MicroRNA-3666 inhibits breast cancer cell proliferation by targeting sirtuin 7

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Abstract. The abnormal expression of microRNAs (miRNAs) is associated with cancer initiation and progression. miRNAs functioning as oncogenes or tumor suppressors represent novel biomarkers for cancer diagnosis, prognosis, and serve as therapeutic tools. MiR-3666 has been reported as a tumor suppressor in various types of cancer; however, its role in breast cancer remains unknown. In the current study, the aim was to investigate the potential role of miR-3666 in breast cancer. It was identified that miR-3666 was decreased in breast cancer cell lines and that the overexpression of miR-3666 inhibited breast cancer cell proliferation. Furthermore, miR-3666 promotes cell apoptosis of breast cancer cells. Bioinformatics analysis and dual-luciferase reporter assay demonstrated that miR-3666 targeted the 3'-untranslated region of sirtuin 7 (SIRT7) which was recognized as an oncogene. Overexpression of miR-3666 decreased SIRT7 expression levels, and knockdown of SIRT7 suppressed proliferation and promoted apoptosis of breast cancer cells. A rescue assay demonstrated that the restoration of SIRT7 expression markedly reversed the miR-3666-induced anti-tumor effects. Thus, the current study indicates that miR-3666 suppresses breast cancer cell proliferation by targeting SIRT7, and propose miR-3666 as a potential candidate for breast cancer therapy.

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Abbreviations: miRNAs, microRNAs; SIRT7, sirtuin7; UTR, untranslated region; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BrdU, Bromodeoxyuridine; RT-qPCR, reverse transcription-quantitative polymerase chain reaction

Key words: breast cancer, miR-3666, sirtuin 7, cell proliferation, cell apoptosis

Introduction

Breast cancer is one of the most prevalent types of malignancy in women worldwide (1). Despite advances in cancer therapeutic strategies, the clinical outcomes and prognosis for breast cancer patients remain particularly poor (2). Numerous genetic and epigenetic alterations have been proposed to contribute to breast cancer pathogenesis and progression (3); however, the precise molecular mechanism remains poorly understood. Therefore, investigating the molecular mechanisms, and developing novel and effective therapeutic targets for breast cancer therapy are of great importance.

MicroRNAs (miRNAs) are non-coding, small RNAs (length, ~22 nucleotides) which post-transcriptionally regulate gene expression (4,5). miRNAs induce mRNA degradation or translational repression by targeting the 3'-untranslated region (UTR) of the target mRNAs (4). miRNAs are important in carcinogenesis, through modulating various biological processes, including cell proliferation, apoptosis, differentiation, migration and invasion (6). Numerous miRNAs are involved in regulating breast cancer pathogenesis and progression (7-9). Furthermore, miRNAs may serve as novel biomarkers for diagnosis, prognosis, and therapeutic tools in breast cancer (10). However, the precise role of miRNAs in breast cancer requires further investigation.

The sirtuins (SIRTs) are nicotinamide adenine dinucleotide oxidized form-dependent deacetylases that contribute significantly to stress responses, inflammation, metabolism, DNA repair and senescence (11-14). To date, seven members, including SIRT1-7 have been characterized in mammals (15). SIRT1 is the most evaluated sirtuin that regulates various cellular and metabolic processes (16). SIRT2 has been reported as an important regulator for neurodegenerative diseases (17). SIRT3 is the major mitochondrial deacetylase, which regulates global mitochondrial lysine acetylation (18,19). SIRT4 and SIRT5 have been significantly implicated in metabolic processes (20,21). SIRT6 predominantly regulates DNA damage and genome integrity (22,23), and SIRT7 is the latest characterized SIRT and evaluation of its function has just begun (24). SIRT7 is important in regulating rDNA transcription and protein synthesis (25,26). Furthermore, SIRT7 has been reported as a response gene in response to hypoxia, low glucose stress, genomic stress and endoplasmic reticulum

stress (27-30). It is involved in cardiac health, hepatic steatosis, ageing and senescence (31,32) and SIRT7 has been suggested as an oncogene in various cancer types, including hepatocellular carcinoma (26) and colorectal cancer (33), representing a potential pharmacologic target for cancer therapy (34). High expression levels have been observed in breast cancer tissues associated with metastasis and adverse outcomes (35,36). However, the regulation of SIRT7 in breast cancer remains poorly understood.

Recent studies reported miR-3666 as a tumor suppressor miRNA in various cancer types (37-39). However, the role of miR-3666 in breast cancer remains unknown. According to the reported features of miR-3666, it was hypothesized that miR-3666 exerts a tumor suppressor role in breast cancer. The present study aimed to investigate the role and underlying mechanism of miR-3666 in regulating the development and progression of breast cancer.

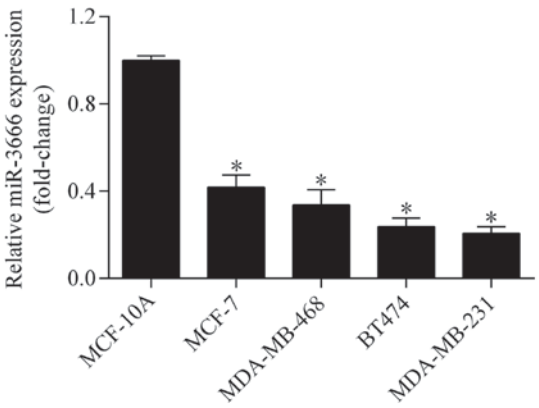

Figure 1. Expression levels of miR-3666 in breast cancer cell lines was detected by reverse transcription-quantitative polymerase chain reaction. Four breast cancer cell lines, including MCF-7, MDA-MB-468, BT474 and MDA-MB-231 were used. The normal breast epithelial cell line MCF-10A served as a control. *P<0.05 vs. MCF-10A. miR, microRNA.

Materials and methods

Cell lines and culture. Human breast cancer cell lines (MCF-7, BT474, MDA-MB-231, and MDA-MB-468), normal breast epithelial cell line MCF-10A and 293T cells were purchased from the American Type Culture Collection (Manassas, VA, USA). BT474 and MDA-MB-231 cells were cultured in Hyclone RPMI-1640 medium (GE Healthcare Life Sciences, Logan, UT, USA) while MCF-7, MDA-MB-468, MCF-10A and 293T cells were cultured in Hyclone Dulbecco's modified Eagle's medium (GE Healthcare Life Sciences). All cells were grown in medium containing 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences) supplemented with 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) in a humidified atmosphere of 5% CO_2 at 37˚C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNAs were extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). To detect miR-3666 expression, cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase (Takara Biotechnology, Co., Ltd., Dalian, China). To detect SIRT7 expression levels, cDNA was synthesized using a miScript Reverse Transcription kit (Qiagen GmbH, Hilden, Germany). The RT-qPCR was conducted using a SYBR-Green master mix kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with an Applied Biosystems AB7500 Real Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the following procedure: 94˚C for 5 min; 30 cycles of 94˚C for 20 sec, 55˚C for 25 sec, and 72˚C for 35 sec; and 72˚C for 10 min. Small nuclear RNA U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the internal controls. The relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method as compared with U6 or GAPDH (40). The fold-change of gene expression was obtained by normalization against the control group. The primers used were as follows: Forward, 5'-ACACTCCAGCTGGGCAGTCAAGTGTAGA-3' and reverse, 5'-TGGTGTCGTGGAGTCG-3' for miR-3666; forward, 5'-CGCTTCGGCAGCACATATACTAA-3' and reverse, 5'-TATGGAACGCTTCACGAATTTGC-3' for U6; forward, 5'-GTGGACACTGCTTCAGAAAG-3' and reverse, 5'-CACAGTTCTGAGACACCACA-3' for SIRT7; and forward, 5'-CCATGTTCGTCATGGGTGTG-3' and reverse, 5'-GGTGCTAAGCAGTTGGTGGTG-3' GAPDH.

Cell transfection. The miR-3666 mimics and negative control (miR-NC) were obtained from Origene Technologies, Inc. (Beijing, China) and transfected into cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at a final concentration of 50 nM. SIRT7 small interfering RNA (siRNA) and negative control (NC siRNA) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and transfected into cells according to the manufacturer's instructions. SIRT7-overexpressing vector was generated by cloning SIRT7 cDNA without a 3'-UTR into a pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.). The pcDNA3.1-SIRT7 vector was transfected into cells using Lipofectamine 2000.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded into 96-well plates at a density of $1x10^4$ cells/well and cultured overnight. Cells were then transfected with miR-3666 mimics and cultivated for 48 h, and 20 μl of MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to each well and incubated at 37˚C for 4 h. Subsequently, the culture media were discarded and 200 μl dimethyl sulfoxide was added to each well. Optical density values at a wavelength of 490 nm were detected using an ELISA reader (Bio-Rad Laboratories, Inc.).

Bromodeoxyuridine (BrdU) assay. The BrdU assay was performed using a BrdU cell proliferation assay kit (Cell Signaling Technology, Inc., Danvers, MA, USA) in accordance with the manufacturer's instructions. Briefly, cells were seeded into 96-well plates ($1x10^4$ cells/well) and transfected with miR-3666 mimics for 48 h. Thereafter, 10 μl BrdU solution was added to each well and cultured for 2 h. Following removal of the culture media, 150 μl denaturing solution was added to each well and incubated at room temperature for 1 h. Subsequently, peroxidase conjugated anti-BrdU was added

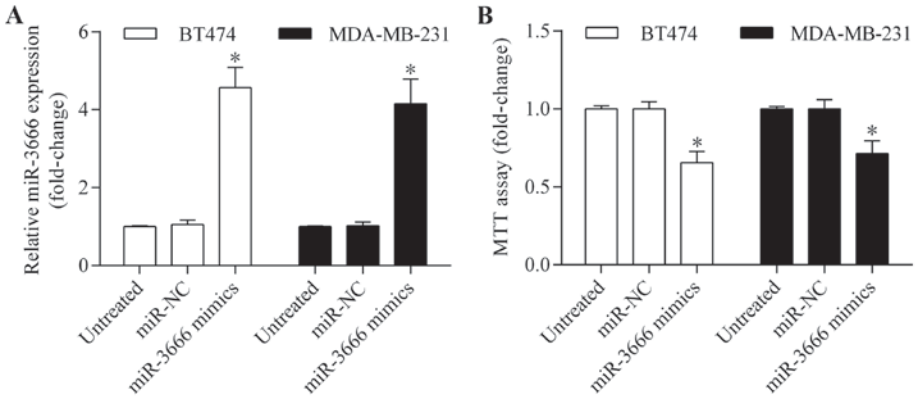

Figure 2. miR-3666 inhibits breast cancer cell growth. BT474 and MDA-MB-231 cells were transiently transfected with miR-3666 mimics or miR-NC for 48 h. (A) Overexpression of miR-3666 was detected by reverse transcription-quantitative polymerase chain reaction. (B) Effect of miR-3666 overexpression on cell viability and growth was measured by MTT assay. *P<0.05 vs. Untreated and miR-NC. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; miR, microRNA; NC, negative control.

and incubated for 1 h at room temperature. The optical density values at a wavelength of 450 nm were measured by an ELISA reader (Bio-Rad Laboratories, Inc.).

Caspase-3 activity assay. Caspase-3 activity assay was performed using a commercial kit (Roche Applied Science, Madison, WI, USA) according to the manufacturer's instructions. Briefly, following treatment, cells were lysed and the supernatant was harvested followed by incubation with DEVD-pNA substrate (Roche Applied Science) at 37˚C for 2 h. Optical density values at a wavelength of 405 nm were determined using an ELISA reader (Bio-Rad Laboratories, Inc.).

Dual-luciferase reporter assay. miRNA targets were predicted using the algorithms of TargetSan (https://www.targetscan.org) (41). The 3'-UTR of SIRT7 containing the wild-type or mutant binding sites of miR-3666 were cloned into pmirGLO vector (Promega Corporation, Madison, WI, USA) followed by transfection into 293T cells with miR-3666 mimics using Lipofectamine 2000. After 48 h of incubation, cells were harvested and detected using a Dual-Luciferase assay kit (Promega Corporation). The relative luciferase activity was calculated according to the following formula: Firefly luciferase/*Renilla* luciferase.

Western blot analysis. Total protein was extracted using RIPA buffer (Sigma-Aldrich; Merck KGaA). Equal quantities (40 μg) of proteins were loaded onto 10% sodium dodecyl sulfate polyacrylamide gels (Sangon Biotech Co., Ltd., Shanghai, China) for separation. The separated proteins were then transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA) followed by incubation with 3% nonfat milk for 1 h. The membrane was incubated with primary antibodies at 4˚C overnight. Anti-SIRT7 (cat. no. sc-135055; dilution, 1:500) and anti GAPDH (cat. no. sc-367714; dilution, 1:800) primary antibodies were both purchased from Santa Cruz Biotechnology, Inc. Subsequently, the membrane was washed with Tris-buffered saline containing 0.1% Tween-20 three times and then blotted with horseradish peroxidase conjugated secondary antibodies (cat. no. A0208; dilution, 1:1,000, Beyotime Institute of Biotechnology, Haimen, China) for 1 h at 37˚C. The protein bands were visualized using enhanced chemiluminescence (EMD Millipore). The intensity of the bands on the membrane was analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). Relative protein expression was calculated by normalization against GAPDH. The fold-change of protein expression was obtained by normalization with the control group.

Statistical analysis. All values are presented as means ± standard deviation and the statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Differences were analyzed by one-way analysis of variance with a Bonferroni correction. P<0.05 was considered to indicate a statistically significant difference.

Results

MiR-3666 is downregulated in breast cancer cell lines. To investigate the potential relevance of miR-3666 in breast cancer, its expression was examined in breast cancer cell lines using RT-qPCR. The results demonstrated that the expression level of miR-3666 was significantly downregulated in breast cancer cell lines (MCF-7, MDA-MB-468, BT474 and MDA-MB-231) compared with the normal breast epithelial cell line, MCF-10A (Fig. 1), indicating a tumor suppressive role of miR-3666 in breast cancer.

Overexpression of miR-3666 inhibits proliferation and promotes apoptosis of breast cancer cells. As miR-3666 demonstrated a lower expression level in BT474 and MDA-MB-231 cells, these two cell lines were selected for subsequent experiments. To investigate the potential biological effect of miR-3666 in breast cancer, gain-of-function experiments were performed by transiently transfecting miR-3666 mimics into BT474 and MDA-MB-231 cells. The expression level of miR-3666 was markedly upregulated by miR-3666 transfection, as detected by RT-qPCR (Fig. 2A). The effect of miR-3666 overexpression on cell viability and growth was then examined by MTT assay. It was observed that miR-3666 overexpression significantly suppressed breast cancer cell growth

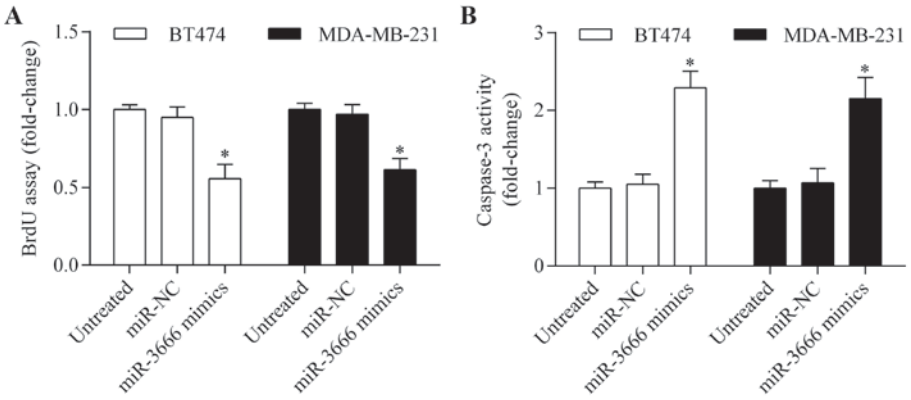

Figure 3. miR-3666 inhibits proliferation and promotes apoptosis of breast cancer cells. BT474 and MDA-MB-231 cells were transiently transfected with miR-3666 mimics or miR-NC for 48 h. (A) Cell proliferation was detected by BrdU assay. (B) Cell apoptosis was detected by caspase-3 activity assay. *P<0.05 vs. Untreated and miR-NC. BrdU, bromodeoxyuridine; miR, microRNA; NC, negative control.

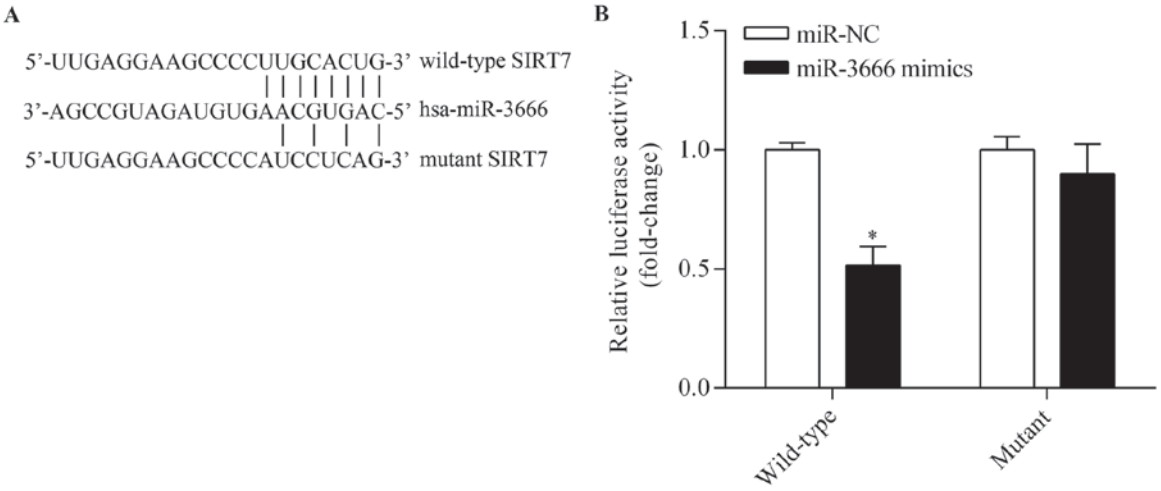

Figure 4. SIRT7 is a direct target of miR-3666. (A) Schematic representation of the miR-3666-binding sites and mutant binding sites in the 3'-UTR of SIRT7. (B) Co-transfection with miR-3666 mimics and luciferase reporter containing wild-type or mutant 3'-UTR of SIRT7 into 293T cells. *P<0.05 vs. miR-NC. SIRT7, sirtuin 7; miR, microRNA; UTR, untranslated region; NC, negative control.

(Fig. 2B). In addition, BrdU assay indicated that proliferation of BT474 and MDA-MB-231 cells was markedly inhibited by miR-3666 overexpression (Fig. 3A). Furthermore, overexpression of miR-3666 significantly promoted apoptosis of BT474 and MDA-MB-231 cells (Fig. 3B). These results indicate that miR-3666 functions as a tumor suppressor.

SIRT7 is a direct target of miR-3666. To elucidate the molecular mechanism by which miR-3666 regulates breast cancer cell proliferation, bioinformatic analyses were performed using TargetScan to predict potential target genes. Among these target genes, SIRT7, which is a novel oncogene, was notable. The putative binding sites of miR-3666 within the 3'-UTR of SIRT7 are presented in Fig. 4A. To verify whether SIRT is a direct target of miR-3666, a Dual-Luciferase reporter system containing either wild-type or mutant 3'-UTR of SIRT7 was used. Co-transfection with miR-3666 mimics markedly inhibited the luciferase activity of the reporter containing the wild-type 3'-UTR (Fig. 4B). However, miR-3666 overexpression demonstrated no significant effect on mutant 3'-UTR of SIRT7 (Fig. 4B). Subsequent RT-qPCR and western blot analysis indicated that SIRT7 expression levels were significantly suppressed in BT474 and MDA-MB-231 cells following transfection with the miR-3666 mimics (Fig. 5A and B). Taken together, these results indicate that SIRT7 is a direct target of miR-3666.

Knockdown of SIRT7 by siRNA inhibits proliferation and promotes the apoptosis of breast cancer cells. To investigate whether SIRT7 is involved in regulating breast cancer, SIRT7 was silenced by transfecting SIRT7 siRNA (Fig. 6A), and its effect on cell proliferation and apoptosis was detected. It was found that silencing SIRT7 using siRNA significantly inhibited proliferation (Fig. 6B) and promoted apoptosis (Fig. 6C) of breast cancer cells. The results indicate that SIRT7 is involved in regulating breast cancer cell proliferation and apoptosis.

Overexpression of SIRT7 reverses the miR-3666-induced anti-tumor effects. To investigate whether miR-3666 induced its anti-tumor effect via SIRT7, a rescue assay was performed using MDA-MB-231 cells. Recombinant SIRT7 lacking the 3'-UTR sequence (pcDNA3.1/SIRT7) was

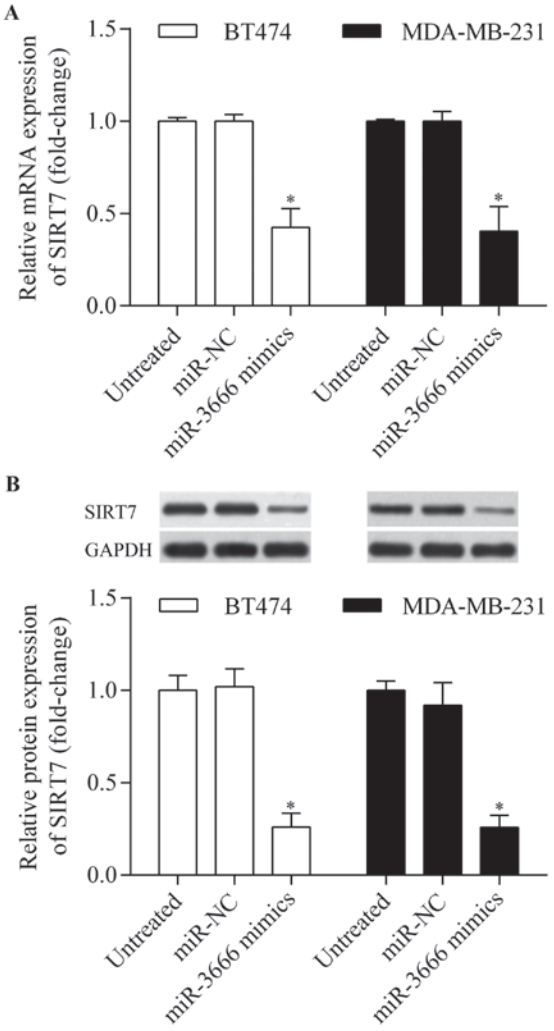

Figure 5. miR-3666 inhibits SIRT7 expression. BT474 and MDA-MB-231 cells were transfected with miR-3666 mimics or miR-NC for 48 h. The (A) mRNA and (B) protein expression of SIRT7 were detected by reverse transcription-quantitative polymerase chain reaction and western blot analyses, respectively. *P<0.05 vs. Untreated and miR-NC. miR, mircroRNA; SIRT7, sirtuin 7; NC, negative control; mRNA, messenger RNA.

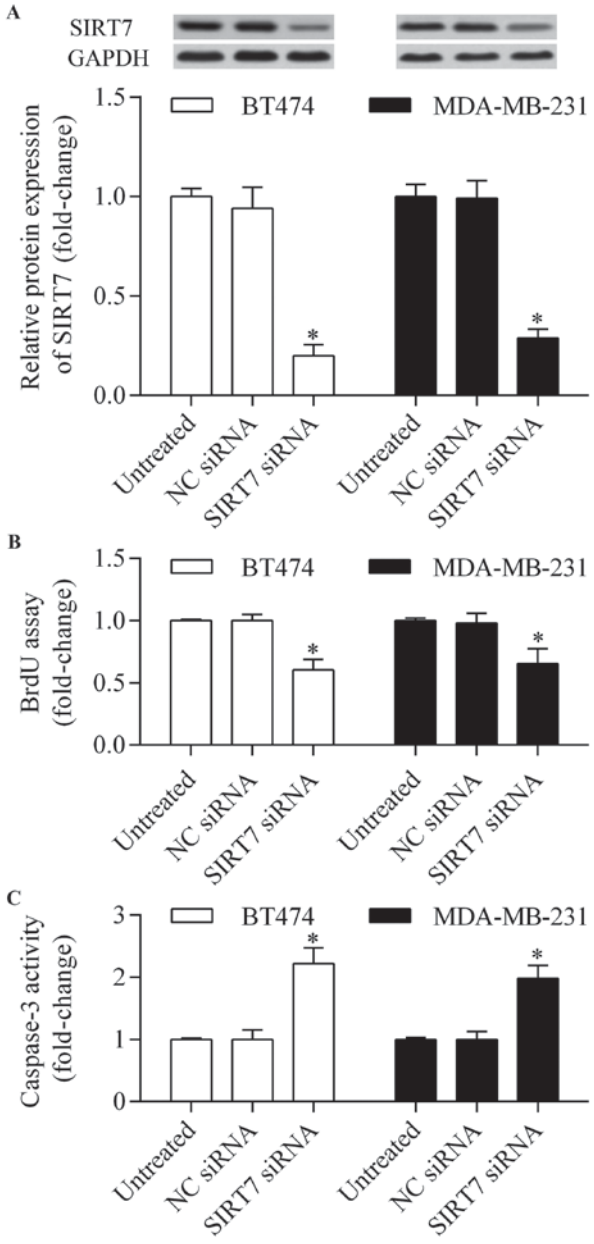

Figure 6. Knockdown of SIRT7 inhibits proliferation and promotes apoptosis of breast cancer cells. BT474 and MDA-MB-231 cells were transfected with SIRT7 siRNA or NC siRNA and incubated for 48 h. (A) The decreased SIRT7 protein expression level was confirmed by western blot analysis. (B) The effect of SIRT7 knockdown on cell proliferation was detected by BrdU assay. (C) The effect of SIRT7 knockdown on cell apoptosis was detected by caspase-3 activity assay. *P<0.05 vs. Untreated and NC siRNA. SIRT7, sirtuin 7; siRNA, small interfering RNA; NC, negative control; BrdU, bromodeoxyuridine.

exogenously expressed in MDA-MB-231 cells. Western blotting demonstrated that co-transfection of pcDNA3.1/SIRT7 and miR-3666 mimics restored the decreased protein expression level induced by miR-3666 overexpression (Fig. 7A). Overexpression of SIRT7 significantly reversed the inhibitory effect of miR-3666 overexpression on cell proliferation (Fig. 7B and C). Furthermore, the increased apoptosis induced by miR-3666 overexpression was markedly reversed by SIRT7 overexpression (Fig. 7D). Thus, these results indicate that miR-3666 exerts its tumor suppressive role via SIRT7.

Discussion

Dysregulation of miRNAs is involved in the initiation and progression of breast cancer, and miRNA-based therapeutic strategies present as a potential therapeutic strategy for breast cancer (10). In the current study, it was demonstrated that miR-3666 is a novel miRNA involved in regulating breast cancer progression. miR-3666 expression was observed to be downregulated in breast cancer cell lines. The overexpression of miR-3666 significantly inhibited proliferation and promoted apoptosis of breast cancer cells. SIRT7 was identified as the target gene of miR-3666, which contributed to the miR-3666-mediated anti-tumor effect. Thus, these findings revealed a novel microRNA-based mechanism for breast cancer pathogenesis.

Various miRNAs have been identified to be dysregulated in breast cancer, which function as oncogenes or tumor suppressors, and are involved in the development of breast cancer (42-45). Recent studies have reported that miR-3666 functions as a tumor suppressor (37-39). MiR-3666 is

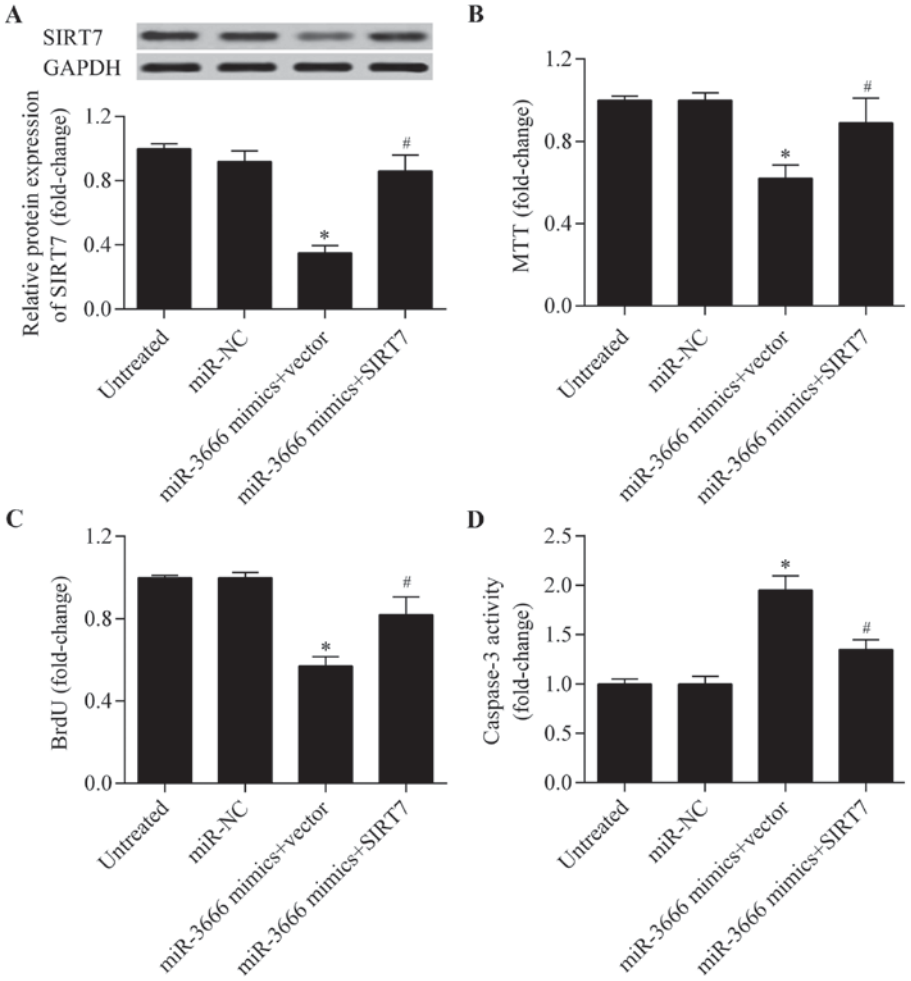

Figure 7. Overexpression of SIRT7 restores the miR-3666-induced anti-tumor effects. MDA-MB-231 cells were co-transfected with pcDNA3.1/SIRT7 and miR-3666 mimics for 48 h. Untreated, cells without treatment; miR-NC, cells treated with miR-NC; miR-3666 mimics + vector, cells treated with miR-3666 mimics and pcDNA3.1 empty vector; miR-3666 mimics + SIRT7, cells treated with miR-3666 mimics and pcDNA3.1/SIRT7 vector. (A) SIRT7 protein expression levels were detected by western blot analysis. Cell proliferation was detected by (B) MTT and (C) BrdU assays. (D) Cell apoptosis was detected by caspase-3 activity assay. *P<0.05 vs. Untreated and miR-NC; #P<0.05 vs. miR-3666 mimics + vector. SIRT7, sirtuin 7; miR, mircroRNA; NC, negative control; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BrdU, bromodeoxyuridine.

reportedly decreased in cervical cancer and inhibits cervical cancer cell metastasis (37). Wang *et al* (38) reported that low miR-3666 expression levels were observed in thyroid carcinoma and associated with poor survival rate. Furthermore, *in vitro* experiments revealed that miR-3666 inhibited thyroid carcinoma cell proliferation (38). A more recent study reports that miR-3666 inhibits the growth of non-small cell lung cancer cells (39). Consistent with these findings, the present study supported a tumor suppressor role of miR-3666 in breast cancer. miR-3666 expression levels were decreased in breast cancer cell lines in the present study, and overexpression of miR-3666 inhibited breast cancer cell proliferation. However, the underlying molecular mechanism requires further elucidation.

To elucidate the molecular mechanism by which miR-3666 inhibits breast cancer cell proliferation, bioinformatic analyses were conducted and SIRT7 was identified as the functional target gene of miR-3666. SIRT7 is a lysine deacetylase that selectively catalyzes the deacetylation of lysine 18 on histone H3 that maintains oncogenic transformation (46). Increasing evidence indicates SIRT7 as an oncogene in various types of cancer (34). SIRT7 is overexpressed in hepatocellular carcinoma, cervical, ovarian, colorectal, gastric and lung cancer, and is involved in regulating cancer cell proliferation, apoptosis and metastasis (25,33,39,47-49). Ashraf *et al* (35) demonstrated that SIRT7 was significantly increased in breast cancer associated with node-positive breast cancer. Aljada *et al* (50) reported that high expression levels of SIRT7 were associated with early stage breast cancer (50). Furthermore, a high level of SIRT7 expression has been suggested as a predictor of adverse outcomes in breast cancer (36). These findings indicate an oncogenic role of SIRT7 in breast cancer. However, the precise biological role of SIRT7 in breast cancer remains unclear. In the present study, knockdown of SIRT7 inhibited proliferation and promoted apoptosis of breast cancer. In addition, SIRT7 was observed to be regulated by miR-3666. The decreased miR-3666 expression level may contribute to the high expression levels of SIRT7 in breast cancer. It has been reported that miR-3666 inhibits tumor progression by targeting zinc finger E-box binding homeobox 1 (37) or met proto-oncogene (38). In the current study, SIRT7 was identified to be a functional target gene of miR-3666. These findings are consistent with a recent study, which demonstrated that miR-3666 inhibits lung cancer cell growth by targeting SIRT7 (39).

The regulation of SIRT7 by miRNAs has been widely reported (51,52). miR-93 regulates adiposity by targeting SIRT7 (51), miR-152 induces human dental pulp stem cell senescence by targeting SIRT7 (53), and miR-125b is reported to inhibit tumor development by targeting SIRT7 in hepatocellular carcinoma (26,54) and bladder cancer (55). These studies indicate that SIRT7 undergoes epigenetic regulation via miRNAs, which is important for pathological processes.

In conclusion, the data presented by the present study indicates that miR-3666 is an important regulator of breast cancer development. The overexpression of miR-3666 inhibits breast cancer cell proliferation by inhibiting SIRT7. These findings indicate that miR-3666 may serve as a potential candidate for the development of miRNA-based anti-cancer therapeutic strategies.

Acknowledgements

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