MicroRNA-181 serves an oncogenic role in breast cancer via the inhibition of SPRY4

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Abstract. Numerous microRNAs (miRs) have been implicated in breast cancer; however, the molecular mechanism is not fully understood. The present study examined the function and regulatory mechanism of miR-181 in breast cancer. Reverse transcription-quantitative polymerase chain reaction and western blot analysis were used to examine the RNA and protein expression. MTT assay, wound healing assay and transwell assay were conducted to study cell proliferation, migration and invasion. Luciferase reporter gene assay was used to confirm targeting relationship. The results suggested that the miR-181 expression levels were significantly higher in breast cancer cell lines and clinical tissue samples. The increased expression of miR-181 was markedly associated with higher clinical stage and lymph node metastasis. The patients with high miR-181 expression demonstrated worse prognosis compared with those with a low expression of miR-181. Small interfering RNA-induced miR-181 downregulation significantly inhibited breast cancer cell proliferation, migration and invasion \textit{in vitro}, and tumor growth \textit{in vivo}. Protein sprouty homolog 4 (SPRY4), downregulated in breast cancer tissues and cell lines, was observed to be a novel target gene of miR-181. Downregulation of SPRY4 was significantly associated with breast cancer progression in addition to poor prognosis. Knockdown of SPRY4 rescued the inhibitory effects of miR-181 downregulation on the malignant phenotypes of breast cancer cells. Thus, the present study demonstrated that miR-181 serves a promoting role in breast cancer at least in part through the inhibition of SPRY4 expression. The present results expand the understanding of the miR-181/SPRY4 axis’ function during for the malignant progression of breast cancer.

Introduction

Breast cancer, one of the most common malignant tumors in women, causes a large number of mortalities every year (1). In previous years, the prognosis of patients with advanced breast cancer has been unsatisfactory, despite the considerable efforts that have been made in surgical resection combined with chemotherapy and radiotherapy (1). Therefore, elucidating the molecular mechanisms of breast cancer development and progression is strongly warranted, which will be beneficial for the development of effective therapeutic strategies (2-4).

MicroRNAs (miRs/miRNAs), a class of small non-coding RNAs, serve key roles in the regulation of gene expression through interactions with the 3’ untranslated region (UTR) of the target mRNAs, which eventually cause translation repression or mRNA degradation (5,6). Large numbers of miRs have been reported to be involved in various cellular biological processes, including cell proliferation, survival, differentiation, apoptosis, migration and invasion, as well as tumourigenesis (7-10). Recently, certain miRs, including miR-133, miR-153 and miR-429, have been reported to function as oncogenes or tumor suppressors in breast cancer (11-14).

miR-181 has been reported to demonstrate different functions in different types of cancer (15,16). For instance, miR-181 expression levels were significantly reduced in non-small cell lung cancer and miR-181 exerted suppressive effects on NSCLC cell migration by targeting high-mobility group box 1 (15). In addition, miR-181 downregulates NOVA alternative splicing regulator 1, which inhibits cell proliferation, migration and invasion, and promotes apoptosis in astrocytoma (16). By contrast, the oncogenic role of miR-181 has been observed in breast cancer (17,18). For example, miR-181 is upregulated in more aggressive forms of breast cancer and may inhibit the DNA damage response in breast cancer by affecting the expression and activity of the stress-sensor kinase ataxia telangiectasia mutated gene (17). Furthermore, Yoo et al (18) reported that miR-181b-5p promoted epithelial-mesenchymal transition in breast cancer cells by inhibiting tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein γ expression. In addition, miR-181b may promote chemoresistance in breast cancer by targeting Bim (19). miR-181 may also target other genes and serve important roles in breast cancer.

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Key words: breast cancer, microRNA, protein sprouty homolog 4, proliferation, migration, invasion
Protein sprout homolog 4 (SPRY4), a member of a family of cysteine- and proline-rich proteins, has been revealed to exert suppressive effects on receptor-transduced mitogen-activated protein kinase signaling, which serves an important role during cancer progression (20,21). Previously, SPRY4 was reported to function as a tumor suppressor in breast cancer (22); however, the regulatory mechanism underlying SPRY4 expression in breast cancer has yet to be thoroughly elucidated.

Accordingly, the molecular mechanism of miR-181 in the malignant progression of breast cancer was investigated in the present study.

Materials and methods

Tissue collection. Breast cancer tissues and matched adjacent non-tumor tissues were obtained from 78 female patients with breast cancer during routine surgery at Xiangya Hospital of Central South University (Changsha, China) from May 2010 to March 2012. These 78 female patients were between 31 and 66 years old. Inclusion and exclusion criteria: The patients did not receive radiotherapy or chemotherapy prior to surgical resection. The present study was approved by the Ethics Committee of Xiangya Hospital of Central South University, and written informed consent was obtained.

Cell culture and transfection. Human breast cancer cell lines, including SK-BR-3 (cat. no. TCHu225), MCF7 (cat. no. SCSP-531) and MDA-MB-231 (cat. no. TCHu227), and a normal human breast cell line Hs 578Bst (cat. no. GNHU16) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). A human breast cancer cell line MDA-MB-361 (cat. no. CL-0553) was purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., USA). The cells were cultured to full confluence in a 6-well plate. The cell culture medium consisted of DMEM supplemented with 10% FBS and 1% penicillin-streptomycin.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues and cell lines using TRIzol® Reagent (Thermo Fisher Scientific, Inc.). To detect miR expression, a Mir-X™ miRNA RT-qPCR SYBR® kit (Takara Biotechnology Co., Ltd., Dalian, China) was used for RT-qPCR, according to the manufacturer’s protocol. To detect mRNA expression, a OneStep RT-PCR kit (Qiagen, Inc., Valencia, CA, USA) was used for the RT-qPCR, which was performed in an ABI 7500 (Thermo Fisher Scientific, Inc.). The thermocycling conditions were 95°C for 5 min, and 40 cycles at 95°C for 15 sec and 60°C for 15 sec. U6 was used as the internal reference for miR-181, and GAPDH was used as the internal reference for SPRY4. The primer sequences were as follows: SPRY4 forward, 5'-TCTGACCAACGGCCTCTTAGAC-3' and reverse, 5'-GTGCCCATATGGACGACACAGTC-3'; and GAPDH forward, 5' -ACA ACT TTG GTA TCG TAT C-3' and reverse, 5'-GCCATCACGCGCATTTGC-3'. The primers for miR-181 and U6 were obtained from Guangzhou Fulengen Co., Ltd. (Guangzhou, China; miR-181: HmiRQP0232; U6: HmiRQ9001). The relative expression was determined using the 2^ΔΔCt method (23).

Western blotting. The cells were lysed in radioimmunoprecipitation assay buffer with protease inhibitor (both Thermo Fisher Scientific, Inc.). The protein concentration was determined using a Bicinchoninic Protein Assay kit (Thermo Fisher Scientific, Inc.). The protein concentration was determined using the 2,4-DNP method (23).

Cell proliferation assay. Transfected SK-BR-3 and MCF7 cells (1x10^4 cells) were incubated at 37°C for different time points (0, 24, 48 and 72 h). Subsequently, 0.5% MTT solution was added to the cells. Following incubation at 37°C for 4 h, 150 µl dimethyl sulfoxide was added to each well to dissolve the formazan. The optical density (570 nm) was determined using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Transwell assay. Transfected SK-BR-3 or MCF7 cells (1x10^4 cells/well) in DMEM were added to the upper chamber of transwell plates that were pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). DMEM supplemented...
with 10% FBS was added to the lower chamber. Cells were incubated at 37˚C for 24 h. The cells that did not invade the membrane of the insert were removed, and the invading cells were stained at room temperature with gentian violet (Thermo Fisher Scientific, Inc.) for 10 min., and imaged under an inverted light microscope (magnification, x200).

Tumor growth in nude mice. The animal experiments performed in the present study were approved by the Ethics Committee of Xiangya Hospital of Central South University. A total of 6 male BALB/c-nu/nu nude mice (10 weeks; 20-22 g; n=3/group) were obtained from the Beijing Experimental Animal Research Center (Beijing, China) and maintained under specific pathogen-free condition at the Animal Center of Central South University. The temperature was 22-25˚C, the humidity was 40 -60% and a 12 -h light/dark cycle was used. The mice had free access to food and water. Transfected SK-BR-3 cells (10^6 cells) were injected into the dorsal flank of the animals. The control mice were injected with SK-BR-3 cells transfected with NC inhibitor. Subsequently, 60 days following cell injection, the mice were sacrificed under anesthesia, and the tumor weights and volumes were determined.

Bioinformatics analysis. The putative target genes of miR-181 were predicted using TargetScan 7.1 online software (www.targetscan.org).

Luciferase reporter gene assay. The psiCHECK-2 luciferase reporter plasmid containing the wild-type (WT) or mutant type (MT) of SPRY4 was obtained from Yearthbio (Changsha, China). SK-BR-3 and MCF7 cells were co-transfected using Lipofectamine 2000® with the WT or MT SPRY4 plasmids, and miR-181 or miR-NC mimics, respectively. The luciferase activity was detected using the Dual Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) at 48 h following transfection. The activity of firefly luciferase was normalized with Renilla luciferase activity.

Statistical analysis. The experiments were repeated three times. Data are expressed as mean ± standard error. SPSS 18 (SPSS, Inc., Chicago, IL, USA) was used to perform the statistical analysis. One-way analysis of variance was used for multiple-group comparisons followed by Tukey's post hoc test. Student's t-test was used for two-group comparisons. The chi-square test was employed to analyze the associations between gene expression and the clinical characteristics of patients with breast cancer. The Kaplan-Meier method was used to conduct survival analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Upregulation of miR-181 in breast cancer is associated with tumor progression and poor prognosis. To study the role of miR-181 in breast cancer, its expression levels in 78 breast cancer tissues were examined. The results demonstrated that miR-181 was significantly upregulated in breast cancer tissues compared with adjacent non-tumor tissues (Fig. 1A). Additionally, the expression levels of miR-181 were significantly increased in breast cancer cell lines compared with Hs 578Bst cells (P<0.01; Fig. 1B). Therefore, miR-181 may be upregulated in breast cancer. The 78 patients were...
characterized into low and high miR-181 groups using the mean expression value as the cut-off. It was observed that the upregulation of miR-181 was significantly associated with advanced clinical stage, lymph node metastasis and distant metastasis (P<0.01; Table I). The association between miR-181 expression and patient prognosis was further studied. It was identified that patients with breast cancer with high miR-181 expression exhibited a shorter survival time when compared with those with low miR-181 expression (Fig. 1C). Therefore, upregulation of miR-181 may contribute to breast cancer progression and poor prognosis.

Knockdown of miR-181 suppresses breast cancer in vitro. SK-BR-3 and MCF7 cells were used in subsequent experiments as they demonstrated the highest expression of miR-181. These two cell lines were transfected with NC inhibitor or miR-181 inhibitor, respectively. As demonstrated in Fig. 2A, the expression of miR-181 was significantly reduced in the miR-181 inhibitor groups when compared with the NC inhibitor groups (P<0.01). Furthermore, cell proliferation was markedly downregulated in the miR-181 inhibitor groups when compared with the NC inhibitor groups (Fig. 2B). Consistently, cell migration and invasion were also significantly downregulated following miR-181 downregulation (P<0.01; Fig. 3A and B). Therefore, it was demonstrated that knockdown of miR-181 inhibits breast cancer in vitro.

To further confirm these results, SK-BR-3 cells were stably transfected with an NC inhibitor or miR-181 inhibitor lentiviral plasmid. Following transfection, miR-181 expression was significantly downregulated in the miR-181 inhibitor group compared with the NC inhibitor group (P<0.01; Fig. 4A). Subsequently, the stably transfected cells were subcutaneously injected into nude mice, which were sacrificed at 60 days following injection. It was observed that downregulation of miR-181 significantly reduced tumor growth, weight and volume in nude mice injected with SK-BR-3 cells (P<0.01; Fig. 4B-E).

SPRY4 is a novel target gene of miR-181 in breast cancer cells. Target genes of miR-181 were investigated using TargetScan software, and SPRY4 was revealed to be a putative target of miR-181. To confirm the association, WT and MT SPRY4 3’UTR luciferase reporter plasmids were generated (Fig. 5A). The luciferase reporter gene assay demonstrated that co-transfection with miR-181 mimics and WT SPRY4 plasmid significantly reduced the luciferase activity, which was unaltered with co-transfection of miR-181 mimics and MT SPRY4 plasmid (Fig. 5B and C). Therefore, SPRY4 was identified to be a target gene of miR-181 in SK-BR-3 and MCF7 cells.

The regulatory role of miR-181 in the expression of SPRY4 in SK-BR-3 and MCF7 cells was examined. SPRY4 was observed to be markedly upregulated in breast cancer cells following transfection with miR-181 inhibitor (Fig. 6A and B). Subsequently, SK-BR-3 and MCF7 cells were transfected with miR-181 mimics to upregulate the expression of miR-181. Following transfection, miR-181 was upregulated in the miR-181 groups when compared with the miR-NC groups (Fig. 6C). Further investigation demonstrated that SPRY4 was downregulated in the miR-181 groups compared with the miR-NC groups (Fig. 6D and E). Therefore, miR-181 downregulates the expression of SPRY4 in breast cancer cells.

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*P<0.05; †P<0.01. TNM, tumor, node, metastasis.
Downregulation of SPRY4 in breast cancer. SPRY4 expression was measured in breast cancer. The qPCR data indicated that the mRNA expression of SPRY4 was significantly reduced in breast cancer tissues when compared with the matched adjacent non-tumor tissues (P<0.01; Fig. 7A). Furthermore, the expression of SPRY4 was also significantly downregulated in breast cancer cell lines when compared with normal breast Hs 578Bst cells (Fig. 7B and C). Based on these results, the
reduced expression of SPRY4 may be due to the increased expression of miR-181 in breast cancer.

Using the mean expression value as the cut-off, the patients with breast cancer were divided into a high SPRY4 expression group and a low SPRY4 expression group. It was observed that low expression of SPRY4 was significantly associated with advanced clinical stage and lymph node metastasis (P<0.05; Table II). Furthermore, the patients with low expression of SPRY4 exhibited worse prognosis (Fig. 7D). Therefore, downregulation of SPRY4 may contribute to breast cancer progression and poor prognosis.

Inhibition of SPRY4 impairs the inhibitor effects of miR-181 downregulation in breast cancer cells. It was hypothesized that SPRY4 acts as a downstream effector in the miR-181-mediated malignant phenotype of breast cancer in vitro. Breast cancer cells were co-transfected with a miR-181 inhibitor and NC siRNA (miR-181 in+siNC), or with a miR-181 inhibitor and SPRY4 siRNA (miR-181 in+siSPRY4). As demonstrated in Fig. 8A and B, SPRY4 expression was decreased in the miR-181 in+siSPRY4 group compared with the miR-181 in+siNC group (Fig. 8A and B). As demonstrated in Figs. 8C and 9, cell proliferation, migration and invasion
Figure 5. (A) SPRY4 was predicted to be a target of miR-181, and the WT and MT luciferase reporter plasmids were generated. A luciferase reporter gene assay was performed in (B) SK-BR-3 and (C) MCF7 breast cancer cells. **P<0.01 vs. WT SPRY4 3'UTR. miR, microRNA; NC, negative control; SPRY4, protein sprouty homolog 4; WT, wild-type; MT, mutant; UTR, untranslated region.

Figure 6. (A and B) SPRY4 was upregulated in breast cancer cells transfected with miR-181 inhibitor when compared with cells transfected with NC inhibitor. (C) miR-181 was upregulated in breast cancer cells transfected with miR-181 mimics compared with cells transfected with miR-NC. **P<0.01 vs. NC inhibitor. (D and E) SPRY4 was downregulated in breast cancer cells transfected with miR-181 mimics when compared with cells transfected with miR-NC. **P<0.01 vs. miR-NC. SPRY4, protein sprout homolog 4; miR, microRNA; NC, negative control.
were markedly upregulated in the miR-181 in+siSPRY4 group compared with the miR-181 in+siNC group. These results indicated that SPRY4 may be involved in miR-181-mediated breast cancer in vitro.

Discussion

The regulatory mechanism of miR-181 in breast cancer has yet to be thoroughly elucidated. The aim of the present study was to examine the molecular mechanism of miR-181 in breast cancer progression. The results suggested that miR-181 was significantly upregulated in breast cancer. High expression of miR-181 was associated with breast cancer progression and a worse prognosis in patients. siRNA-induced miR-181 downregulation significantly inhibited breast cancer cell proliferation, migration and invasion in vitro, and tumor growth in vivo. SPRY4, downregulated in breast cancer tissues and cell lines, was identified to be a novel target gene of miR-181. Downregulation of SPRY4 was significantly associated with breast cancer progression in addition to poor prognosis. Knockdown of SPRY4 rescued the inhibitory effects of miR-181 downregulation on breast cancer in vitro.

Previous studies have demonstrated the oncogenic function of miR-181 in breast cancer (20-22). Taylor et al (20) reported that transforming growth factor-β is able to upregulate the expression of miR-181 to promote breast cancer metastasis. Niu et al (21) demonstrated that genotoxic treatments induced the expression of miRNA-18 in breast cancer cells, which promoted chemotherapeutic resistance and tumor metastasis. In the present study, it was observed that the miR-181 levels were upregulated in breast cancer tissues and cell lines. Furthermore, it was identified that high expression of miR-181 was markedly associated with the malignant progression of breast cancer in addition to a shorter survival time of patients with breast cancer. Therefore, upregulation of miR-181 is likely to contribute to breast cancer progression. Indeed, knockdown of miR-181 exerted suppressive effects on breast cancer in vitro and in vivo. Similarly, Neel et al (22) reported
Table II. Association between SPRY4 expression and clinicopathological characteristics in breast cancer.

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*P<0.05. TNM, tumor, node, metastasis.

Figure 8. SK-BR-3 and MCF7 cells were co-transfected miR-181 inhibitor and NC siRNA (miR-181 in+siNC group), or miR-181 inhibitor and SPRY4 siRNA (miR-181 in+siSPRY4 group), respectively. The (A) mRNA and (B) protein expressions of SPRY4 were examined. (C) Cell proliferation was examined using an MTT assay. **P<0.01 vs. the corresponding miR-181 in+siNC. miR, microRNA; NC, negative control; si-, small interfering RNA; in, inhibitor; OD, optical density.
that miR-181 exhibits pro-migratory and pro-invasive effects in breast cancer cells. In addition, the serum levels of miR-181 were markedly decreased in patients with early stage breast cancer following surgical resection (24). Although miR-181 functions as an intracellular oncogene in breast cancer, whether it additionally serves oncogenic roles in a circulating form in breast cancer requires further investigation.

SPRY4 was identified as a novel target of miR-181. SPRY4 generally serves a suppressive role in human cancer (25,26). For example, Zhou et al (27) demonstrated that SPRY4 was significantly downregulated in colorectal cancer and predicted a poor prognosis, and they determined that SPRY4 upregulation significantly inhibited colorectal cancer cell proliferation (28). Previously, Vanas et al (28) reported that ectopic expression of SPRY4 inhibited breast cancer cell proliferation independently of its endogenous expression levels, while inhibition of SPRY4 caused accelerated growth. In addition, Jing et al (29) demonstrated that downregulation of SPRY4 enhanced the cancer stem cell properties of breast cancer cells. In the present study, it was observed that downregulation of SPRY4 was significantly associated with advanced malignant phenotypes and shorter survival time of patients with breast cancer. Therefore, SPRY4 may be used as a promising therapeutic candidate for this disease. Furthermore, as miR-181 may negatively regulate the expression of SPRY4 in breast cancer cells, SPRY4 may be associated with the oncogenic function of miR-181 in breast cancer. As it was observed that knockdown of SPRY4 abolished the inhibitory effects of miR-181 downregulation on breast cancer cells, it was hypothesized that SPRY4 may be a downstream effector of miR-181. In addition to miR-181, miR-411-5p has additionally been reported to promote the proliferation and differentiation of rhabdomyosarcoma cells via direct targeting of SPRY4 and the activation of p38 mitogen-activated protein kinase signaling (21).

In conclusion, the present study demonstrated that miR-181 functions as an onco-miR in breast cancer, at least partly, by targeting SPRY4. Therefore, miR-181 may become a novel therapeutic target for breast cancer.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors’ contributions
YT wrote the manuscript. LS designed the study and revised the manuscript. YT, XF, QL, YW, DF, QZ and WK performed all experiments.

Ethics approval and consent to participate
The human and animal experiments performed in the present study were approved by the Ethics Committee of Xiangya Hospital of Central South University; Written informed consent was obtained from all of the patients recruited.
Patient consent for publication

Written informed consent to publication was obtained from all of the patients recruited.

Competing interests

The authors declare that they have no competing interests.

References


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