Overexpression of HDAC9 Promotes Invasion and Angiogenesis of Triple Negative Breast Cancer by regulating microRNA-206

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Abstract

Triple negative breast cancer (TNBC) is among the most aggressive breast cancer subtypes with poor prognosis. The purpose of this study is to better understand the molecular basis of TNBC as well as develop new therapeutic strategies. Our results demonstrate that HDAC9 is overexpressed in TNBC compared to non-TNBC cell lines and tissues. Furthermore, we show that HDAC9 overexpression is inversely proportional with miR-206 expression levels. Subsequent HDAC9 siRNA knockdown was then shown to restore miR-206 while also decreasing VEGF and MAPK3 levels. This study highlights HDAC9 as a mediator of invasion and angiogenesis in TNBC cells through VEGF and MAPK3 by modulating miR-206 expression and suggests that selective inhibition of HDAC9 may be an efficient route for TNBC therapy.

Keywords

HDAC9; triple negative breast cancer; microRNA; invasion; angiogenesis

1. Introduction

Two classes of enzymes impact the acetylation state of histone proteins — histone acetyltransferases (HATs) and histone deacetylases (HDACs) [1, 2]. The HDAC family of enzymes is involved in various biological processes including transcriptional control, growth arrest, and cell death, particularly in tumor development and proliferation [3–7]. A number of HDAC inhibitors have been characterized that inhibit tumor growth *in vitro* and *in vivo* at amounts that have little to no toxicity [8–15]. The results from these studies and their proposed mechanisms indicate that HDACs are excellent targets for cancer treatment. However, clinical benefits of selective versus broad HDAC inhibitors are unknown and the

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appropriateness of inhibitor may depend on tumor HDAC enzyme expression, enzyme selectivity of the inhibitor, and the desired effects. Although there are several published studies [16, 17] and numerous ongoing clinical trials involving HDAC inhibitors [18], very little is known about the roles of individual HDAC enzymes, and a formal assessment of HDAC enzyme expression is not routinely done. Emerging data suggests that the currently used HDAC inhibitors may differ significantly with regard to target selectivity. In addition, the expression of HDAC enzymes may vary considerably between normal and tumor tissues and likely between different phenotypes of tumor tissues. HDAC9 is thought to regulate gene expression through epigenetic modulation of the chromatin structure by catalyzing the deacetylation of histone proteins [19]. HDAC9 is also known to target non-histone proteins, such as forkhead box protein 3, ataxia telangiectasia group D-complementing protein (ATDC), and glioblastoma 1 protein, which are members of pathways implicated in carcinogenesis [20, 21]. More recently, aberrant HDAC9 expression has been observed in several types of cancers, including medulloblastoma [21], acute lymphoblastic leukemia [22], glioblastoma [23], osteosarcoma [24], and breast cancer [25]. HDAC9 has been shown to promote the growth of these tumors.

Triple negative breast cancer (TNBC) is one of the most clinically aggressive subtypes of breast cancer and is associated with overall poor prognosis [26–28]. TNBC lacks expression of the estrogen receptor (ER), progesterone receptor (PR) or human epidermal growth factor receptor 2 (her2/neu) [29, 30]; as a result, conventional therapies targeting each of these receptors proves to be unsuccessful in TNBC. 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. Emerging studies have highlighted microRNAs (miRNAs) as critical mediators of tumorigenesis, as their involvement have been well-established across several types of cancers, including breast, prostate, ovarian, and head and neck cancers [31–34]. Previous work from our group identified miR-206 as a critical tumor suppressor in TNBC and showed that downregulation of miR-206 lead to an upregulation of VEGF, MAPK3, and SOX9, crucial drivers of invasion and angiogenesis [35]. In this study, we demonstrate that overexpression of HDAC9 promotes the invasion and angiogenesis in TNBC by directly modulating miR-206 and provides a selective basis for TNBC treatment.

2. Materials and methods

2.1. Breast cancer cell lines and culture

The human breast cancer cell lines MDA-MB-231, MDA-MB-1739, HCC1395, MDA-MB-361, and SKBR3, 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% CO_2. MCF-7, an ER-expressing breast cancer cell line, was cultured in DMEM medium containing 10% FBS plus 10 μg/ml of insulin. SKBR3 is a Her2/neu-expressing human breast cancer cell line, and MDA-MB-361 is positive for ER, PR, and Her2/neu. MDA-MB-231, MDA-MB-1739, and HCC1395 are all triple negative cell lines.

2.2. Tissue samples and immunohistochemical staining

A formalin-fixed and paraffin-embedded breast cancer tissue array was obtained from US Biomax (Derwood, Maryland, USA). This is a breast cancer and matched metastatic carcinoma tissue array, including TNM and pathology grade, with ER, PR and Her-2 (neu) IHC results, 50 cases/100 cores. The sources and characteristics of archived breast tumor breast samples are summarized in Table 1. HDAC9 antibody (Abcam, Cat No. ab70954) was applied on slides at 1:500 and incubated for 1 hour at room temperature after deparaffinized, antigen-retrieved (DAKO, Cat No., S1699), and endogenous peroxidase block with 3% Hydrogen Peroxide (DAKO, Cat No., S2003,). Visualization and detection were established using DAKO EnVision+ Dual (mouse and rabbit) Link System-HRP (Cat No., K4061) with an incubation time of 30 minutes. The detailed staining procedure and semi-quantitative method of immunohistochemical staining of these tissue sections for HDAC9 are described in our previous paper [36] (DAKO, Cat No., K4061).

Quantitative real-time RT-PCR

Regular and quantitative RT-PCR were performed following our previous protocol [37]. Primer sequences of *miR-206*, and *U6 snRNA* have been described in our previous report [35]. Primer sequences of HDAC9 are as follows: (GeneBank accession number BC152405), 5'- CAGCAACGAAAGACACTCCA-3' and 5'- CAGAGGCAGTTTTTCGAAGG-3'. SYBR Green quantitative PCR reaction was carried out in a 15 μl reaction volume containing 2× PCR Master Mix (Applied Biosystems) per our previous reports [38, 39].

2.4. Construction of HDAC9 siRNAs and Transfection.

We designed and purchased the small interfering RNA (siRNA) duplexes against HDAC9 (Genbank accession no., BC152405) from ThermoFisher Scientific (Grand Island, NY, USA). The target sequence of HDAC9 siRNA is 5′- UAAAAUCUUCCUGCCCACCdTdT-3′. The nonspecific control siRNA duplexes were purchased from ThermoFisher Scientific with the same GC content as HDAC9 siRNAs. The siRNA or control oligonucleotides were transfected into TNBC MDA-MB-231 cells at a final concentration of 100 nM with Lipofectamine 2000 per the instructions.

2.5. Matrigel Invasion Assay

The invasion assay was performed by using a Matrigel invasion chamber from Corning Biocoat (Tewksbury, MA) as previously described [40]. 5×10^4 HDAC9 inhibitor-transfected or control oligonucleotide-transfected TNBC MDA-MB-231 cells were added into the top chambers. The Matrigel invasion chambers were then incubated for 20 hours 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 the *in vivo* angiogenesis assay (Matrigel plug assay), 2×10^5 MDA-MB-231 cells were mixed with 0.5 ml of growth factor-reduced Matrigel (Corning, Tewksbury, MA) and implanted subcutaneously into the flanks of nude mice. The following day, six mice in each group were treated with 100 μg/kg HDAC9 siRNAs or control oligonucleotides via daily

subcutaneous injections between the two plugs on the back of the mice. For selective HDAC IIa inhibitor, TMP269, mice were administered with TMP269 by subcutaneous injections every other day at 15 mg/kg mice body weight 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 [41].

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. HDAC9 is overexpressed in TNBC and is inversely correlated with miR-206

Expression levels of HDAC1–11 in three TNBC cell lines, MDA-MB-231, MDA-MB-1739, and HCC70 compared to those in three non-TNBC cell lines, MCF-7, MDA-MB-361, and SKBR3 were profiled with qRT-PCR analysis. Compared to non-TNBC cells, TNBC cells expressed much higher HDAC9 (Fig. 1A). Quantitative RT-PCR results reveal that expression levels of miR-206 are notably lower while HDAC9 levels are inversely higher in TNBC cell lines than those in non-TNBC cell lines (Fig. 1B). Furthermore, we analyzed the expression levels of HDAC9 proteins determined by immunohistochemical staining in breast cancer tissue samples. TNBC tissues express higher levels of HDAC9 compared to non-TNBC tissue samples (Fig. 1C). These results demonstrate that expression levels of HDAC9 are upregulated in TNBC tissues in comparison to non-TNBC tissue samples and are inversely correlated with the levels of miR-206.

3.2. Inhibition of HDAC9 blocks the invasion of TNBC cells in vitro

To investigate whether selective HDAC9 inhibition blocks the invasion of TNBC cells, TNBC MDA-MB-231 cells were treated with TMP269, a selective class IIa HDAC inhibitor, or HDAC9 siRNA prior to Matrigel invasion assay. The invasive cells from 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 treated with selective class IIa HDAC inhibitor TMP269 was only 28% of that of the control (Fig. 2B). Similarly, HDAC9 siRNA treatment significantly blocked TNBC cell invasion. These results suggest that selective HDAC9 inhibition efficiently blocks the invasion of TNBC cells.

3.3. Knockout of HDAC9 inhibits the angiogenesis of TNBC tumors

To determine the effect of selective HDAC9 inhibition on angiogenesis *in vivo*, Matrigel plug assay was performed in nude mice. Briefly, a mixture of 2×10^5 TNBC MDA-MB-231 cells in 0.5 ml of growth factor reduced Matrigel was implanted at two subcutaneous sites. The mice in two groups were treated with 100 μg/kg HDAC9 siRNAs or control oligonucleotides via daily subcutaneous injections. For other two groups, mice received 15 mg/kg TMP269 or vehicle every other day by intraperitoneal injection between the two

plugs on the back of the mice. Ten days after the Matrigel implant, the mice were sacrificed. The Matrigel plugs were excised, photographed, and processed to measure hemoglobin content by using Drabkin's solution following manufacturer' instructions. When MDA-MB-231 cells successfully promote neovasculature formation within the Matrigel plug, these neovasculatures allow tumor cells to proliferate much better than those without neovasculatures. Therefore, the control group with better angiogenesis in the Matrigel plug showed more red cells than the treated group (Fig. 3A). Fig. 3B summarizes the quantification of percentage of antiangiogenic efficacy based on hemoglobin content in 12 Matrigel plugs per group. In comparison to their controls, TMP269 treatment or HDAC9 siRNA transfection shows an obvious antiangiogenic effect with 76 % and 72% inhibition of angiogenesis, respectively. These results suggest that selective inhibition of HDAC9 effectively blocks TNBC angiogenesis.

3.4. HDAC9 regulates expression of VEGF and MAPK3 through miR-206 miRNA

To determine whether HDAC9 regulates miR-206 expression, HDAC9 was silenced with HDAC9 siRNA and then miR-206 expression levels were measured by quantitative RT-PCR analysis. The results show that selective HDAC9 inhibition significantly upregulated miR-206 expression (Fig. 4A). Furthermore, the results of a search for the predicted targets with TargetScan revealed that *VEGF* and *MAPK3* are predicted targets of miR-206 (Fig. 4B). To confirm these results *in vitro*, TNBC MDA-MB-231 cells were treated with HDAC9 siRNA and then protein levels of VEGF and MAPK3 by Western blot analysis. The results demonstrated that selective inhibition of HDAC9 decreased the expression levels of VEGF and MAPK3 compared to their controls (Fig. 4C). These results demonstrated that selective HDAC9 inhibition increased the expression of VEGF and MAPK3 via upregulating miR-206 expression.

4. Discussion

It is now widely accepted that aberrant expression or activity of HDAC enzymes may lead to carcinogenesis and that specific HDAC enzymes are associated with particular malignancies; in turn, the inhibition of HDAC enzymes can result in therapeutic benefits in certain cancer types [42]. However, clinical benefits of selective versus broad HDAC inhibitors are unknown and the appropriateness of inhibitor may depend on tumor HDAC enzyme expression, enzyme selectivity of the inhibitor, and the desired effects. Although there are several published studies [16, 17] and numerous ongoing clinical trials involving HDAC inhibitors [18], very little is known about the roles of individual HDAC enzymes, and a formal assessment of HDAC enzyme expression is not routinely done. Emerging data suggests that the currently used HDAC inhibitors may differ significantly with regard to target selectivity. In addition, the expression of HDAC enzymes may vary considerably between normal and tumor tissues and likely between different phenotypes of tumor tissues. HDAC9 is thought to regulate gene expression through epigenetic modulation of the chromatin structure by catalyzing the deacetylation of histone proteins [19]. More recently, aberrant HDAC9 expression has been observed in several types of cancers, including medulloblastoma [21], acute lymphoblastic leukemia [22], glioblastoma [23], osteosarcoma [24], and breast cancer [25]. HDAC9 has been shown to promote the growth of these tumors.

Our results demonstrate that HDAC9 is overexpressed in TNBC compared to non-TNBC cell lines and tissues. Furthermore, our data showed that selective inhibition of HDAC9 repressed the invasion and angiogenesis of TNBC cells. These findings reveal that HDAC9 promotes the invasion and angiogenesis of TNBC cells besides proliferation.

Several investigations have demonstrated that decreased miR-206 expression is involved in breast cancer proliferation [43, 44]. Our previous studies show that miR-206 is antagonistically involved in TNBC invasion and angiogenesis through modulating VEGF and MAPK3 [35]. Numerous investigations have demonstrated that VEGF actively promotes angiogenesis, metastasis, and chemoresistance [45–47]. MAPK overexpression has been shown to be associated with advanced stages and short survival in patients with some cancers [48, 49]. However, miR-206 has not been characterized as the targets of HDAC9 regulation. Our findings demonstrated that the selective inhibition of HDAC9 in siRNA-transfected TNBC MDA-MB-231 cells not only increased expression of miR-206, but also repressed the expression of VEGF and MAPK proteins and further inhibited TNBC cell invasion and angiogenesis. Here, we report HDAC9 for the first time as a mediator to modulate VEGF-mediated invasion and angiogenesis of TNBC tumor cells via modulating miR-206 expression. The results further confirmed that miR-206 actively regulates the expression of VEGF and MAPK3 in TNBC cells.

In conclusion, higher expression levels of HDAC9 are inversely correlated with miR-206 expression in TNBC cells. Furthermore, the selective inhibition of HDAC9 not only modulated the expression of miR-206, VEGF and MAPK3, but also particularly inhibited TNBC invasion and angiogenesis. Our findings suggested that HDAC9 overexpression in TNBC cells promotes the invasion and angiogenesis through VEGF and MAPK3 via regulating miR-206. These findings may be beneficial for better understanding TNBC regulation and designing personalized therapies for breast cancer patients.

Acknowledgements

Abbreviations:

HDAC9 HDAC	histone deacetylase
TNBC	Triple negative breast cancer
miRNA	microRNA
VEGF	Vascular endothelial growth factor
MAPK	Mitogen-activated protein kinase
siRNA	small interfering RNA
RT-PCR	reverse transcription polymerase chain reaction

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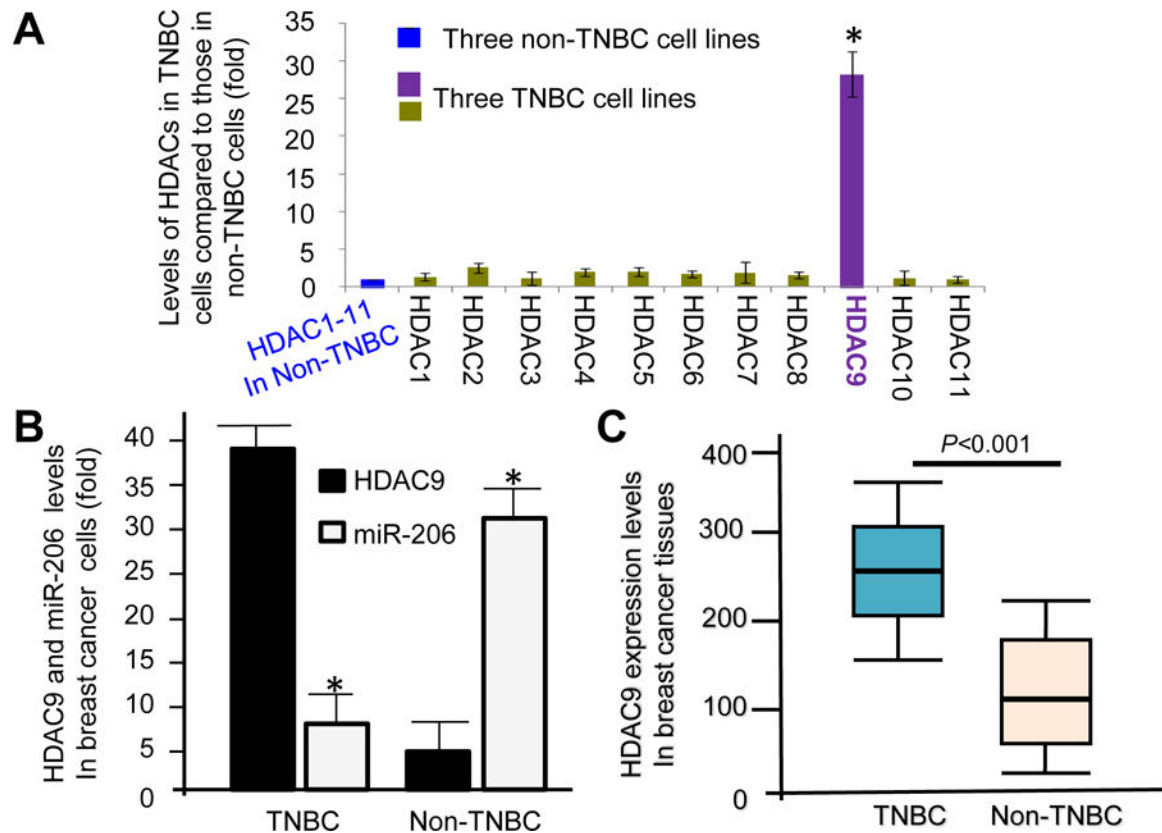
Fig. 1.
HDAC9 is overexpressed in TNBC cells and tissues. (A) Expression levels of HDAC1–9 mRNAs in three TNBC cell lines, MDA-MB-231, MDA-MB-1739, and HCC70 compared to those in three non-TNBC cell lines, MCF-7, MDA-MB-361, and SKBR3 determined by quantitative RT-PCR analysis. * $P<0.001$. (B) HDAC9 expression levels in triple negative breast cancer cell lines are inversely consistent with those of miR-206. * $P<0.001$. (C) A box and whisker plot diagram showing the comparison of HDAC9 levels in triple negative breast cancer tissues (n = 41), and non-TNBC breast cancer tissues (n =59) determined with HDAC9 IHC staining. Horizontal lines in the boxes represent the median HDAC9 value of each group ($P<0.001$). Top and bottom edges of the boxes indicate the score values of the 25th and the 75th percentile, respectively. Whiskers represent the highest and lowest values. The range is shown as a vertical line.
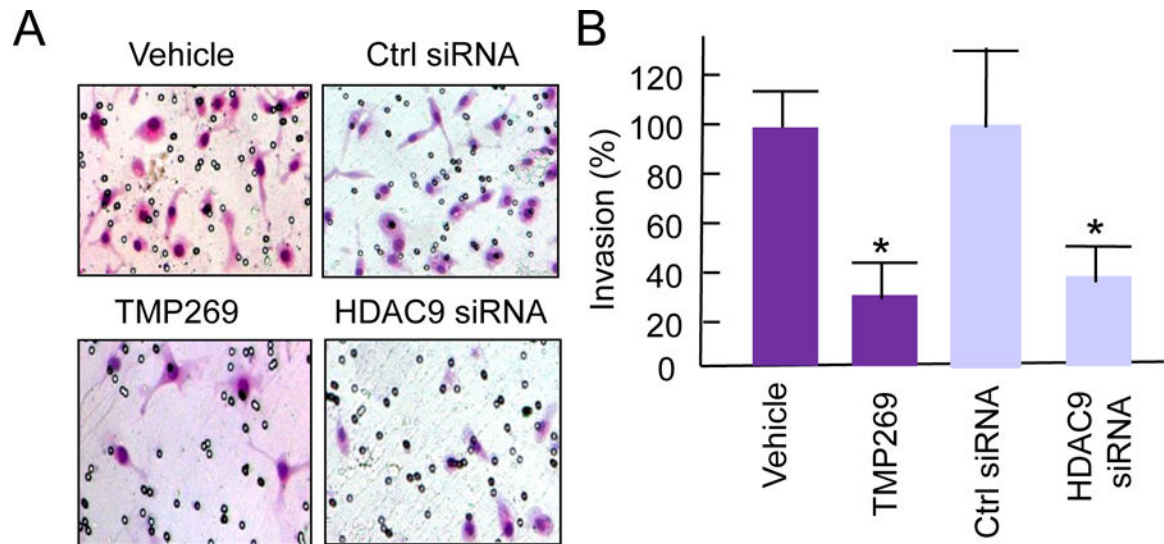
Fig. 2.
Selective HDAC9 inhibition blocks invasion of human TNBC MDA-MB-231 cells. (A)
Representative photograph for invasion cells from HDAC9 siRNA-treated or TMP269-
treated and their control groups. (B) Quantitative comparison of invasion cells of selective
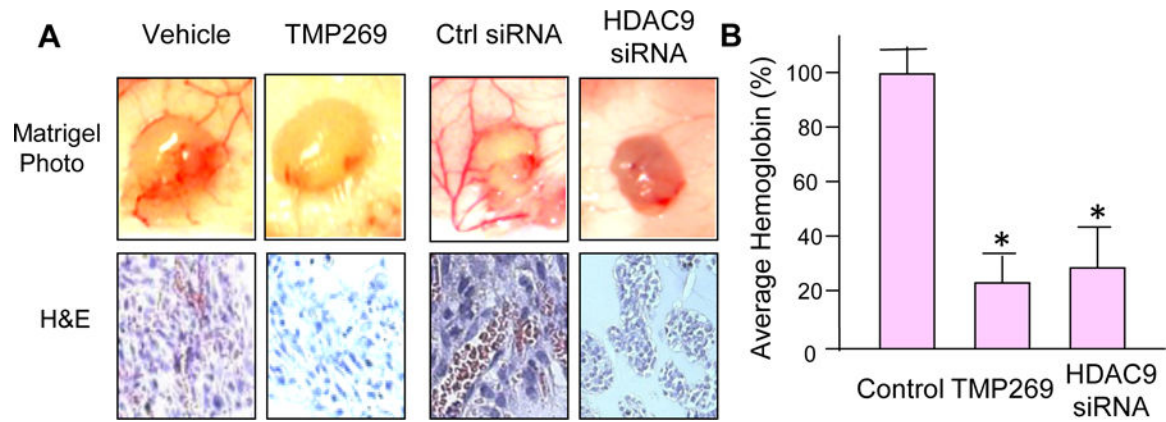HDAC9 inhibition and their controls by Matrigel Invasion Assay. * P<0.01.

Fig. 3.
Efficacies of HDAC9 inhibition on Matrigel plug angiogenesis *in vivo*. (A) Microphotograph of Matrigel plug representatives from the control and the treated groups and representative pictures of H&E staining for Matrigel plug sections. (B) The column graph shows the comparison of average hemoglobin in plugs from the control and HDAC9-inhibited groups. * *P*<0.001.
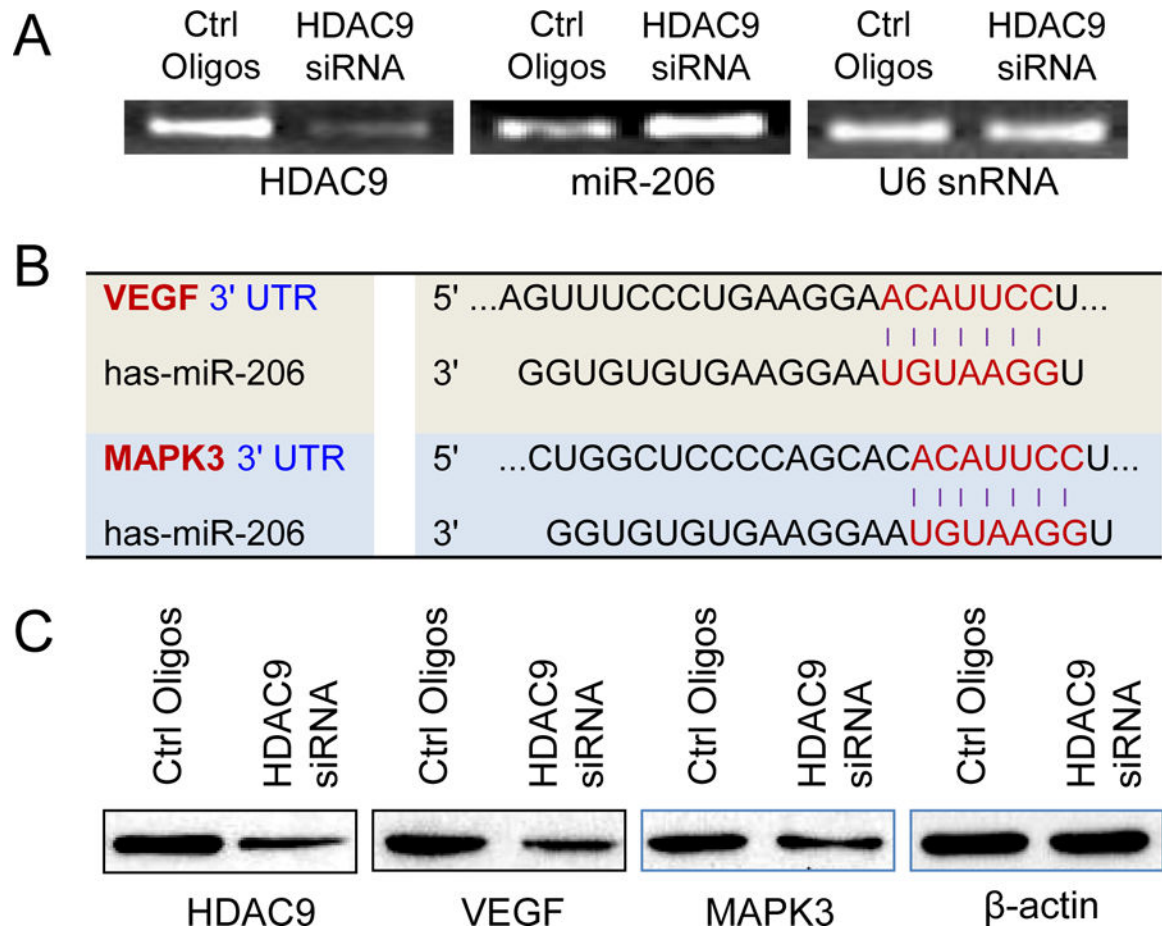
Fig. 4.
HDAC9 inhibition restores the miR-206 levels in TNBC cells and decreases VEGF and MAPK3 levels. (A) MiR-206 expression levels in TNBC MDA-MB-231 cells treated by HDAC9 siRNA. (B) Targeting sequences of miR-206 on 3'UTRs of *VEGF* and *MAPK3*. (C) HDAC9 inhibition decreased expression levels of VEGF and MAPK3 determined Western blot analysis.

Table 1

Characteristics and HDAC9 expression of tissue specimens of breast tumors.

	Total	T stage		Sex		Age	
		T1&T2	T3T4	Male	Female	<50	50
Triple Negative	41	25	16	0	41	22	19
Non-triple negative	59	48	11	0	59	28	31
Total	100	73	27	0	100	50	50