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Original Article

MiR-29a regulated FOXO3 expression and promoted the cell proliferation of human cervical cancer

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Abstract: Objective: Cervical cancer is one of the most common cancers and a leading cause of cancer-related mortality in women worldwide. MicroRNA-29a (miR-29a) has been reported to play a critical role in tumor pathogenesis of several human cancers. The purpose of this study was to explore the biological functions of miR-29a in cervical cancer. Materials and methods: The expression of miR-29a in cervical cancer tissue samples and cell lines was detected by quantitative real-time PCR (qRT-PCR). CCK-8 and colony formation assays were used to measure the viabilities of cervical cancer cells. The effect of miR-29a on cell cycle was determined by using flow cytometry. Moreover, luciferase reporter assay were used to identify the target gene of miR-29a in cervical cancer. Results: In the current study, we found that miR-29a expression were significantly upregulated in cervical cancer tissues as well as cell lines. Overexpression of miR-29a in HeLa cells transfected with miR-29a mimics promoted cell proliferation, while downregulation of miR-29a induced the opposite effect. In addition, FOXO3 is a novel direct target of miR-29a in cervical cancer cells; knockdown of FOXO3 expression counteracted the cell proliferation arrest by miR-29a inhibitor. Conclusions: These findings demonstrated that miR-29a promoted cervical cancer cell proliferation by targeting FOXO3; miR-29a might serve as a potential molecular therapeutic target for cervical cancer.

Keywords: MicroRNA, miR-29a, cervical cancer, FOXO3

Introduction

Cervical cancer is the third most common cancer and the fourth leading cause of cancer-related mortality in women worldwide [1]. There are over 500,000 new cervical cancer cases and 270,000 related deaths annually according to a recent global cancer statistics [2]. The age standardized mortality rate in developing countries is about 10/10000, which is more than three times higher than in developed countries [3]. It is well known that human papillomavirus (HPV) infection is the most important cause of cervical cancer. Surgery, radiotherapy, or a combination of radiotherapy and chemotherapy are primary treatment strategies for cervical cancer depending on the stage [4]. However, the molecular mechanism underlying the initiation and progression of cervical cancer remains unclear. Therefore, identification of novel potential therapeutic targets is necessary for improving the management of cervical cancer.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules that regulate gene expression by directly binding to the 3'-untranslated region (UTR) of target messenger RNAs (mRNAs) and resulting in mRNA degradation or translation repression. MiRNAs modulate a variety of important biological processes, including cellular development, proliferation, differentiation, apoptosis, immune regulation, and tumorigenesis [5, 6]. Accumulating studies have shown that the expression of miRNAs were dysregulated in many types of human cancers including cervical cancer, and play important roles in the processes of tumor initiation, progression and metastasis [7-10].

Among these functional miRNAs, miRNA-29a (miR-29a) has been reported to be downregulated in several types of human cancers including prostate cancer, pancreatic cancer, gastric cancer, lung adenocarcinoma and acts as a tumor suppressor [11-14]. Moreover, miR-29a was significantly upregulated in breast cancer

compared with non-tumor tissues, promoted breast cancer cell proliferation and EMT by targeting ten eleven translocation 1 [15]. This demonstrated that miR-29a acts as a tumor activator in breast cancer. The functional role of miR-29a is extremely complex as it may act as an oncogene or a tumor suppressor depending on the cellular contexts. However, the expression and function of miR-29a in the development of cervical cancer remains un clear.

In the present study, we detected the expression of miR-29a in cervical cancer tissues and cell lines, and investigated the effects of miR-29a on cervical cancer cell proliferation. Furthermore, we demonstrated that FOXO3 is a direct target for miR-29a in cervical cancer cells and involved in the functional effects of miR-29a on cervical cancer cell proliferation. This study provides a novel molecular mechanism involved in the progression of cervical cancer and may be useful for the development of therapeutic and diagnostic strategies for cervical cancer.

Materials and methods

Human tissue samples

A total of 30 cervical cancer tissue samples and matched adjacent non-tumor tissues were obtained from patients who received surgery without preoperative systemic therapy at the Department of Gynecology, The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology between 2012 and 2015. The tissue samples were immediately frozen using liquid nitrogen. The study was performed with the approval of the Clinical Research Ethics Committee of The First Affiliated Hospital, College of Clinical Medicine of Henan University of Science and Technology, and all patients written informed consent for the use of the tissue samples for research purposes.

Cell lines and transfection

Five human cervical cancer cell lines (HeLa, SiHa, C33A, HCC94 and CaSki) and the non-tumorigenic immortalized keratinocytes (HaCaT) were obtained from Chinese Academy of Sciences (Shanghai, China). Cell lines were cultured in DMEM medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA) at a humidified atmosphere incubator of 37°C and 5% CO_2. The miR-29a mimics, inhibitor, and corresponding negative controls were synthesized and purchased from RiboBio Company (Guangzhou, China). The specific small interfering RNA (siRNA) for FOXO3 and corresponding negative control were synthesized and purchased from GenePharma Company (Shanghai, China). Transfection was performed by using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

RNA extraction and real-time quantitative PCR

Total RNA was extracted from cervical cancer tissues and cell lines by Trizol Reagent (Invitrogen, USA) according to the manufacturer's protocol. Reverse transcription and quantitative real-time PCR were performed by using the One Step PrimeScript miRNA cDNA Synthesis Kit (Takara, Japan) according to the manufacturer's protocol on ABI 7500 Fast Real-Time PCR system (Applied Biosystems, USA). GAPDH and U6 were used as internal controls respectively for normalization and quantification of FOXO3 and miR-29a expression. The relative expression of genes was analyzed and calculated by using the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation assay

Cell proliferation was measured by using Cell Counting Kit-8 (CCK-8) (Beyotime, Shanghai, China) according to the manufacturer's protocol. Cells were seeded in a 96-well plate at a concentration of 5×10^3 cells/well for 24 hours, then transfected with the indicated miRNA mimics, inhibitor, or siRNA and further incubated for 24, 48, 72 and 96h respectively. At different time points, CCK-8 reagents were added to each well and incubated in dark at 37°C for another 2 h. The absorbance was assessed at the wavelength of 450 nm by a microplate reader.

Cell cycle analysis

For cell cycle analysis, cells were harvested and washed using cold Phosphate Buffered Saline (PBS), fixed in 70% ethanol overnight at 4°C. Fixed cells were re-suspended in cold PBS and then incubated with RNaseA (20 μg/ml) for 30 minutes followed by propidium iodide (PI, 50 μg/ml) (Sigma, USA) at room temperature for 1 h. The stained cells were analyzed for DNA con-

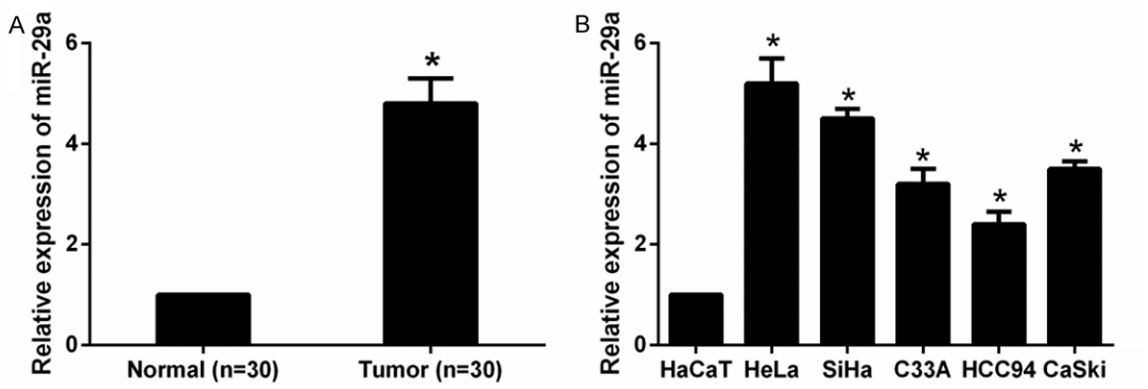

Figure 1. Expression levels of miR-29a in human cervical cancer tissues and cell lines. A. MiR-29a expression was significantly upregulated in 30 human cervical cancer tissues compared with that in matched adjacent non-tumor tissues by using real-time quantitative PCR. B. MiR-29a expression was markedly increased in five cervical cancer cell lines (HeLa, SiHa, C33A, HCC94 and CaSki) compared with that in non-tumorigenic immortalized keratinocytes (HaCaT). *P<0.05.

tent by using a FACSCalibur flow cytometer (BD Biosciences, USA).

Colony formation assay

For colony formation assay, cells were plated in 6-well plates with 1×10^3 cells in each well after transfection and incubated in DMEM medium containing 10% FBS at 37°C for 14 days. Then the colonies were washed twice with PBS and fixed with 10% formalin, stained with 0.5% crystal violet (Sigma, USA). Colonies were counted and this assay was repeated in triplicates.

Luciferase reporter assay

The 3'UTR of FOXO3 containing the wild-type or mutant miR-29a binding site were designed and cloned into the pGL3 plasmid (Invitrogen, USA) according to the manufacturer's protocol. Cells were seeded in 24-well plates and co-transfected with plasmid pGL3 FOXO3-3'UTR-WT or pGL3 FOXO3-3'UTR-MUT and miR-29a mimics or negative control by using Lipofectamine 2000. Cells were collected 48 h after transfection, and relative luciferase activities were measured by a dual-luciferase reporter assay (Promega, USA) according to the manufacturer's instruction. Renilla luciferase was used for normalization.

Western blot

Proteins were extracted by RIPA lysis buffer and the concentrations were detected by using BCA Protein Assay Kit (Beyotime, Shanghai, China). Equal amounts of protein samples were fractionated by using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto PVDF membranes and blocked with 5% non-fat milk for 30 minutes at room temperature. Membranes were probed with primary antibodies against FOXO3 and GAPDH (Abcam, MA, USA) at 4°C overnight, followed by incubation with HRP-conjugated secondary antibodies. GAPDH was used as an endogenous protein for normalization. Results were detected by using the Odyssey Scanning system (Li-Cor, Lincoln, USA).

Statistical analysis

All statistical analyses were performed by using SPSS 18.0 software. Data were presented as mean ± SD of three separate experiments. Differences between groups were analyzed by using student's t test or one-way ANOVA analysis. A value of $P < 0.05$ was considered statistically significant.

Results

MiR-29a was significantly upregulated in cervical cancer tissues and cell lines

To our knowledge, the expression of miR-29a in cervical cancer has not been examined. In this study, we first detected the expression levels of miR-29a in cervical cancer tissues and cell lines by qRT-PCR. MiR-29a expression was significantly upregulated in cervical cancer tissues

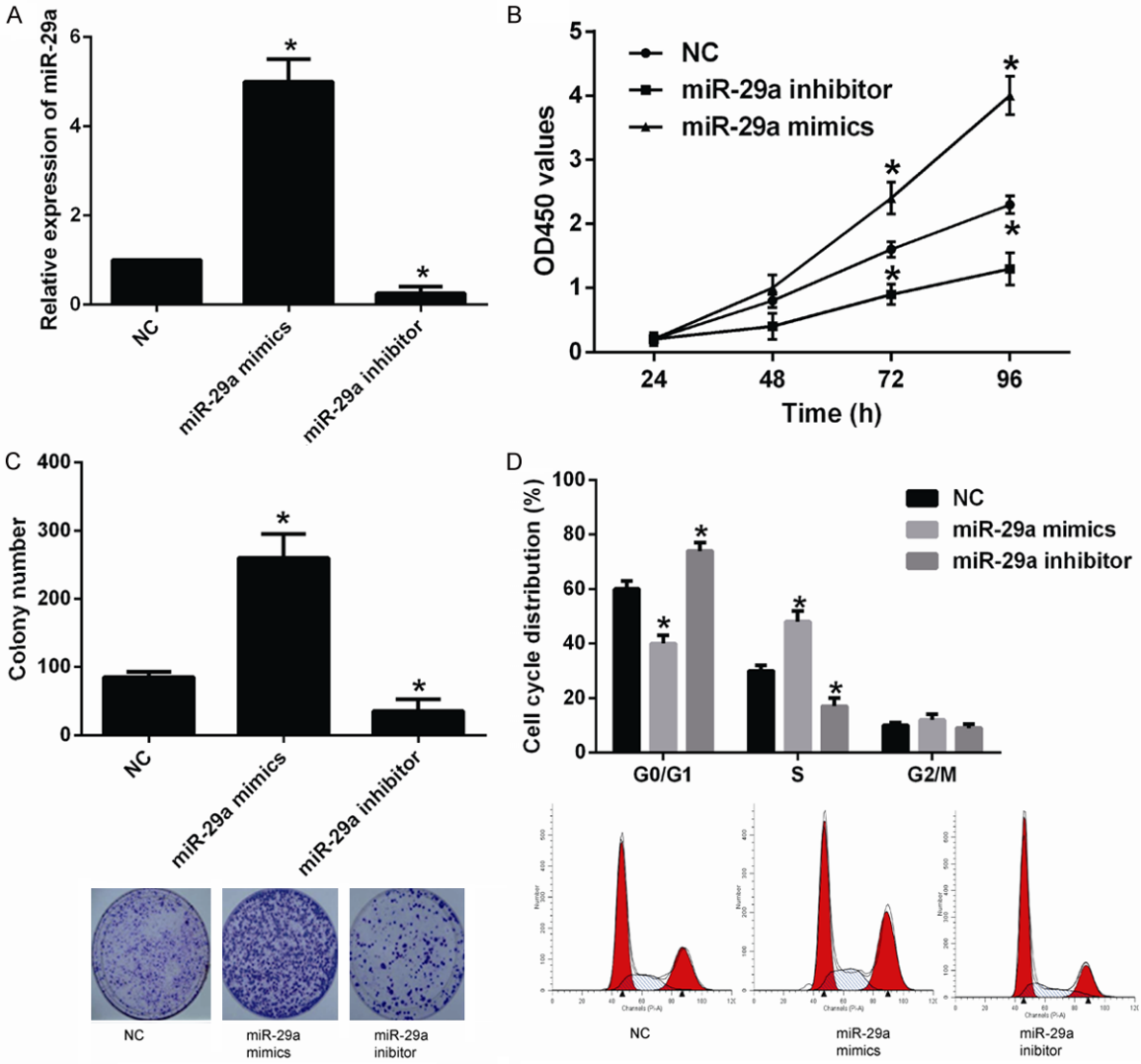

Figure 2. MiR-29a promoted the cell proliferation of cervical cancer cells. A. The miR-29a mimics, inhibitor and corresponding negative controls were transfected into HeLa cells, and the miR-29a expression was then detected by using qRT-PCR. B. Upregulation of miR-29a in HeLa cells transfected with miR-29a mimics significantly promoted the cell growth by using CCK-8 assay, while downregulation of miR-29a induced the opposite effect. C. Upregulation of miR-29a in HeLa cells significantly promoted the cell proliferation by using colony formation assay, while downregulation of miR-29a induced the opposite effect. D. Cell cycle distributions of HeLa cells transfected with the miR-29a mimics or inhibitor were analyzed by flow cytometry. *P<0.05.

compared with that in matched adjacent non-tumor tissues (**Figure 1A**). Moreover, miR-29a expression was also significantly increased in five cervical cancer cell lines compared with that in HaCaT cells (**Figure 1B**).

MiR-29a promoted cell proliferation of cervical cancer

To investigate the potential role of miR-29a in cell proliferation of cervical cancer, miR-29a mimics, inhibitor, and corresponding negative controls were transfected into HeLa cells. As expected, miR-29a expression was obviously upregulated in HeLa cells transfected with miR-29a mimics compared with that transfected with negative control, miR-29a expression was significantly downregulated in HeLa cells transfected with miR-29a inhibitor (**Figure 2A**). The effects of miR-29a on cervical cancer cell proliferation were determined by using CCK-8 and colony formation assays. The results showed that compared with the control group, cell proliferation was significantly enhanced in HeLa cells transfected with miR-29a mimics, on the

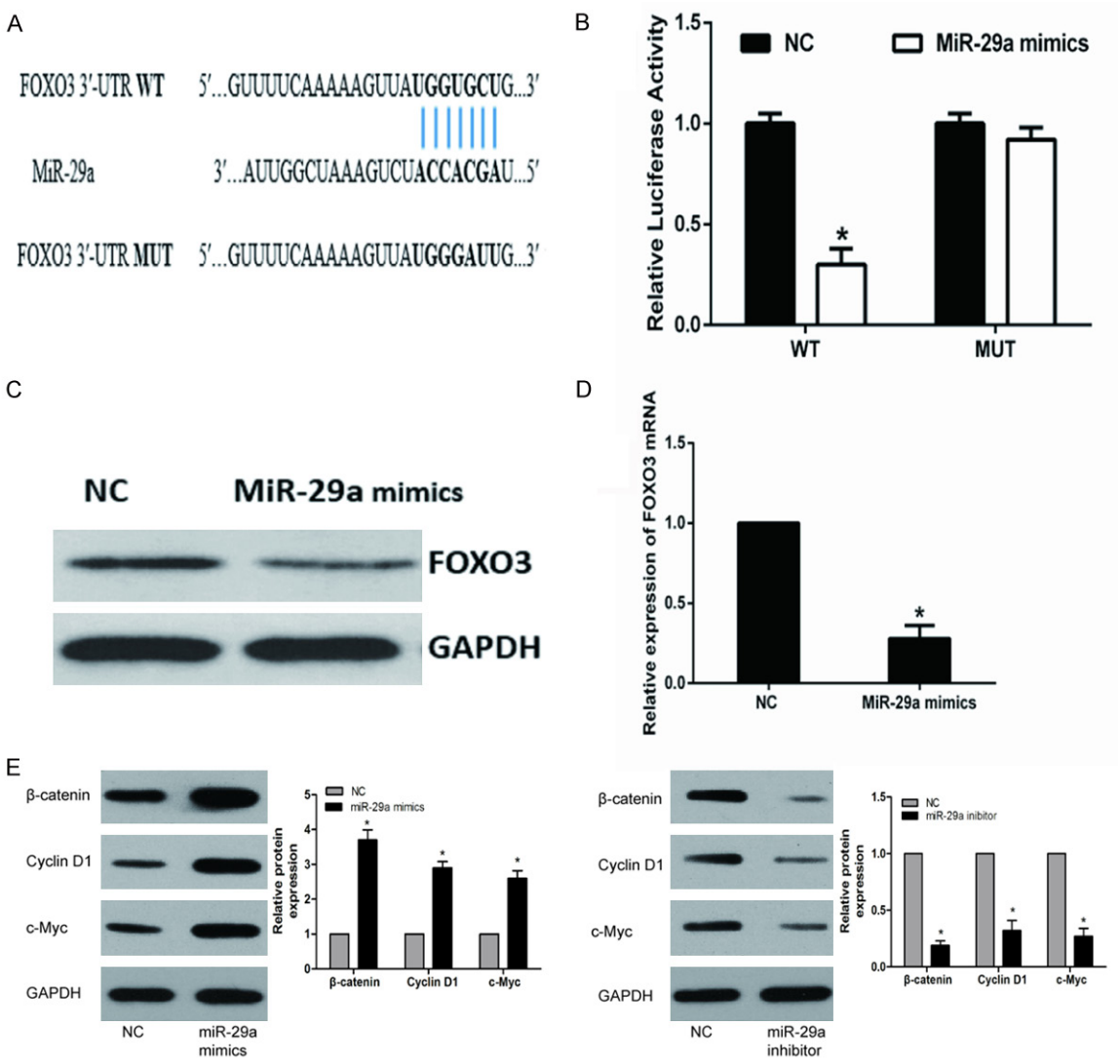

Figure 3. FOXO3 is a novel direct target of miR-29a in cervical cancer. A. The potential miR-29a binding sites of FOXO3 mRNA 3'-UTR and the construct information of mutant FOXO3 3'-UTR. B and C. The expression of FOXO3 protein and mRNA were significantly reduced in Hela cells transfected with miR-29a mimics by western blot and qRT-PCR analyses. D. Wild-type or mutant FOXO3 3'-UTR vector and miR-29a mimics or negative control were co-transfection into HeLa cells, the luciferase activity was measured by Luciferase reporter assay. E. Western blot explored the protein expression of β-catenin, cyclin D1 and c-myc after miR-29a overexpression or miR-29a knockdown. GAPDH was used as the loading control *P<0.05.

contrary, and cell proliferation was significantly repressed in HeLa cells transfected with miR-29a inhibitors (**Figure 2B**, **2C**).

To further explore the mechanisms mediating this anti-proliferative effect, we then investigated whether miR-29a had an effect on cell cycle progression of cervical cancer by using flow cytometry. The results revealed that overexpression of miR-29a in HeLa cells led to a significantly decrease in the cellular percentage in G0/G1 phase but an increase in the cellular percentage in S phase, while downregulated expression of miR-29a in HeLa cells induced the opposite effect (**Figure 2D**). Taken together, miR-29a promoted the cervical cancer cell proliferation and cell cycle progression from the G1/G0 to S phase in cervical cancer cells.

FOXO3 is a direct target of miR-29a in cervical cancer cells

To investigate the underlying molecular mechanisms of miR-29a in cervical cancer progres-

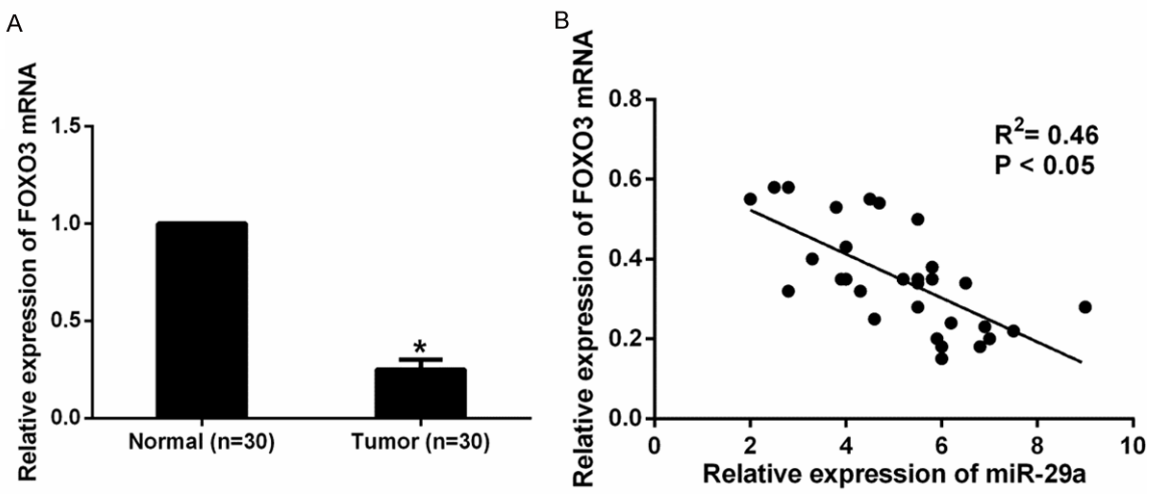

Figure 4. Inverse relationship between FOXO3 and miR-29a expression in cervical cancer. A. The expression of FOXO3 was significantly downregulated in 30 human cervical cancer tissues compared with that in adjacent non-tumor tissues. B. The expression of FOXO3 was inversely associated with the expression of miR-29a in cervical cancer tissues. *P<0.05.

sion, we tried to predict the potential target genes of miR-29a by using bioinformatic tools (TargetScan and miRanda), which identified that the 3'UTR of FOXO3 mRNA contained a target site for miR-29a (**Figure 3A**). To determine whether FOXO3 is a target gene of miR-29a, HeLa cells were transfected with miR-29a mimics or negative control, and the expression of FOXO3 was detected by using real-time quantitative PCR and western blot. The results showed that the mRNA and protein levels were all significantly decreased in HeLa cells transfected with miR-29a mimics compared with cells transfected with negative control (**Figure 3B**, **3C**). To further confirm whether FOXO3 is a direct target of miR-29a in HeLa cells, luciferase reporter assay was performed. We constructed luciferase reporter vector containing wild-type FOXO3 3'-UTR with miR-29a binding site (pGL3-FOXO3-wt) or containing the mutant FOXO3 3'-UTR (pGL3-FOXO3-mut). Co-transfection with miR-29a mimics significantly decreased the luciferase activity of pGL3-FOXO3-wt vector in HeLa cells compared with negative control, however, miR-29a mimics did not have effect on luciferase activity of pGL3-FOXO3-mut vector (**Figure 3D**). Taken together, our data indicated that FOXO3 is a direct target of miR-29a in cervical cancer cells.

Previous reports showed that FOXO3 was associated with Wnt/β-catenin signaling pathway. We tested the protein expression levels of β-catenin, Cyclin D1 and c-Myc. We found that expression levels of β-catenin, Cyclin D1 and c-Myc wad increased in HeLa cells transfected with miR-29a mimics, but reduced in the cells transfected with miR-29a inhibitor, relative to control cells (**Figure 3E**). Altogether, we suggested that miR-29a functionally modulates cellular proliferation regulators by repressing FOXO3 expression, and then modulating Wnt/β-catenin signaling.

Inverse relationship between FOXO3 and miR-29a expression in cervical cancer tissues

We then detected the expression levels of FOXO3 in 30 paired cervical cancer tissues and adjacent non-tumor tissues by using real-time quantitative PCR. FOXO3 expression was significantly downregulated in cervical cancer tissues compared with that in matched normal tissues (**Figure 4A**). Furthermore, the expression level of FOXO3 was inversely correlated with the expression level of miR-29a in cervical cancer tissues (**Figure 4B**).

FOXO3 downregulation counteracted the proliferation arrest by miR-29a inhibitor

We further determined whether FOXO3 is involved in the effects of miR-29a on cervical cancer cell proliferation. FOXO3 siRNA or negative control was transfected into miR-29a inhibitor-transfected HeLa cells. Results revealed that the mRNA and protein levels of FOXO3

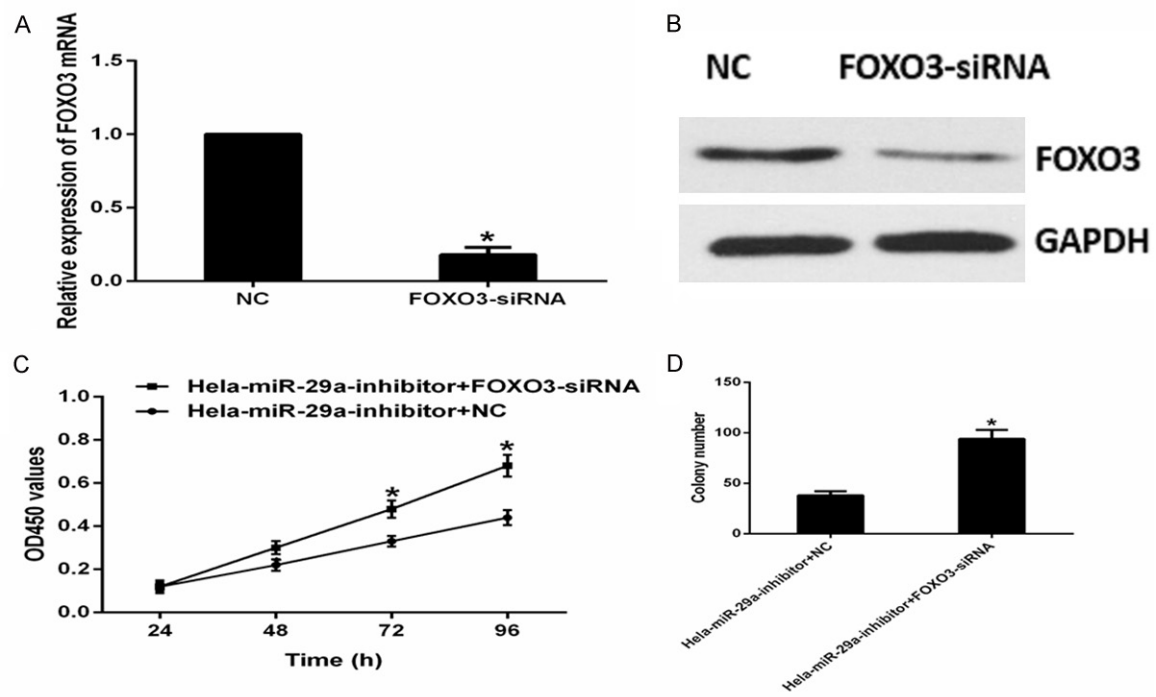

Figure 5. Downregulation of FOXO3 counteracted the cell proliferation arrest by miR-29a inhibitor in cervical cancer. A, B. qRT-PCR and western blot analyses revealed that knockdown of FOXO3 by FOXO3 siRNA significantly decreased the mRNA and protein levels in miR-29a inhibitor-transfected Hela cells. C. Knockdown of FOXO3 by siRNA significantly promoted the cell growth of miR-29a inhibitor-transfected Hela cells by using CCK-8 assay. D. Knockdown of FOXO3 by siRNA significantly promoted the cell proliferation of miR-29a inhibitor-transfected Hela cells by using colony formation assay.

were markedly decreased in FOXO3 siRNA group compared with negative control (**Figure 5A**, **5B**). CCK-8 and colony formation assays demonstrated that knockdown of FOXO3 by siRNA effectively counteracted the proliferation arrest by miR-29a inhibitor (**Figure 5C**, **5D**).

Discussion

In the present study, we found that the expression of miR-29a was significantly upregulated in cervical cancer tissues and cell lines compared with that in matched adjacent non-tumor tissues and HaCaT cells. Overexpression of miR-29a in HeLa cells transfected with miR-29a mimics could promote cell proliferation of cervical cancer, while downregulated expression of miR-29a in HeLa cells transfected with miR-29a inhibitor suppressed the cell proliferation of cervical cancer. In addition, FOXO3 was a direct and functional target of miR-29a in cervical cancer cells, FOXO3 involved in miR-29a-induced cell proliferation of cervical cancer. Taken together, these results demonstrated that miR-29a may play an important role in carcinogenesis and progression of cervical cancer.

Cervical cancer is one of the most common cancers and a leading cause of cancer-related mortality in women worldwide. Although many recent studies have reported a series of new treatment strategies for cervical cancer, unfortunately, the poor survival rate has not been substantially improved. Exploration of the molecular mechanisms underlying tumorigenesis and progression of cervical cancer helps to develop novel diagnostic and therapeutic approaches. Accumulating evidence have indicated that the aberrant expression of miRNAs contribute to tumorigenesis and are considered as biomarkers for diagnosis and therapy in various cancers, including cervical cancer. For example, Su et al reported that miR-140-5p was downregulated in cervical cancer specimens and the downregulation of miR-140-5p was associated with cervical cancer poor prognosis, furthermore, miR-140-5p significantly suppressed cervical cancer cell proliferation and metastasis by targeting insulin like growth

factor 2 [16]. Xu et al also indicated that the levels of miR-181a were evidently enhanced in cervical cancer cell lines compared with normal cervical epithelium cells, inhibition of miR-181a suppressed cell proliferation and invasion and promote apoptosis [17].

However, different miRNAs could play different roles in cervical cancer, so we still need to clarify the clinical significance and function of certain specific miRNA. MiR-29a has been reported to be dysregulated in several cancers and plays an important role in cancer progression. For example, miR-29a was reported to be significantly downregulated in both cholangiocarcinoma tissues and tumor cell lines, furthermore, overexpression of miR-29a reduced the cell proliferation and metastasis capacity of cholangiocarcinoma cell lines [18]. On the other hand, miR-29a was upregulated in pancreatic tumor tissues and cell lines and positively correlated with metastasis, miR-29a promoted cell proliferation and invasion of pancreatic cancer by inhibiting tristetraprolin [19]. However, little is known of miR-29a expression and its potential function in cervical cancer. In this study, we examined the expression of miR-29a in cervical cancer tissues and cell lines, and also investigated the effects of miR-29a on cervical cancer cell proliferation. Our results demonstrated that miR-29a was significantly upregulated in cervical cancer tissues and cell lines, miR-29a enhanced cell proliferation and functioned a tumor promoter gene in cervical cancer.

To investigate the underlying mechanisms of miR-29a in cervical cancer, bioinformatics methods were used to predict the potential target genes for miR-29a. We found that FOXO3 was one of the cancer-associated genes, whose mRNA contained a target site for miR-29a on the 3'-UTR. FOXO3 is a key transcription factor from the forkhead box (FOX) protein family and considered as a tumor suppressor, which regulates the transcription of a number of genes that are critical in a variety of cellular process including cell cycle [20], cell apoptosis [21], differentiation [22], angiogenesis [23], and oxidative stress responses [24]. Emerging evidence has revealed that FOXO3 acts as a suppressor of prostate cancer metastasis and inhibits epithelial-to-mesenchymal transition by modulate WNT/β-catenin signaling in prostate cancer cells [25]. High expression of FOXO3 has been reported to be associated with low tumor stages, better survival in breast cancer patients and suppress cell proliferation and tumorigenesis of estrogen-dependent breast cancer [26, 27]. Li et al also found that miR-96 can promote invasion and inhibit apoptosis in non-small cell lung cancer cells by targeting FOXO3 [28]. In the current study, our results revealed that FOXO3 expression was significantly downregulated and inversely correlated with the miR-29a expression in cervical cancer tissues. FOXO3 acts as a direct functional target of miR-29a in cervical cancer cells. Furthermore, we found that protein expression of β-catenin, cyclinD1 and c-Myc were all markedly upregulated by overexpression of miR-29a and downregulated by miR-29 inhibitor. Taken together, our findings indicated that miR-29a may act as a tumor promoter in cervical cancer by targeting FOXO3, and then modulate Wnt/β-catenin signaling pathway.

In conclusion, our study found that miR-29a was significantly downregulated in cervical cancer. Downregulation of miR-29a could inhibit cervical cancer cell proliferation, while overexpression of miR-29a promoted cervical cancer cell proliferation. Furthermore, FOXO3 is a direct functional target of miR-29a in cervical cancer cells, miR-29a promotes the cell proliferation of cervical cancer by targeting FOXO3 and then modulating Wnt/β-catenin signaling pathway. These results suggested that miR-29a might serve as a potential therapeutic target for cervical cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

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