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MiR-183 maintains canonical Wnt signaling activity and regulates growth and apoptosis in bladder cancer via targeting AXIN2

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Abstract. – OBJECTIVE: Previous investigations have shown that miR-183 is upregulated in bladder cancer (BC); however, its biological significance is not fully investigated. The goal of the current study is to analyze the function of miR-183 in BC development and progression.

PATIENTS AND METHODS: 23 pairs of BC tumor and adjacent tissues were analyzed for miR-183 and c-Myc expression using Real-time polymerase chain reaction (PCR). MiR-183 expression was modulated by transfection of miR-183 or miR-183 inhibitor (miR-183-in). Protein expression of AXIN2, c-Myc and Cyclin D1 was determined by western blot. Cell growth activity and apoptotic potential were evaluated by cell viability assay and flow cytometry assay, respectively. Luciferase activity assay was conducted to determine whether AXIN2 is a direct target of miR-183.

RESULTS: The expression of miR-183 is upregulated in BC tissues and cell lines, and is positively correlated with the expression of the Wnt target gene, c-Myc. MiR-183 positively regulated Wnt signaling activity by directly suppressing its negative feedback regulator, AXIN2. Overexpression of miR-183 promoted cell growth and inhibited apoptosis. Inhibition of miR-183 attenuated cell growth and enhanced apoptosis. The effect of miR-183 on cell growth and apoptosis can be abolished by knockdown of AXIN2.

CONCLUSIONS: MiR-183 functions as an oncomiR in BC and upregulates Wnt signaling activity by directly suppressing AXIN2 expression.

Key Words:
Bladder cancer, Wnt signaling, AXIN2, MiR-183, Apoptosis, Proliferation.

Introduction

Bladder cancer (BC) is a common cancer type in urinary tract[1,2]. Although it is estimated that 75-80% of BC cases are non-muscle-invasive BC that can be safely managed with surgical treatment followed by intravesical chemotherapy and immunotherapy, they are under a high risk of recurrence after the conventional treatments3. More advanced therapeutics would possibly improve the clinical management for BC. Despite its prevalence, we currently know little about the pathogenesis of BC. Thus, it is necessary for us to investigate the molecular basis that underlies the cancer cell behaviors in BC.

MicroRNAs (miRs) are a class of non-coding oligonucleotides, which are about 22 nt in length. The multiple functions of miRs are achieved by its base-pairing directed binding with mature mRNAs, which subsequently leads to degradation or translation blockage of targeted mRNAs4. Negative regulations of gene expression by miRs in cancer cells often lead to changes of cellular characteristics including proliferation, differentiation and programmed cell death[5,6]. Thus, identifying the functions of miRs may provide a possible strategy for the control of carcinogenicity. Over the last decades, the multiple roles of miRs in BC have been extensively studied. Numerous miRs have been identified as biomarkers, prognostic markers and therapeutic targets for BC[7]. MiR-183 has been previously shown to play both tumor suppressive and oncogenic roles in various cancer types[8,9]. Intriguingly, its expression has proven to be upregulated in tumor tissues and patient serum in BC, which suggests that it possibly plays an oncogenic role[10,11]. However, the precise functions and mechanisms of miR-183 in BC have not been experimentally investigated.

To study the possible role of miR-183 in BC development and progression, we studied its expression level in BC tissues and compared it with that

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in matched normal tissues. We detected an upregulation of this microRNA. Moreover, miR-183 expression is positively associated canonical Wnt signaling activity. MiR-183 exerts an oncogenic action by promoting proliferation and inhibiting apoptosis. We further demonstrated that miR-183 positively regulates cellular oncogenicity and Wnt signaling through inhibiting the expression of AXIN2, a negative feedback regulator in Wnt pathway. Our study provides miR-183 as a novel target for the control of cellular growth activity in BC.

Patients and Methods

Patients

The tissue samples for BC tumor tissues and matched adjacent normal tissues were obtained during July 2016 and February 2018 from the 2nd Affiliated Hospital of Fujian Medical University. Tissues were collected during standard surgical treatment. Patients did not receive any adjuvant therapy before surgery. The surgical tissues were snap frozen in liquid nitrogen and stored in -70°C refrigerator until use. Patients were informed for the use of the tissue samples, and the consents were obtained. This study was approved by the institutional Ethics Committee of the 2nd Affiliated Hospital of Fujian Medical University.

Real-Time Polymerase Chain Reaction (PCR)

MicroRNA samples were prepared using miRcute miRNA isolation kit (Tiangen, Beijing, China). The RNA samples were reversed transcribed using QuantScript RT Kit (Tiangen) with the Bulge-loop primers purchased from Ribo Biotech Inc. (Guangzhou, China). The amplification step was done on a Bio-Rad CFX-96 (Hercules, CA, USA) system using 2X SYBR Green master mix (TaKaRa, Dalian, China). U6 was used as a normalizing control for miR-183, and its primer was purchased from Ribo Biotech Inc. For detection of c-Myc mRNA, the following primer was used F- 5'GGCTCCTGGCAAAAGGTCA-3' R 5'-CTGCGTAGTTGTGCTGATGT-3'. GAPDH was used as a normalizing control for c-Myc detection, the primer was purchased from Sangon Biotech (Shanghai, China).

Cell Culture

Normal human bladder epithelial cell SV-Huc1, and bladder cancer cell lines 5637 and T24 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). SV-Huc1 cells were cultured with F12 medium (HyClone, South-Logan, UT, USA), and 5637 and T24 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South-Logan, UT, USA). All cells were cultured under the condition of 10% FBS, 5% CO2, 37 °C and humidified atmosphere.

Transfection

To inhibit or overexpress miR-183 in BC cells, miR-183 mimics and inhibitors (all purchased from Ribo Biotech. Inc., Guangzhou, China) were transfected into BC cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specification. Briefly, for 6-well plates, 100 nmol/L oligonucleotides and 8 μl Lipofectamine 2000 reagents were mixed and incubated for 20 min, and then applied to each well. The medium was then refreshed with complete medium 24 h after transfection.

Luciferase Activity Assay

The 3' untranslated region (UTR) of AXIN2 mRNA were amplified using standard PCR and the sequence was then subcloned into a pmiRGLO construct (Promega, Madison, WI, USA) in the multiple cloning sites downstream the firefly luciferase gene. For dual luciferase assay, miR-183 mimics or miR-183 inhibitor was transfected along with the luciferase construct into BC cells. The luciferase activity was then detected 24 h post-transfection using a Dual-Luciferase Detection System (Promega, Madison, WI, USA).

Western Blot

Cells were treated with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) and centrifuged at 15000 g to remove the cell debris. The supernatant was collected and subjected to BCA assay to test the protein concentration. Next, 30 μg total protein was added to each lane to perform electrophoresis. The protein samples were then transferred onto a polyvinylidene difluoride (PVDF) membrane, blocked with 5% skimmed milk and then incubated with antibodies against AXIN2 (Abcam, Cambridge, MA, USA), c-Myc (Abcam, Cambridge, MA, USA), Cyclin D1 (Cell Signaling Technology, Danvers, MA, USA) and GAPDH (Cwbiotech, Beijing, China) overnight at 4°C. On the following day, membranes were incubated with horseradish peroxidase (HRP)-linked secondary antibodies (Cwbiotech) at room tem-

perature. The membranes were then visualized with BeyoECL Plus kit (Beyotime, Shanghai, China). The band density was analyzed using Image J software.

Cell Viability Assay

After transfection, cells were seeded into 96-well plates at the concentration of 2×105 /ml. These cells were incubated for 4 h before taken out from incubator for detection; the thiazolyl blue tetrazolium bromide (MTT) solution (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. The medium was then removed, and DMSO was supplied to each well for color development. The absorbance value at 490 nm was obtained, and relative cell viability was calculated.

Apoptosis Assay

For detection of apoptosis, cells were treated with Annexin V-FITC apoptosis detection kit (Beyotime) according to the manufacturer's protocol. The double stained cells were then analyzed with the FACS Calibur flow cytometer system (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical Analysis

Data were expressed as means± standard errors of the mean (S.E.M.) and at least 3 repeats were performed for each experiment. Spearman correlation test was used for analyzing relationship between miR-183 and c-Myc. Pairwise comparison was determined by t-test or one-way ANOVA with Tukey's post-hoc test. A *p*-value less than 0.05 was considered to be statistically significant.

Results

MiR-183 Expression in BC Tissues and Cell Lines and its Correlation with Wnt Signaling Activity

In the first part of our study, we compared the expression level of miR-183 in BC tumor tissues with their matched normal tissues using Real-time PCR analysis. Consistent with previous reports, we also found that miR-183 is significantly upregulated in tumor samples (Figure 1A). *In vitro*, we found that BC cancer cell lines 5637 and T24 express a significantly higher level of miR-183 when comparing with the normal bladder epithelial cell SV-Huc1 (Figure 1B), T24 cell has a highest expression level. We also detected that he proto-oncogene, c-Myc, which is a Wnt target gene, was increased in the tumor tissues (Figure 2C). Interestingly, miR-183 expression is in a good correlation with c-Myc expression, suggesting that miR-183 may be an oncomiR which is involved in regulation of Wnt signaling activity in BC cells.

Validation of AXIN2 as a Direct Target of miR-183

We searched the www.microRNA.org database, and found that the tumor suppressor and negative feedback regulator of Wnt signaling, AXIN2, contains a putative binding site of miR-183 in its mRNA 3'UTR (Figure 3A). We reasoned that miR-183 directly influence Wnt signaling by suppressing AXIN2 expression. We constructed luciferase reporter carrying the 3'UTR of AXIN2, and cotransfected the reporter with miR-183 or miR-183 inhibitor (miR-183-in). In both cell lines, miR-183 decreased the reporter activity, whereas miR-183-in enhanced the reporter activity (Figure 3B). Western blot analyses showed that miR-183 inhibited AXIN2 expression in 5637 and T24 cells, whereas miR-183-in promoted AXIN2 expression (Figure 3C and D). These results confirmed that AXIN2 is a direct target of miR-183 in BC. The expression of canonical Wnt signaling target genes c-Myc and Cyclin-D1 was also determined by Western blot analyses. In both cell lines, miR-183 enhanced their expression, whereas miR-183- was repressed (Figure 3E and F). These results suggest that miR-183 modulates Wnt signaling activity through suppressing AXIN2 in BC.

The Effect of miR-183 on Cell Growth and Apoptosis

To understand the functional significance of miR-183 upregulation in BC cells, we manipulated miR-183 expression by transfection of miR-183 or miR-183-in again. Since T24 cell has the highest miR-183 expression among the detected cell lines, all the following studies were conducted in this cell line. Cell viability assay indicated that overexpression of miR-183 promoted cellular growth activity (Figure 2A). However, transfection of miR-183-in attenuated cell growth (Figure 2A). Importantly, flow cytometry analysis results showed that overexpression of miR-183 decreased apoptotic potential (Figure 2B and C). In contrast, inhibition of miR-183 promoted apoptosis. These results are in accordance with the cell viability assay data. Taken

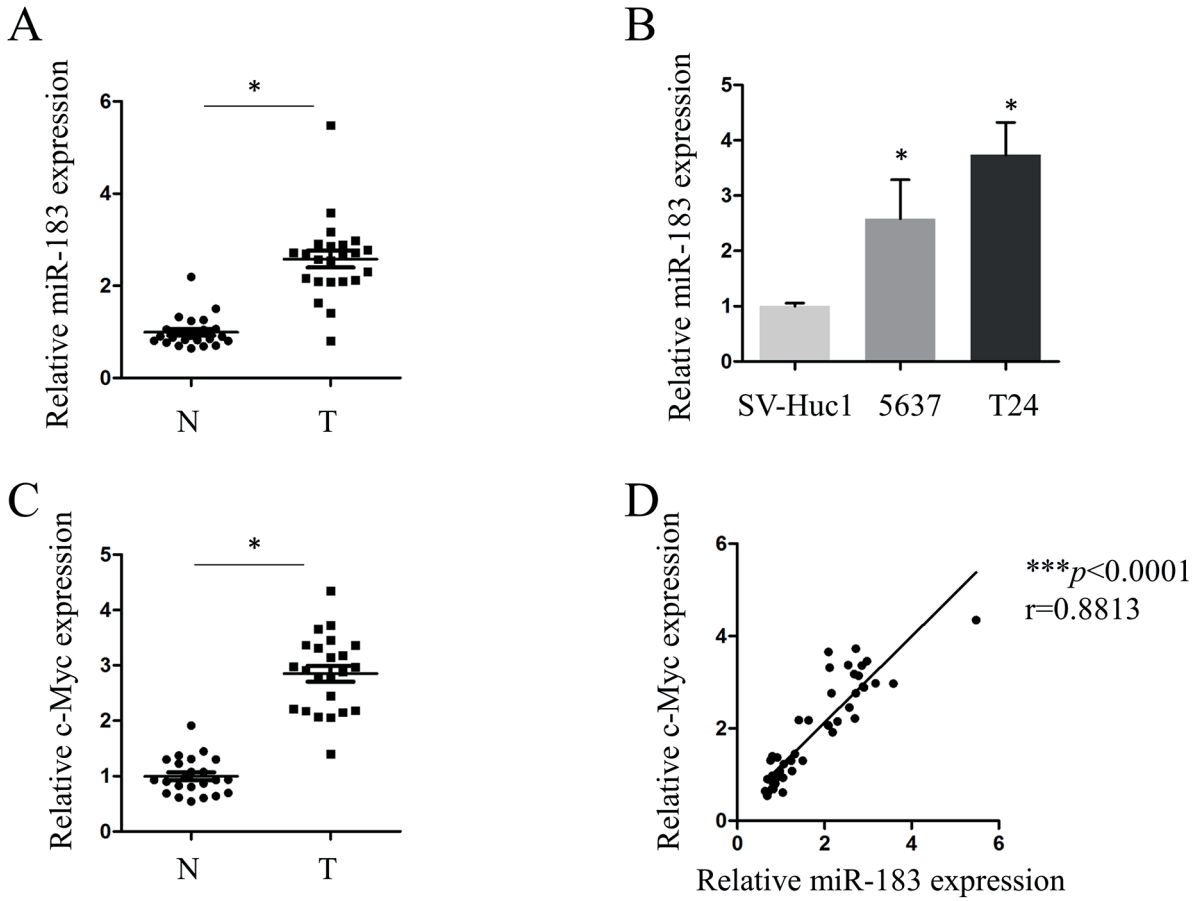

Figure 1. MiR-183 expression in BC tissues and cell lines and its correlation with Wnt signaling activity. ***(A)*** The expression of miR-183 in BC tumor tissues (T) and normal adjacent tissues (N). ***(B)*** The expression of miR-183 in normal bladder epithelial cell SVC-Huc1 and cancer cell lines 5637 and T24. ***(C)*** The expression of c-Myc in BC tumor tissues and normal adjacent tissues. ***(D)*** The correlation analysis of the expressions of c-Myc and miR-183. $^{*}p$ <0.05 vs. N in (A) and ***(C)***, $^{*}p$ < 0.05 vs. SV-Huc1 in ***(B)***.

together, we confirm that miR-183 serves as an oncomiR in BC.

The Effect of AXIN2 Inhibition on miR-183 Mediated Cellular Processes

Finally, we tested whether the effect of miR-183 on cell growth and apoptosis was mediated by AXIN2 inhibition. We employed small interfering RNA of AXIN2 (si-AXIN2) to knockdown its expression. Attenuation of cell growth caused by miR-183 inhibition was abolished by si-AXIN2 transfection (Figure 4A). Consistent with this result, apoptosis induced by miR-183-in was also abolished by AXIN2 knockdown (Figure 4B and C). These data strongly support that miR-183 regulates cell growth and apoptosis through suppression of AXIN2. Thus, AXIN2 serves as a functional target of miR-183 in BC cells.

Discussion

The current understanding on the molecular pathogenesis of BC is still far from clear. In the present work, we have focused our attention on microRNA-based regulatory mechanisms. We have identified miR-183 as a novel regulator of cellular oncogenicity in BC cells by regulating the canonical Wnt signaling. By comparing the expression of miR-183 in BC tissues and normal adjacent tissue, we showed that it is significantly upregulated in tumor site, suggesting a tumor-promoting role. Importantly, the expression of Wnt target gene, c-Myc, is positively correlated with miR-183 expression in BC cells, suggesting that miR-183 may be a critical regulator of canonical Wnt signaling. Overexpression of miR-183 promoted cell growth activity and inhibited apoptosis, and inhibition of miR-183 has

an opposite effect. We further showed that the tumor suppressor AXIN2 is a direct target of miR-183. Through targeting AXIN2, miR-183 is able to maintain a high Wnt signaling activity, thereby promoting cellular malignancy of BC cells. Our study may provide a clue for microRNA-based therapy for BC.

In recent years, microRNAs have emerged as critical regulators in multiple aspects of tumor biology of BC cells by targeting thousands of targets[3,6,10]. Thanks to the development of the next generation sequencing technique, many microRNAs have been identified to be differentially expressed in BC tissues, which perhaps indicated their important functional significance. Intriguingly, many microRNAs tend to have the potential to target a set of genes that have a similar tumor suppressive/promoting function. For example, miR-21 has been identified to be upregulated in BC and target a batch of tumor suppressors including PDCD4, PTEN and TP53[12,13]; upregulation of miR-23a/b in BC triggers subsequent repression of downstream tumor promoting genes such as MAPK1, FGFR3, EGFR and ZEB[13 14,15]. Previous studies have shown that the miR-183 cluster plays an oncogenic role in various cancer types such as breast cancer, tongue squamous cell carcinoma, and endometrial cancer[16-18]. However, in some other cancer types such as non-small cell lung cancer, renal cancer and osteosarcoma, the miR-183 family is reported to be a tumor suppressor[19-22], which differs from the above-mentioned reports. Several recent transcriptome analyses have shown that miR-183 is upregulated in both in the tumor tissue but also in the circulating serum[10, 11,] indicative of its tumor suppressive function. To test our hypothesis that miR-183 regulates cellular malignancy of BC cells, we employed experiments using gain and loss of function techniques. To our expectation, miR-183 indeed exerted a promoting role in cell growth. Importantly, cell apoptosis was significantly inhibited by miR-183, suggesting that miR-183 has pleiotropic effects. Our study firstly addressed the oncogenic role of miR-183 in BC cells.

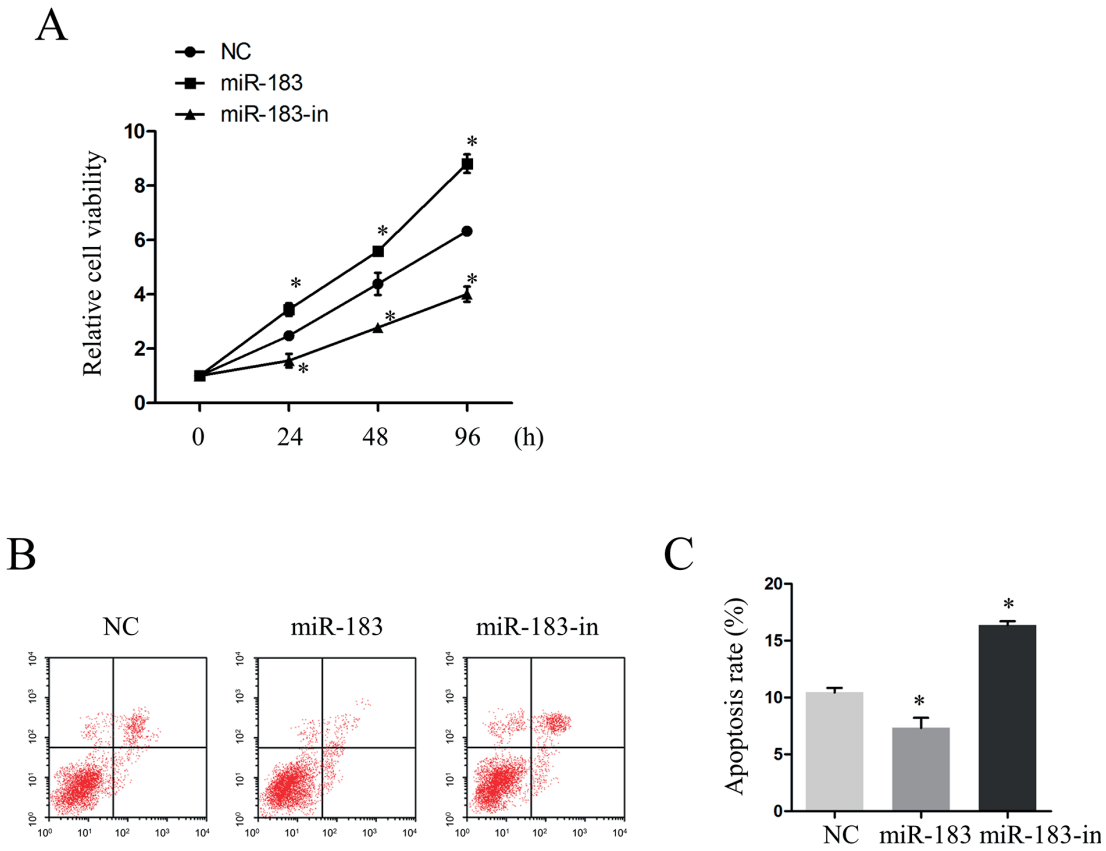

Figure 2. Validation of AXIN2 as a direct target of miR-183. ***(A)*** The effect of miR-183 and miR-183-in on cell proliferation as determined by MTT assay. ***(B)*** The effect of miR-183 and miR-183-in on cell apoptosis as determined by flow cytometry assay. ***(C)*** The statistical analysis of the apoptosis determined by flow cytometry assay. $^{*}p<0.05$ vs. NC. NC, negative control.

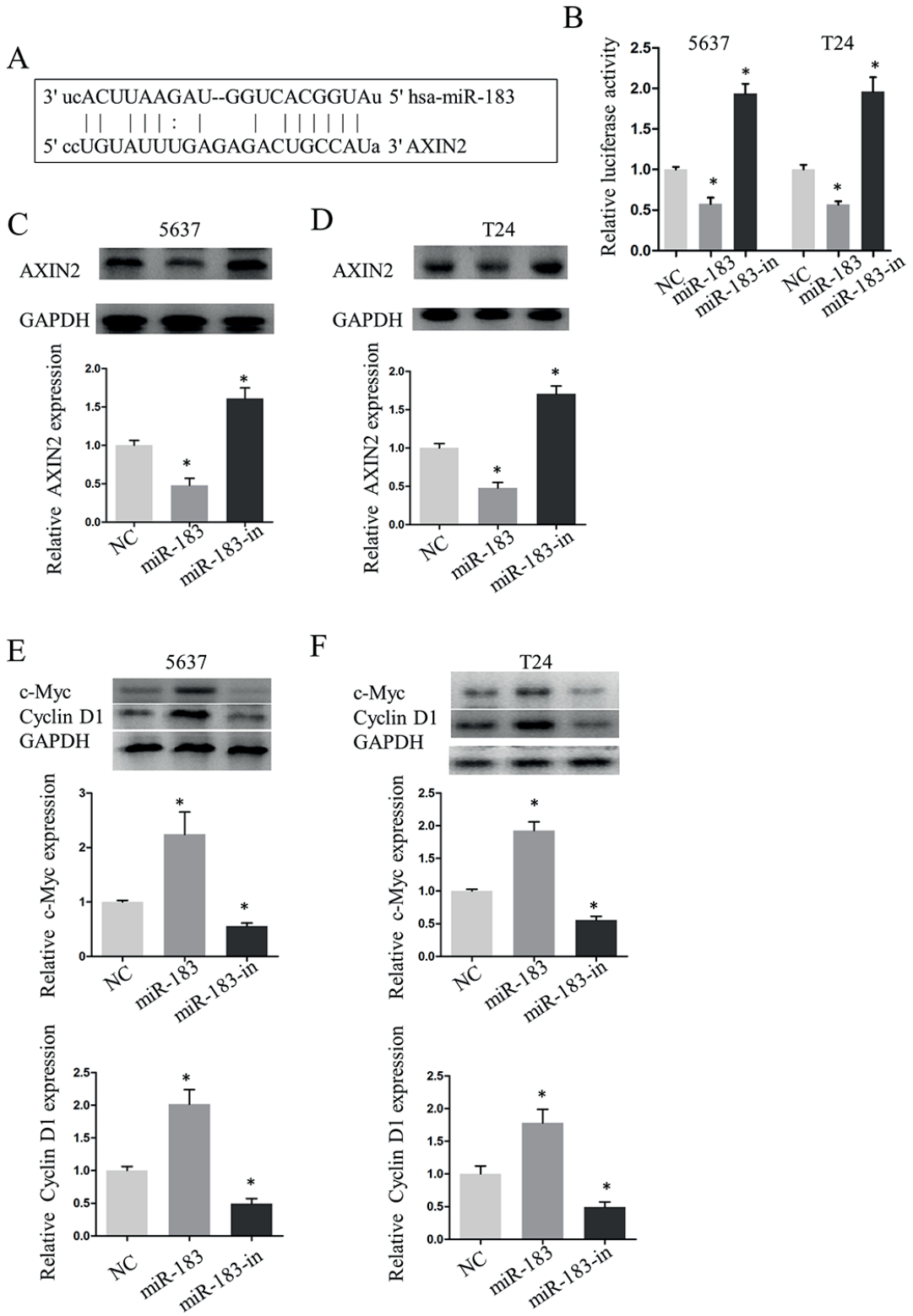

Figure 3. The effect of miR-183 on cell growth and apoptosis. ***(A)*** The schematic diagram showing the binding of miR-183 and AXIN2. ***(B)*** The relative luciferase activity after miR-183 or miR-183 inhibitor (miR-183-in) transfection. ***(C)*** The expression of AXIN2 after miR-183 or miR-183-in transfection in 5637 cell. ***(D)*** The expression of AXIN2 after miR-183 or miR-183-in transfection in T24 cell. ***(E)*** The expression of c-Myc and Cyclin-D1 after miR-183 or miR-183-in transfection in 5637 cell. ***(F)*** The expression of c-Myc and Cyclin-D1 after miR-183 or miR-183-in transfection in T24 cell. $^{*}p < 0.05$ vs. NC. NC, negative control.

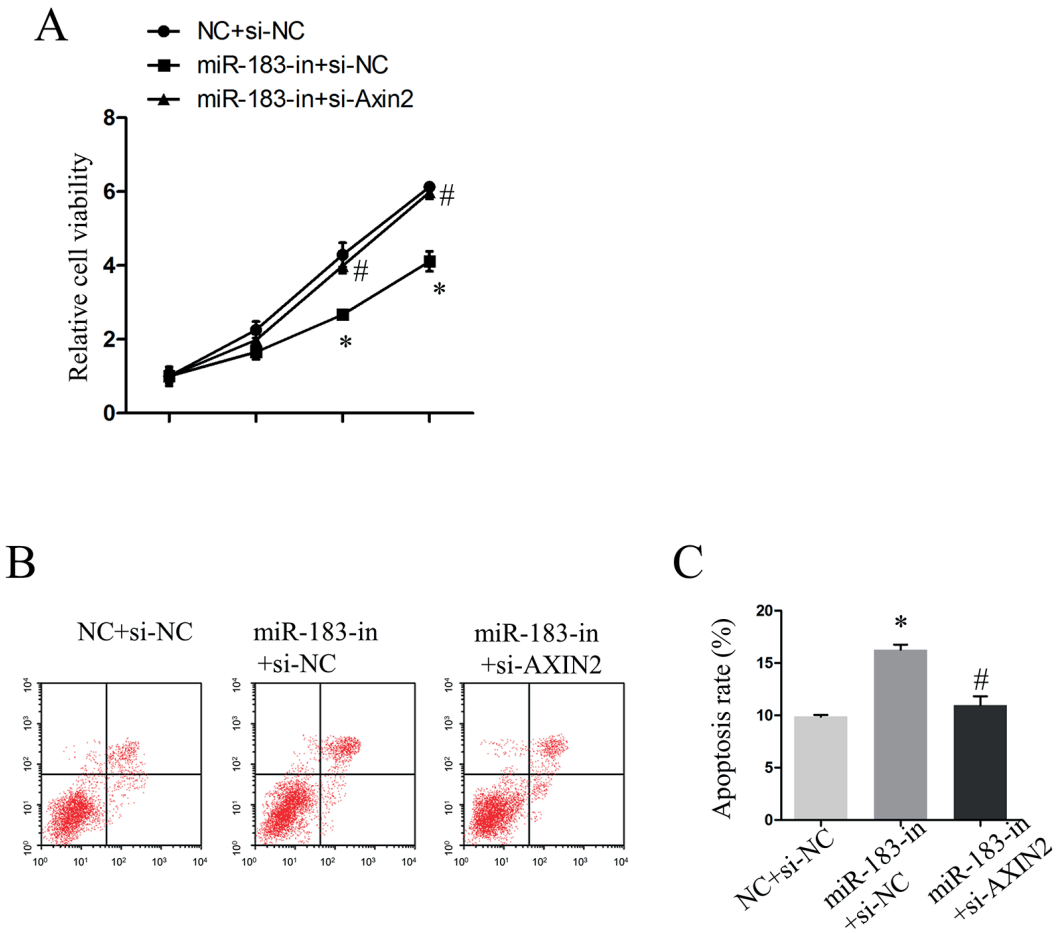

Figure 4. The effect of AXIN2 inhibition on miR-183 mediated cellular processes. ***(A)*** The effect of miR-183-in and miR-183-in + si-AXIN2 on cell proliferation as determined by MTT assay. ***(B)*** The effect of miR-183-in and miR-183-in + si-AXIN2 on cell apoptosis as determined by flowcytometry assay. ***(C)*** The statistical analysis of the apoptosis determined by flow cytometry assay. $^{*}p<0.05$ vs. NC+si-NC, $^{\#}p<0.05$ vs.miR-183-in+si-NC. NC, negative control.

In identifying the molecular mechanism of the tumor promoting action of miR-183, we were more focusing on Wnt signaling pathway, because it has been recently recognized as a critical pathway for BC development and progression[23]. The canonical Wnt pathway target gene c-Myc can be a reliable readout of Wnt signaling activity. The present results showed that miR-183 expression is positively correlated with c-Myc expression, which suggested its critical function in modulating Wnt signaling activity. We later identified that the 3'UTR of AXIN2 possesses a potential binding site for miR-183. This interaction was indeed confirmed by luciferase activity assay. AXIN2 is a critical component of APC complex for degradation of β-catenin, which negatively regulates Wnt signaling activity. AXIN2 is a well-established tumor suppressor in many cancer types[24]. Our results therefore provided compelling evidence that miR-183 may function as a tumor promoting microRNA that directly suppresses AXIN2 to upregulate Wnt signaling activity. Corroborating with our study, Wnt/catenin β1 expression showed a same trend as that of miR-183, and they may serve as a prognostic marker for colorectal cancer[25]. Intriguingly, given the recent reports showing the positive regulation of miR-183 expression by Wnt/β-Catenin signaling in hepatocellular carcinoma[26], it is presumable that a positive feedback circuit of miR-183-AXIN2- Wnt/β-Catenin signaling may exist in the development and progression of BC. In prostate cancer, miR-183 also functions as an oncogene, the effect of which is through

targeting the Wnt inhibitory protein, DKK-3 and SMAD4[27]. Thus, AXIN2 may not be the only target of miR-183 that mediates the hyperactivation of Wnt signaling in BC. Alternatively, it may regulate a set of genes that directly or indirectly modulate Wnt signaling. Nonetheless, miR-183-AXIN2 interaction may partially confer its effects on cell growth and apoptosis, since silencing AXIN2 can partially reverse the effects of miR-183 inhibitor.

Conclusions

We identified a novel microRNA regulator of Wnt signaling activity in BC. Upregulation of miR-183 in BC cells confers the hyperactivity of Wnt signaling, which permits a high cell proliferation rate and a low level of apoptosis. These effects may be partially mediated by the direct miR-183-AXIN2 interaction. Our study may provide a clue for miR-183-based therapy for BC.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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