen receptor (ER), progesterone receptor (PR) or human epidermal growth factor receptor 2 (her2/neu) [1, 2]. TNBC is one of clinically aggressive subtypes and associated with overall poor prognosis [3–5]. Patients with TNBC have an increased likelihood of metastasis and distant recurrence compared to patients with other breast

*To whom correspondence should be addressed: Dr. Zhongxing Liang, zliang@emory.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

cancers [5, 6]. In addition, patients with TNBC are more likely to have grade III tumors [5]. It is diagnosed more frequently in African-American women [7, 8]. ER, PR, and Her2/neu are known to enable cancer development, and the most successful breast cancer treatments are hormone-based drugs that directly target these receptors. Unfortunately, Tamoxifen and Herceptin, two of the most effective medications of targeted therapy for breast cancer fail to work for TNBC patients. Therefore, there is a major unmet need to better understand the molecular basis of this type of breast cancer as well as develop new therapeutic strategies against it.

To improve our understanding of the molecular mechanisms of the metastatic process and benefit prognosis and therapy of advanced breast cancer, it is critical to investigate whether microRNAs (miRNAs) are involved in TNBC growth and progression. Vascular endothelial growth factor (VEGF), the major mediator of angiogenesis and tumor proliferation [9, 10], is frequently overexpressed in a variety of cancers [11, 12]. Numerous studies have shown that VEGF actively contributes to cancer progression through angiogenesis, metastasis, and chemotherapeutic resistance [13, 14]. MiR-206 expression levels have been reported as downregulated in a few types of cancer tissues compared to normal tissues [15–17], and low expression levels are correlated with poor overall survival [18] and metastasis of lung cancer [19]. The roles of miR-206 in tumor progression, especially in TNBC progression, are not well understood so far. In the present study, we show for the first time that miRNA-206 is a potential therapeutic target for triple negative breast cancer through modulating contributors to angiogenesis, such as VEGF.

2. Materials and methods

2.1. Breast cancer cell lines and culture

The human TNBC cell lines MDA-MB-231, MDA-MB-435, and HCC1395 were grown in RPMI1640 medium containing 10% FBS, 100 U/ml of penicillin sodium, and 100 µg/ml of streptomycin sulfate at 37 °C in a humidified atmosphere of 5% CO₂. MCF-7, an ER-expressing breast cancer cell line, was cultured in DMEM medium containing 10% FBS plus 10 µg/ml insulin. SKBR3, a Her2/neu-expressing human breast cancer cell line, and MDA-MB-361 expressing ER, PR, and Her2/neu, were incubated with RPMI1640 containing 10% FBS plus 100 U/ml of penicillin sodium and 100 µg/ml of streptomycin sulfate.

2.2. Tissue samples and immunohistochemical staining

The sources and characteristics of archived breast tumor and normal breast samples are summarized in Table 1. These samples were obtained from the Avon Tissue Bank for Translational Genomics Research at Grady Memorial Hospital in Atlanta, GA. The detailed staining procedure and semi-quantitative method of immunohistochemical staining of these formalin-fixed and paraffin-embedded tissue sections for VEGF were described in our previous paper [20].

2.3. RNA isolation from formalin-fixed and paraffin-embedded tissues and quantitative real-time RT-PCR

Total RNA was extracted from formalin-fixed paraffin-embedded breast cancer tissue sections after deparaffinization by using Trizol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions following our previous description [20, 21]. Regular and quantitative RT-PCR were performed following our previous descriptions [21]. Primer sequences of *miR-206*, and *U6 snRNA* are as follows: *miR-206* (GeneBank accession number NR029713), 5'-CGAGGCCACATGCTTCTTTA-3' and 5'-CCGAAACCACACACTTCCTT-3'; *U6 snRNA* (GeneBank accession number NM_012321), 5'-CGGGTTTGTGTTTGCATTCT-3' and 5'-AGTCCCAGCATGAACAGCTT-3'. SYBR Green quantitative PCR reaction was carried out in a 15 µl reaction volume containing 2× PCR Master Mix (Applied Biosystems) following our previous reports [22, 23].

2.4. Transfection of hsa-miR-206 mimics and Western blotting analyses

The hsa-miR-206 mimics were purchased from Dharmacon (Lafayette, CO). The mimics or control oligonucleotides were transfected into TNBC MDA-MB-231 cells and an estrogen positive human breast cancer MCF-7 cells at a final concentration of 100 nM. The transfected cells were collected at 48 hours post-transfection. Proteins were resolved in an SDS/PAGE gel and subjected to immunoblot analysis using monoclonal antibodies against VEGF (EMD Millipore, Billerica, MA), MAPK3 (Abcam, Cambridge, MA), SOX9 (Abcam, Cambridge, MA), or β-actin (Sigma-Aldrich, St. Louis, MO). All antibodies were used at 1 µg/ml of working concentration in PBS with 5% dried milk. Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL; Pierce Biotechnology Inc., Rockford, IL).

2.5. Tumor Cell Invasion Assay

The invasion assay was performed by using a Matrigel invasion chamber from BD Biocoat Cellware (San Jose, CA) as previously described [24]. The 2×10^4 of miR-206 mimic-transfected or control oligonucleotides-transfected TNBC MDA-MB-231 and non-TNBC MCF-7 cells were added into the top chambers. The Matrigel invasion chambers were then incubated for 16 hrs in a humidified tissue culture incubator. Invading cells at the bottom was determined by counting the H&E-stained cells.

2.6. Matrigel plug assay and hemoglobin assay

For an *in vivo* angiogenesis assay (Matrigel plug assay), 2×10^5 MDA-MB-231 or MCF-7 cells were mixed with 0.5 ml of growth factor-reduced Matrigel (BD Biosciences) and implanted subcutaneously into the flanks of nude mice. From the following day, six mice in each group were treated with 100 µg/kg miR-206 mimics or control oligonucleotides via daily subcutaneous injections between the two plugs on the back of the mice. The animals were sacrificed and the Matrigel plugs were excised 10 days after Matrigel injection. The excised plugs were homogenized and subjected to measure hemoglobin content with 100 µL of Drabkin's solution (Sigma, St. Louis, MO) following manufactural instruction and our previous description [25].

2.7. Statistical analysis

Real-time RT-PCR reaction was run in triplicate for each sample and repeated at least 2 times, and the data were statistically analyzed with a Student T-test.

3. Results

3.1. MiR-206 was prominently downregulated in TNBC tissues and inversely correlated with VEGF

Quantitative RT-PCR results show that expression levels of miR-206 are obviously lower in TNBC cell lines than those in non-TNBC cell lines (Fig. 1A). Similarly, TNBC tissues express prominently lower levels of miR-206 compared to non-TNBC tissue samples and normal breast tissues (Fig. 1B). It is worth noting that non-TNBC tissues expressed lower miR-206 compared to normal breast tissues but miR-206 levels in non-TNBC tissues were higher than those in TNBC tissues (Fig. 1B). Furthermore, we analyzed the expression levels of VEGF protein determined by immunohistochemical staining in breast cancer tissue samples. VEGF expression levels were inversely correlated with those of miR-206 in breast cancer tissues (Fig. 1C). These results demonstrate that expression levels of miR-206 are predominantly downregulated in TNBC tissues in comparison to non-TNBC tissues and normal breast tissue samples and are inversely correlated with the levels of VEGF.

3.2. MiR-206 mimics predominantly inhibit the invasion of TNBC cells in vitro

To investigate whether miR-206 mimics block the invasion of TNBC cells, miR-206 mimics or control oligonucleotides were transfected into TNBC MDA-MB-231 and non-TNBC MCF-7 cells. The invasive cells of treated groups were determined and compared to their controls by Matrigel invasion assay. Fig 2A shows representatives of invasive cell photographs from individual groups. Matrigel Invasion assay shows that the invasion of TNBC cells transfected with miR-206 mimics was only 12% of that of the control (Fig. 2B). These results demonstrate that miR-206 inhibits the invasion of 88% of TNBC cells. In addition, Fig.2 shows that less invasive non-TNBC MCF-7 cells were observed in comparison to TNBC MDA-MB-231 cells. MiR-206 mimics blocked 62% invasive MCF-7 cells, though it was less than 88% invasive inhibition of TNBC MDA-MB-231 cells (Fig. 2B). These data suggest that miR-206 mimics efficiently inhibit the invasion of breast cancer cells, particularly TNBC cells.

3.3. MiR-206 mimics predominantly inhibit the angiogenesis of TNBC tumors by Tumor Angiogenesis Assay

To determine the effect of the miR-206 mimics on angiogenesis *in vivo*, Matrigel plug assay was performed in nude mice. When MDA-MB-231 cells successfully promote the neovasculture formation within the Matrigel plug, these neovasculatures allow tumor cells to proliferate much better than those without neovasculatures. Microphotographs of the miR-206 mimic-treated Matrigel plugs in the left panel of Fig. 3A show fewer neovasculatures compared to the control. H&E staining shows that fewer red cells present in the Matrigel plug sections from treated groups compared to the control in the right panel of Fig. 3A. Therefore, the control group with better angiogenesis in the Matrigel plugs showed

more red cells than the treated group (Fig. 3A), particularly in the TNBC MDA-MB-231 control groups. Figure 3B showed the percentage of antiangiogenic efficacy based on hemoglobin content in 10 Matrigel plugs per group. MiR-206 mimics show an obvious antiangiogenic effect with 67 % inhibition of angiogenesis compared to the oligonucleotide control. In addition, a 54% inhibition of angiogenesis in the MCF-7 derived Matrigel plugs was observed with miR-206 mimic treatment compared to the control (Fig. 3). These results suggest that miR-206 mimics predominantly inhibit TNBC angiogenesis.

3.4. VEGF, SOX9, and MAPK3 are predicted targets of MiR-206 miRNA

The results of a search for the predicted targets with TargetScan showed that *VEGF*, *MAPK3*, and *SOX9* are predicted targets of miR-206 (Fig. 4A). To verify whether miR-206 directly targets *VEGF*, we performed a luciferase reporter assay. The results showed that luciferase activity from the vector with the *VEGF* 3'-UTR segment containing miR-206 binding sites was inhibited by miR-206 mimics in a dose-dependent manner (Fig. 4B). Furthermore, the miR-206 mimics downregulated expression levels of *VEGF*, *MAPK3*, and *SOX9* proteins in TNBC cells compared to control oligonucleotide-transfected TNBC cells (Fig. 4C).

4. Discussion

The expression levels of miR-206 are predominantly decreased in TNBC cells and tissue samples compared to non-TNBC and normal breast tissue samples. Previous studies suggest that downregulation of miRNAs may play a role in cancer progression [26–28]. Despite strong evidence suggesting that miRNAs can be potential biomarkers for cancer diagnosis and personalized therapy, only a few publications have reported on the involvement of microRNAs in development of triple negative breast cancer. Recently, a few of reports have shown that miR-206 plays a critical role in the growth, migration, and metastasis of breast cancer as a tumor suppressor [29–31]. However, the roles of miR-206 in the growth and progression in TNBC are not well understood so far. In the present studies, our results showed that non-TNBC tissues expressed lower miR-206 compared to normal breast tissues but miR-206 levels in TNBC tissues were particularly lower than those in non-TNBC tissues. The particularly lower expression of miR-206 in TNBC cells and tissues provides not only important evidence for understanding mechanisms of TNBC growth and progression, but also offers the chance to identify new therapeutic targets for the therapy of TNBC.

Our present studies show that miR-206 is antagonistically involved in TNBC invasion and angiogenesis. Numerous studies have shown that VEGF actively contributes to cancer progression through angiogenesis, metastasis, and chemotherapeutic resistance [13, 14, 32]. Dysregulation of Mitogen-activated protein kinase (MAPK) levels in patients has been shown to be associated with advanced stages and short survival in patients with prostate, breast, and liver cancer [33, 34]. SOX9, a transcription factor, has exhibited several pro-oncogenic properties, including the ability to promote proliferation, inhibit senescence, and collaborate with other oncogenes in neoplastic transformation [35]. Bioinformatics analysis shows that VEGF, SOX9, and MAPK are the predicted targets of miR-206. However, VEGF,

SOX9, and MAPK have not been characterized as the targets of miR-206. Our results show that the elevated levels of miR-206 in mimic-transfected TNBC MDA-MB-231 cells not only downregulated expression of VEGF protein, but also repressed the expression of SOX9 and MAPK and further inhibited TNBC cell invasion and angiogenesis. Here, we report for the first time miR-206 as a suppressor to modulate VEGF-mediated invasion and angiogenesis of TNBC tumor cells. Our results demonstrated that the loss of miR-206 in TNBC cells contributed to the overexpression of VEGF, MAPK3, and SOX9.

Novel targeted therapies, such as PARP and EGFR inhibitors for TNBC therapy alone or in combination with chemotherapy, are currently under study. Anti-EGFR antibodies and EGFR tyrosine kinase inhibitors are promising in the treatment of TNBC with EGFR gene amplification [36]. However, resistance of breast cancer cells has been demonstrated to some of these inhibitors [37, 38]. The mechanism of TNBC progression and novel therapeutic approaches needs to be investigated and developed. The advantage of miRNAs is their ability to affect multiple targets with a single hit, thus regulating a whole network of interacting molecules [39]. A recent study showed that miR-206 was downregulated [29] in human breast cancer tumors. Furthermore, miR-206 reduced cell proliferation through targeting 6-Phosphofructo-2-kinase (PFKFB3) to control the rate of glycolysis [29] and inhibited migration in breast cancer cells and T-box transcription factor Tbx3 [29, 31]. In our studies, miR-206 has shown to be downregulated in breast cancer cell lines and tissues, particularly in TNBC cell lines and tissue samples. Furthermore, the efficient inhibition in the invasion and angiogenesis of TNBC cells by miR-206 replacement treatment was observed in this investigation. Taken together, miR-206 may be a robust target for the inhibition of the growth and metastasis of triple negative breast cancer through modulating multifaceted signaling pathways.

In conclusion, we have shown that expression of miR-206 is negatively correlated with VEGF expression, particularly in TNBC cells. Furthermore, the elevated levels of miR-206 by transfection of miR-206 mimics not only down-regulate expression of VEGF, MAPK3, and SOX9, but also particularly inhibit TNBC invasion and angiogenesis. Our findings further contribute to the understanding of TNBC regulation. Additionally, these findings may be beneficial for designing personalized therapy for breast cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was financially supported by a Research Grant from NIH NCI (1R01CA165306). We thank the Avon Tissue Bank for Translational Genomics Research at Grady Memorial Hospital for providing normal breast and breast tumor tissues. The authors thank Ms. Jessica Paulishen and Ms. Amber Feng for proof-reading.

Abbreviations

TNBC	Triple negative breast cancer
miRNA	microRNA

VEGF	Vascular endothelial growth factor
ER	estrogen receptor
PR	progesterone receptor
MAPK	matrix metalloproteinase
SOX-9	sex-determining region Y (SRY)-box 9 transcription factor protein that in humans is encoded by the <i>SOX9</i> gene
siRNA	small interfering RNA
RT-PCR	reverse transcription polymerase chain reaction

References

1. Rakha EA, Ellis IO. Triple-negative/basal-like breast cancer: review. *Pathology*. 2009; 41:40–47. [PubMed: 19089739]
2. Irvin WJ Jr, Carey LA. What is triple-negative breast cancer? *Eur J Cancer*. 2008; 44:2799–2805. [PubMed: 19008097]
3. Bauer KR, Brown M, Cress RD, Parise CA, Caggiano V. Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry. *Cancer*. 2007; 109:1721–1728. [PubMed: 17387718]
4. Schneider BP, Winer EP, Foulkes WD, Garber J, Perou CM, Richardson A, Sledge GW, Carey LA. Triple-negative breast cancer: risk factors to potential targets. *Clin Cancer Res*. 2008; 14:8010–8018. [PubMed: 19088017]
5. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, Lickley LA, Rawlinson E, Sun P, Narod SA. Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin Cancer Res*. 2007; 13:4429–4434. [PubMed: 17671126]
6. Fulford LG, Reis-Filho JS, Ryder K, Jones C, Gillett CE, Hanby A, Easton D, Lakhani SR. Basal-like grade III invasive ductal carcinoma of the breast: patterns of metastasis and long-term survival. *Breast Cancer Res*. 2007; 9:R4. [PubMed: 17217540]
7. Morris GJ, Naidu S, Topham AK, Guiles F, Xu Y, McCue P, Schwartz GF, Park PK, Rosenberg AL, Brill K, Mitchell EP. Differences in breast carcinoma characteristics in newly diagnosed African-American and Caucasian patients: a single-institution compilation compared with the National Cancer Institute's Surveillance, Epidemiology, and End Results database. *Cancer*. 2007; 110:876–884. [PubMed: 17620276]
8. Lund MJ, Trivers KF, Porter PL, Coates RJ, Leyland-Jones B, Brawley OW, Flagg EW, O'Regan RM, Gabram SG, Eley JW. Race and triple negative threats to breast cancer survival: a population-based study in Atlanta, GA. *Breast cancer research and treatment*. 2009; 113:357–370. [PubMed: 18324472]
9. Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discov*. 2004; 3:391–400. [PubMed: 15136787]
10. Lamszus K, Ulbricht U, Matschke J, Brockmann MA, Fillbrandt R, Westphal M. Levels of soluble vascular endothelial growth factor (VEGF) receptor 1 in astrocytic tumors and its relation to malignancy, vascularity, and VEGF-A. *Clin Cancer Res*. 2003; 9:1399–1405. [PubMed: 12684411]
11. Kirkpatrick K, Ogunkolade W, Elkak A, Bustin S, Jenkins P, Ghilchik M, Mokbel K. The mRNA expression of cyclo-oxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) in human breast cancer. *Curr Med Res Opin*. 2002; 18:237–241. [PubMed: 12201625]

12. Latil A, Bieche I, Pesche S, Valeri A, Fournier G, Cussenot O, Lidereau R. VEGF overexpression in clinically localized prostate tumors and neuropilin-1 overexpression in metastatic forms. *International journal of cancer*. 2000; 89:167–171. [PubMed: 10754495]
13. Schuurbiers OC, Kaanders JH, van der Heijden HF, Dekhuijzen RP, Oyen WJ, Bussink J. The PI3-K/AKT-pathway and radiation resistance mechanisms in non-small cell lung cancer. *J Thorac Oncol*. 2009; 4:761–767. [PubMed: 19404218]
14. Jameel JK, Rao VS, Cawkwell L, Drew PJ. Radioresistance in carcinoma of the breast. *Breast (Edinburgh, Scotland)*. 2004; 13:452–460.
15. Yang Q, Zhang C, Huang B, Li H, Zhang R, Huang Y, Wang J. Downregulation of microRNA-206 is a potent prognostic marker for patients with gastric cancer. *Eur J Gastroenterol Hepatol*. 2013; 25:953–957. [PubMed: 23751352]
16. Bao YP, Yi Y, Peng LL, Fang J, Liu KB, Li WZ, Luo HS. Roles of microRNA-206 in osteosarcoma pathogenesis and progression. *Asian Pacific journal of cancer prevention : APJCP*. 2013; 14:3751–3755. [PubMed: 23886177]
17. Li Y, Hong F, Yu Z. Decreased expression of microRNA-206 in breast cancer and its association with disease characteristics and patient survival. *J Int Med Res*. 2013; 41:596–602. [PubMed: 23696595]
18. Missiaglia E, Shepherd CJ, Patel S, Thway K, Pierron G, Pritchard-Jones K, Renard M, Sciort R, Rao P, Oberlin O, Delattre O, Shipley J. MicroRNA-206 expression levels correlate with clinical behaviour of rhabdomyosarcomas. *British journal of cancer*. 2010; 102:1769–1777. [PubMed: 20502458]
19. Wang X, Ling C, Bai Y, Zhao J. MicroRNA-206 is associated with invasion and metastasis of lung cancer. *Anatomical record*. 2011; 294:88–92.
20. Shim H, Lau SK, Devi S, Yoon Y, Cho HT, Liang Z. Lower expression of CXCR4 in lymph node metastases than in primary breast cancers: potential regulation by ligand-dependent degradation and HIF-1 α . *Biochem Biophys Res Commun*. 2006; 346:252–258. [PubMed: 16756955]
21. Liang Z, Brooks J, Willard M, Liang K, Yoon Y, Kang S, Shim H. CXCR4/CXCL12 axis promotes VEGF-mediated tumor angiogenesis through Akt signaling pathway. *Biochem Biophys Res Commun*. 2007; 359:716–722. [PubMed: 17559806]
22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2 $\times$ (-Delta Delta C(T)) Method. *Methods (San Diego, Calif)*. 2001; 25:402–408.
23. Liang Z, Wu H, Xia J, Li Y, Zhang Y, Huang K, Wagar N, Yoon Y, Cho HT, Scala S, Shim H. Involvement of miR-326 in chemotherapy resistance of breast cancer through modulating expression of multidrug resistance-associated protein 1. *Biochemical pharmacology*. 2010; 79:817–824. [PubMed: 19883630]
24. Liang Z, Yoon Y, Votaw J, Goodman M, William L, Shim H. Silencing of CXCR4 blocks breast cancer metastasis. *Cancer research*. 2005; 65:967–971. [PubMed: 15705897]
25. Zhu A, Zhan W, Liang Z, Yoon Y, Yang H, Grossniklaus HE, Xu J, Rojas M, Lockwood M, Snyder JP, Liotta DC, Shim H. Dipyrimidine amines: a novel class of chemokine receptor type 4 antagonists with high specificity. *Journal of medicinal chemistry*. 2010; 53:8556–8568. [PubMed: 21105715]
26. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer research*. 2005; 65:6029–6033. [PubMed: 16024602]
27. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102:13944–13949. [PubMed: 16166262]
28. Singh R, Mo YY. Role of microRNAs in breast cancer. *Cancer biology & therapy*. 2013; 14:201–212. [PubMed: 23291983]
29. Ge X, Lyu P, Cao Z, Li J, Guo G, Xia W, Gu Y. Overexpression of miR-206 suppresses glycolysis, proliferation and migration in breast cancer cells via PFKFB3 targeting. *Biochem Biophys Res Commun*. 2015; 463:1115–1121. [PubMed: 26093295]
30. Sasaki A, Tsunoda Y, Tsuji M, Uda Y, Oyama H, Tsuchiya H, Oguchi K. Decreased miR-206 expression in BRCA1 wild-type triple-negative breast cancer cells after concomitant treatment

- with gemcitabine and a Poly(ADP-ribose) polymerase-1 inhibitor. *Anticancer Res.* 2014; 34:4893–4897. [PubMed: 25202071]
31. Amir S, Simion C, Umeh-Garcia M, Krig S, Moss T, Carraway KL 3rd, Sweeney C. Regulation of the T-box transcription factor Tbx3 by the tumour suppressor microRNA-206 in breast cancer. *British journal of cancer.* 2016; 114:1125–1134. [PubMed: 27100732]
32. Bussink J, van der Kogel AJ, Kaanders JH. Activation of the PI3-K/AKT pathway and implications for radioresistance mechanisms in head and neck cancer. *Lancet Oncol.* 2008; 9:288–296. [PubMed: 18308254]
33. Mukherjee R, McGuinness DH, McCall P, Underwood MA, Seywright M, Orange C, Edwards J. Upregulation of MAPK pathway is associated with survival in castrate-resistant prostate cancer. *British journal of cancer.* 2011; 104:1920–1928. [PubMed: 21559022]
34. Wang C, Cigliano A, Delogu S, Armbruster J, Dombrowski F, Evert M, Chen X, Calvisi DF. Functional crosstalk between AKT/mTOR and Ras/MAPK pathways in hepatocarcinogenesis: implications for the treatment of human liver cancer. *Cell Cycle.* 2013; 12:1999–2010. [PubMed: 23759595]
35. Matheu A, Collado M, Wise C, Manterola L, Cekaite L, Tye AJ, Canamero M, Bujanda L, Schedl A, Cheah KS, Skotheim RI, Lothe RA, Lopez de Munain A, Briscoe J, Serrano M, Lovell-Badge R. Oncogenicity of the developmental transcription factor Sox9. *Cancer research.* 2012; 72:1301–1315. [PubMed: 22246670]
36. Kumar S, Mishra AK, Kolte AP, Arora AL, Singh D, Singh VK. Effects of the Booroola (FecB) genotypes on growth performance, ewe's productivity efficiency and litter size in Garole × Malpura sheep. *Animal reproduction science.* 2008; 105:319–331. [PubMed: 17449205]
37. Yamasaki F, Johansen MJ, Zhang D, Krishnamurthy S, Felix E, Bartholomeusz C, Aguilar RJ, Kurisu K, Mills GB, Hortobagyi GN, Ueno NT. Acquired resistance to erlotinib in A-431 epidermoid cancer cells requires down-regulation of MMAC1/PTEN and up-regulation of phosphorylated Akt. *Cancer research.* 2007; 67:5779–5788. [PubMed: 17575145]
38. Cruz-Lopez O, Conejo-Garcia A, Nunez MC, Kimatrai M, Garcia-Rubino ME, Morales F, Gomez-Perez V, Campos JM. Novel substituted quinazolines for potent EGFR tyrosine kinase inhibitors. *Current medicinal chemistry.* 2011; 18:943–963. [PubMed: 21254978]
39. Iorio MV, Croce CM. MicroRNAs in cancer: small molecules with a huge impact. *J Clin Oncol.* 2009; 27:5848–5856. [PubMed: 19884536]

Highlights

- MiR-206 is downregulated in TNBC compared to non-TNBC cell lines and tissues.
- Decreased levels of miR-206 are inversely consistent with expression levels of VEGF.
- MiR-206 downregulates VEGF expression in the mimic-transfected TNBC cells.
- MiR-206 mimics inhibit TNBC breast cell invasion and angiogenesis.

Fig. 1.

MiR-206 is downregulated in triple negative breast cancer cells and tissues and inversely correlated with VEGF levels. (A) Comparison of miR-206 expression levels in breast cancer cell lines. (B) A box and whisker plot diagram showing the comparison of miR-206 expression levels in triple negative breast cancer tissues (n=18), non-TNBC breast cancer tissues (n=22), and normal breast tissue samples (n=9). MiR-206 expression levels were normalized to expression levels of miR-16 in the same sample. Horizontal lines in the boxes represent the median value of miR-206 of each group. Top and bottom edges of the boxes indicate the score values of the 75th and the 25th percentile, respectively. Whiskers represent

the highest and lowest values. The range is shown as a vertical line. (C) miR-206 expression levels determined by qRT-PCR are conversely correlated with VEGF levels determined by immunohistochemical staining in 40 breast cancer tissue samples.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 2.

MiR-206 mimics predominantly inhibit invasion of human TNBC MDA-MB-231 cells. **(A)** Representative photograph for invasion cells from miR-206 mimics-treated and control oligonucleotide-treated groups. **(B)** Comparison of invasion cells of miR-206 mimics-treated TNBC MDA-MB231 and non-TNBC MCF-7 cells and control oligonucleotides-treated cells by Matrigel Invasion Assay. * $P < 0.01$ compared to their controls.

Fig. 3.

MiR-206 mimics inhibit the angiogenesis *in vivo*. **(A)** Microphotograph of Matrigel plug representatives from the control and miR-206 mimics-treated groups on the left panel and representative pictures of H&E staining for Matrigel plug sections from the control and treated groups on the right panel. The green arrows point to red cells in the picture. **(B)** The column graph shows the comparison of average hemoglobin in plugs from the control and miR-206 mimics-treated groups.

Fig. 4. MiR-206 microRNA directly targets *VEGF*. (A) The putative miR-206 targeted sequences in *VEGF*, *SOX9*, and *MAPK3*, 3' UTRs by TargetScan prediction. (B) Luciferase reporter assays. The luciferase activity in MDA-MB-231 transfected with the vector containing *VEGF* 3' UTR fragment with binding sequence of miR-206 was inhibited by transfection of miR-206 mimics in a dose-dependent manner. (C) Expression levels of VEGF, MAPK3, and SOX9 were downregulated in miR-206 mimics-transfected TNBC cells.

Table 1

Source and characteristics of tissue specimens of breast tumors

	Total	T stage		Sex		Age	
		T1&T2	T3T4	Male	Female	<50	≥50
Triple negative	18	7	11	0	18	4	14
Non-triple negative	22	8	14	0	22	5	17
Total	40	15	25	0	40	9	31