Original Article
MiR-182 promotes cell proliferation, migration and invasion by targeting FoxF2 in endometrial carcinoma cells

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Abstract: miR-182 has been reported to be up-regulated in many tumors, including endometrial carcinoma (EC). However, effects of miR-182 on the development of EC and possible molecular mechanisms have not been fully reported. The purpose of this study was to investigate the function of miR-182 and its potential mechanism in EC cell line RL95-2. At first, the expression of miR-182 in RL95-2 cells was inhibited using miR-182 inhibitor and detected by qRT-PCR. The expression of its target FoxF2 was also changed using lentiviral-mediated over-expression vector or interfering RNA vector. Cell migration and invasion, cell cycle progress and colony formation of RL95-2 cells were examined and statistically analyzed after the inhibition of miR-182 and/or altered expression of FoxF2. The inhibition of miR-182 significantly increased the expression of FoxF2, decreased the numbers of migrated and invaded cells, as well as the number of colonized cells, induced cell cycle arrest to the G1 phase, and suppressed epithelial-mesenchymal transition. Lentiviral-mediated over-expression of FoxF2 showed similar functions to miR-182 inhibition, whereas lentiviral-mediated down-regulation of FoxF2 displayed the opposite effects. The effects of miR-182 inhibitor on cell proliferation, migration, and invasion were reversed when the expression of both miR-182 and FoxF2 were suppressed. Our findings indicate miR-182 acts as an oncogenic miRNA and promotes cell proliferation, migration, and invasion by targeting FoxF2 in EC.

Keywords: Endometrial carcinoma, miR-182, FoxF2, migration, invasion, proliferation

Introduction

Endometrial carcinoma (EC) is one of the three most common malignant tumors in female reproductive system, and the morbidity is increasing in recent years [1, 2]. Surgery is the main strategy for this disease at present while there are also some adjuvant strategies, including radiotherapy and chemotherapy. The therapeutic effect is efficient just in early-stage EC and barely satisfactory in advanced, recurrent, or metastatic EC [3, 4]. In particular, patients with recurrent or metastatic EC usually have extremely poor prognosis. Additionally the pathogenesis of EC is still not completely understood [3]. Therefore, the identification of new potential biomarkers for the early diagnoses and prognoses of this disease and the discovery of additional cellular and molecular mechanisms for the oncogenesis of EC are critical for the establishment of novel effective treatment regimens.

MicroRNAs (miRNAs) are non-coding and single-chain small RNAs, comprised of approximately 22 nucleotides and they can regulate target gene expression by translational repression and transcript cleavage [5]. miRNA function is involved in a wide variety of functions, including cell proliferation, apoptosis, migration, invasion, and epithelial-mesenchymal transition (EMT). Growing evidence has verified that the dysregulation of miRNA expression is closely related to carcinogenesis [4, 6, 7]. miR-182, one member of the miR-182-96-182 cluster, has two alternative mature subtypes, including miR-182-3p and miR-182-5p, and has been shown to be dysregulated in many tumors, including prostate cancer, breast cancer, and ovarian cancer [8-10]. For example, the expres-

sion of miR-182 was at a high level in endometrial carcinoma cell lines and endometrial carcinoma tissues [11, 12], indicating that miR-182 may be implicated in the pathogenesis of EC. However, the function and molecular mechanisms of miR-182 in EC have not been completely investigated.

The capacities of tumor cell migration and invasion directly affect tumor metastasis to distant tissues or organs. For the epithelial cancer, epithelial cells become migratory mesenchymal cells through the activation of EMT, resulting in acquisition ofmigration and invasion ability [13]. The mesenchymal regulator FoxF2 belongs to the transcription factor forkhead box (Fox) superfamily and can regulate the EMT program to maintain tissue homeostasis [14]. Some studies showed that the expression of FoxF2 was down-regulated and acted as a potential tumor suppressor to inhibit EMT and metastasis in breast cancer and prostate cancer [8, 15]. Additionally, it has been verified that FoxF2 is a direct target of miR-182 and that miR-182 can promote development of tumors by down-regulating the expression of FoxF2 [8, 9]. Even so, whether miR-182 exerts its function in EC cell through targeting FoxF2 is barely reported so far.

In the present study, we investigated the function of miR-182 on cell cycle progress, colony formation, migration, invasion and EMT program in EC cell line by suppressing the expression of miR-182 or changing the expression of its target gene FoxF2. We demonstrated that the down-regulation of miR-182 could suppress the capacities of colony formation, cell migration, and invasion, the activation of EMT through up-regulating the expression FoxF2 in RL95-2 cells, and inducing cell cycle arrest to G1 phase.

Materials and methods

Cell culture

The human endometrial carcinoma RL95-2 cell line used in the present study was purchased from Typical Culture Preservation Commission Cell Bank, Chinese Academy of Sciences (Shanghai, China), and the RL95-2 cells were cultured in Gibco Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) (ThermoFisher Scientific, Waltham, MA, USA) supplemented with 1.5 g/L sodium bicarbon-

ate (Sigma-Aldrich, St Louis, MO, USA) and 10% fetal bovine serum (FBS) (Hyclone, Logan, USA). The cells were incubated in a humidified incubator at 37°C with 95% humidified air and 5% CO_2. Subculture of RL95-2 cells was conducted when the cultured cells reached 90% confluence.

Inhibition of miR-182 and lentiviral infection-mediated changes of FoxF2 expression

RL95-2 cells were transfected with a miR-182 inhibitor (ThermoFisher Scientific, Waltham, MA, USA) for which the target sequence was 5'-UGGUUCUAGACUUGCCAACUA-3' or a negative control (miR-NC) according to the manufacturer's instructions. To alter the expression of FoxF2 in RL95-2 cells, RL95-2 cells were infected with the lentiviral-mediated FoxF2-overexpression vector (Lv-expFoxF2 vector) or lentiviral-mediated FoxF2-interfering RNA vectors (Lv-shFoxF2 vectors). During the establishment of lentiviral-mediated FoxF2-overexpression vector, the coding region that expressed FoxF2 protein was amplified by quantitative polymerase chain reaction (PCR) and then the products were inserted in pCDH-EF1-CopGFP-T2A-Puro plasmid (Addgene, Cambridge, MA, USA). During the establishment of Lv-shFoxF2 vectors, three independent short hairpin RNA (shRNA) targeting the coding sequence region of human *FoxF2* gene 5'-GCATCACTCTACTC-CAGTGTG-3' (shFoxF2-1), 5'-GCGGGTCGTATTA-TCACCATC-3' (shFoxF2-2) and 5'-GCGTCTGTC-AGGATATTAAGC-3' (shFoxF2-3) were used and inserted in interfering plasmids pSicoR (Addgene, USA). An empty lentiviral vector (Lv-exp-NC) and a non-targeting shRNA (Lv-shNC) were used as negative controls for Lv-expFoxF2 vector and Lv-shFoxF2 vector, respectively. Then RL-95 cells were infected with lentiviral particles; and the medium mixed with lentiviral particles was replaced with new complete medium the next day. Next the transfected RL95-2 cells were continually cultured for 48 h and monitored under a fluorescence microscope. The efficiency on the expression of FoxF2 was evaluated by quantitative real-time PCR and western blotting.

Quantitative real-time PCR (qRT-PCR)

RNA (total RNA and micro RNA) were exacted from RL95-2 cells using the TriZol reagent (Invitrogen, Garlsbad, CA, USA). The miR-182

expression was detected using the TaqMan microRNA assay kit (Applied Biosystems, Foster City, USA). U6 was used as an internal control to normalize the relative expression of miR-182. For the detection of mRNA expression, total RNA was reverse transcribed to generate cDNA using M-MLV Reverse Transcriptase (Invitrogen) and qRT-PCR was performed using SYBR Green qPCR Master Mix (ThermoFisher Scientific). GAPDH was used as an internal control to normalize the relative expression of FoxF2. The primer sequences for qRT-PCR were as follows: miR-182: forward: 5'-CTCA-ACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCT-CACACT-3', reverse: 5'-GGGTTTGGCAATGGTAG-AA-3'; U6: forward: 5'-CTCGCTTCGGCAGCAC A-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'; FoxF2: forward: 5'-GGATATTAAGCCCTGCGTCA-3', reverse: 5'-TTCAGATTGGGGAACGCTAC-3'; GAPDH: forward: 5'-CCCACTAACATCAAATGG-GG-3', reverse: 5'-CCTTCCACAATGCCAAAGTT-3'. The relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

The RL95-2 cells in different groups were collected and total protein lysates were prepared using RIPA lysis buffer (Beyotime Biotechnology Co., Ltd., Shanghai, China). After protein concentration determination, 20 μg protein was separated by SDS-PAGE and transferred to PVDF membranes (Invitrogen). The membranes were blocked in 5% skim milk for 1.5 h at room temperature and then incubated with the primary antibody dilute solution for FoxF2 (1:2000, Abcam, Cambridge, MA), E-cadherin (1:1000, Abcam), Vimentin (1:2000, Abcam) and GAPDH (1:1000, Cell Signaling Technology, Beverly, MA) at 4°C overnight. The membranes were washed with TBST solution for three times and then incubated with HRP-conjugated secondary anti-rabbit antibody (1:10000, Cell signaling technology) for 1.5 h at room temperature. After the washing for three times, protein bands were detected using the chemiluminescent substrate reagents (Millipore, Massachusetts, USA) and imaged using automatic chemiluminescence analyzer (Tanon, Shanghai, China).

Cell migration and invasion assay

Cell migration and invasion capacities of RL95-2 cells in different groups were detected using non-Matrigel-coated and Matrigel-coated

Transwell inserts (BD Bioscience, San Diego, USA) as described by Luan et al [16]. For the cell migration assay, 500 μL serum-free medium containing 3×10^4 cells from different groups was placed into the upper chamber, while 700 μL cell-free medium containing 20% FBS was added into the bottom chamber. Then the RL95-2 cells were cultured for 48 h. Non-migrative cells on the upper surface were removed with a swab, whereas the cells on the lower surface were fixed in 4% paraformaldehyde for 10 min and stained in a 0.5% crystal violet solution for 30 min. Then the cells in five fields were counted under an inverted microscope. Cell invasion assay was also performed as described above, and the only difference from the cell migration assay was that the upper chambers were covered with 80 μL Matrigel dilution before adding the serum-free medium.

Colony formation assay

RL95-2 cells in different groups were collected and seeded into 6-well plates at a density of 500 cells per well, three reduplicative wells per group. Then the cells were cultured at 37°C in a humidified incubator with 5% CO_2 for two continual weeks and the culture medium was replaced every three days. At the end of experiments, the cells in plates were fixed in 4% paraformaldehyde and stained in a 0.5% crystal violet solution. Then the number of colonies that were a cluster of more than ten cells was counted under an inverted microscope. The experiments were repeated three times.

Cell cycle assay

RL95-2 cells in different groups were digested using 0.25% Trypsin solution to prepare single cell suspension and collected into 15 mL centrifuge tube. After centrifugation, 300 μL PBS containing 10% FBS was added to prepare single cell suspension and 700 μL ethyl alcohol was added into single cell suspension dropwise to fix the cells for 24 h. Then the cells were digested with 1 mg/mL RNase A solution at 37°C and next stained with 50 μg/mL propidium iodide solution (Beyotime Biotechnology Co., Ltd., Shanghai, China) for 10 min. Flow cytometry was used to detect fluorescent signal and the data were analyzed by Beckman Fc500 Mcl software to assess the cell number in G1, S and G2 phases of the cycle.

Statistical analysis

One-way analysis of variance and Student's *t*-test were used for statistical analysis and performed using IBM SPSS statistics 22.0 software. The data were presented as mean ± SD from at least three independent experiments and graphically displayed using GraphPad Prism 5. *P*<0.05 was considered significant.

Results

Down-regulation of miR-182 inhibited cell migration and invasion of RL95-2 cells

In order to investigate the effects of miR-182 on endometrial carcinoma RL95-2 cells, the miR-182 inhibitor was adopted to suppress the expression of miR-182. The result of qRT-PCR has verified that the expression of miR-182 was significantly down-regulated when endometrial carcinoma RL95-2 cells were treated with miR-182 inhibitor (**Figure 1A**). Then the effects of miR-182 on the cell invasion and metastasis capacity of RL95-2 cells were evaluated. As shown in **Figure 1B** and **1C**, cell density and the number of migration and invasion of cells significantly decreased in the miR-182 inhibitor group (*P*<0.05), while there was no significant difference between the control group and the miR-NC group. There data indicated that miR-182 had positive effects on migration and invasion capacities.

Down-regulation of miR-182 suppressed colony formation and cell proliferation in RL95-2 cells

Similarly, colony formation assay in vitro after crystal violet staining showed a significant decrease of colony number in miR-182 inhibitor group, suggesting that cell proliferation was suppressed after the treatment with miR-182 inhibitor (**Figure 1D**). In addition, we detected the cell cycle of RL95-2 cells in different groups. Statistical data displayed that the percentage of cells in G1 phase was significant higher (*P*<0.05) than that in the control group and the miR-NC group, whereas the percentage of cells in S phase was significantly lower (*P*<0.05) (**Figure 1E**), which indicated that RL95-2 cells were induced cell cycle arrest to G1 phase after the treatment with miR-182 inhibitor. Thus, these data demonstrated that miR-182 promoted cell proliferation of RL95-2 cells, and

down-regulation of miR-182 would lead to cell cycle arrest to G1 phase.

Down-regulation of miR-182 increased the expression of FoxF2

To investigate the effects of miR-182 on the expression of target gene FoxF2 in RL95-2 cells, the mRNA and protein expression of FoxF2 were evaluated by qRT-PCR and western blotting. The results showed that mRNA expression of FoxF2 significantly increased when RL95-2 cells were treated with miR-182 inhibitor (**Figure 2A**). Compared with the control group and miR-NC group, the FoxF2 protein level in the miR-182 inhibitor group was at a higher level, which the quantitative analysis of density testified that the differences were significant (*P*<0.05) (**Figure 2B** and **2C**). So it could be concluded that inhibition of miR-182 could raise the expression of FoxF2.

The expression of FoxF2 was altered by Lv-expFoxF2 vector or Lv-shFoxF2 vector
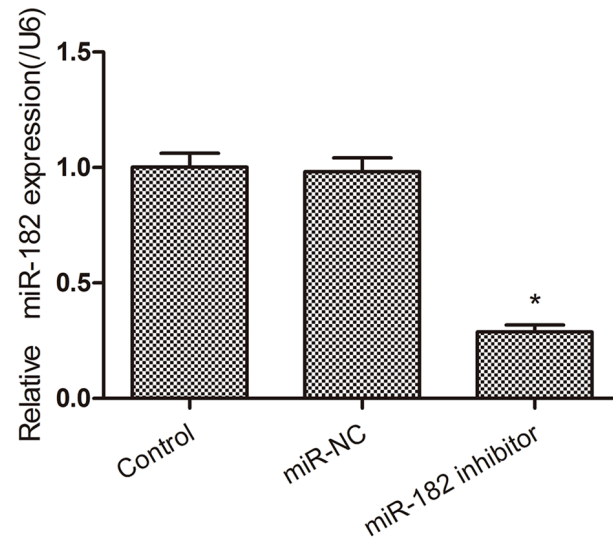
In order to further assess whether the effects of miR-182 was dependent on the expression of FoxF2, the over-expression lentiviral vector (Lv-expFoxF2) and short hairpin RNA (shRNA) lentiviral vector (Lv-shFoxF2) of FoxF2 were constructed and packaged. The effects of Lv-expFoxF2 vector and Lv-shFoxF2 vector on the expression of FoxF2 were detected. As shown in **Figure 2D-I**, the mRNA and protein expressions of FoxF2 were up-regulated when RL95-2 cells were transfected with Lv-expFoxF2 and in contrast, the expression of FoxF2 was down-regulated after transfection with Lv-shFoxF2. Among the three Lv-shFoxF2 vectors, FoxF2 mRNA and protein levels were the lowest after the treatment with Lv-shFoxF2-1 vector, indicating the inhibitory effect of Lv-shFoxF2-1 was the best. Therefore, Lv-shFoxF2-1 vector was used as FoxF2 inhibitor in the following experiments.

The effects of miR-182 on colony formation and cell proliferation in RL95-2 cells are mediated by targeting FoxF2
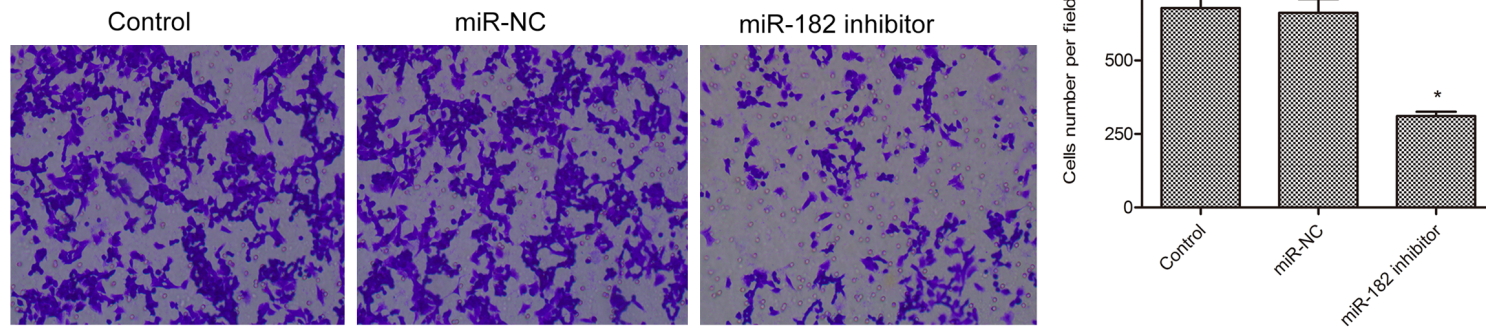
The mechanisms of the effects of miR-182 in RL95-2 cells were further investigated by miR-182 inhibition and FoxF2 over-expression or down-regulation. As shown in **Figure 3A**, compared to the control group, the density of colony
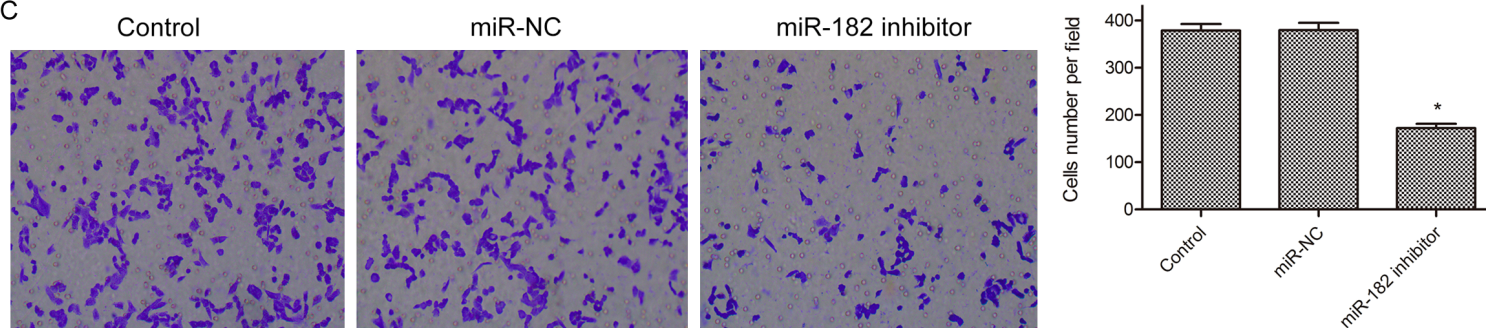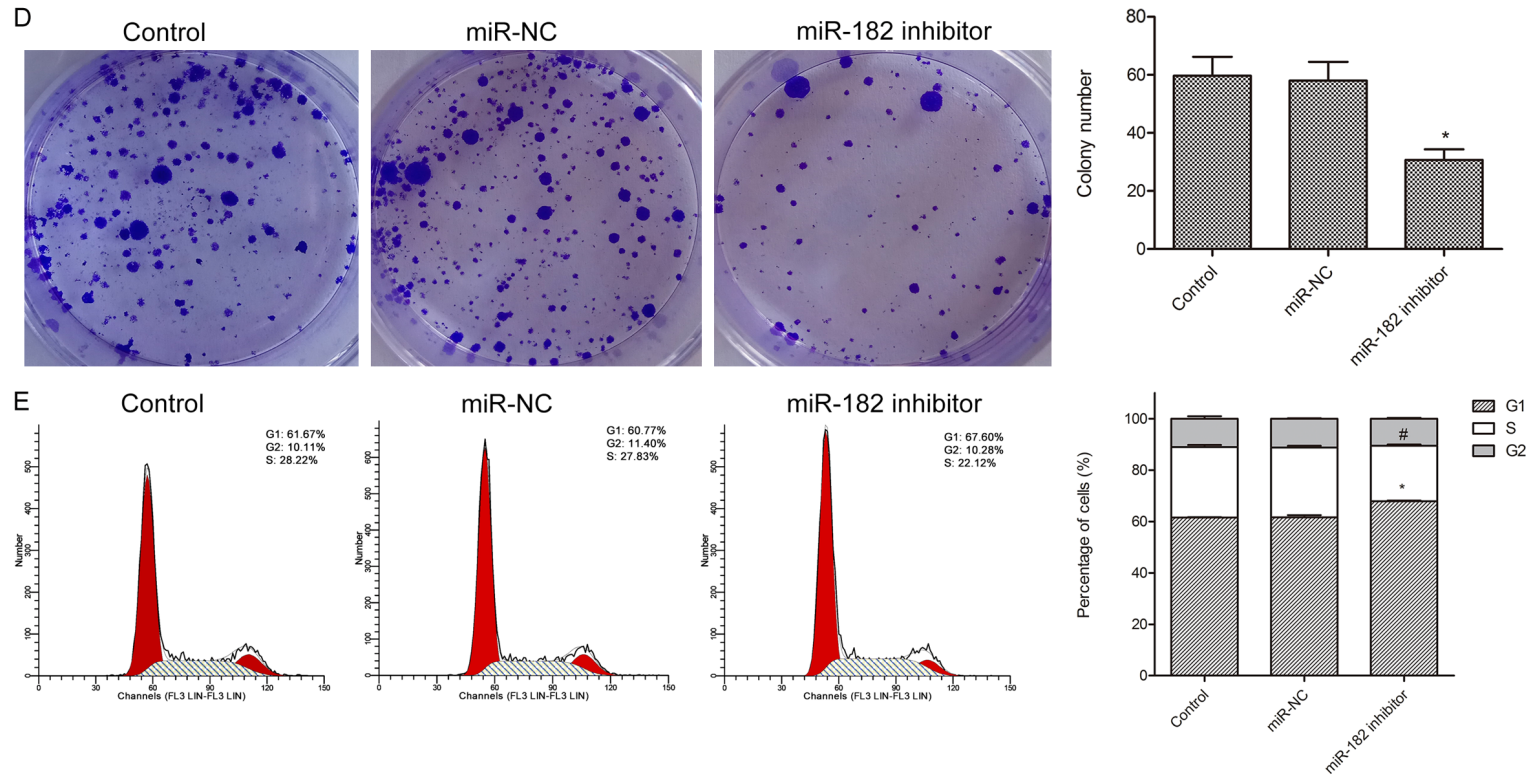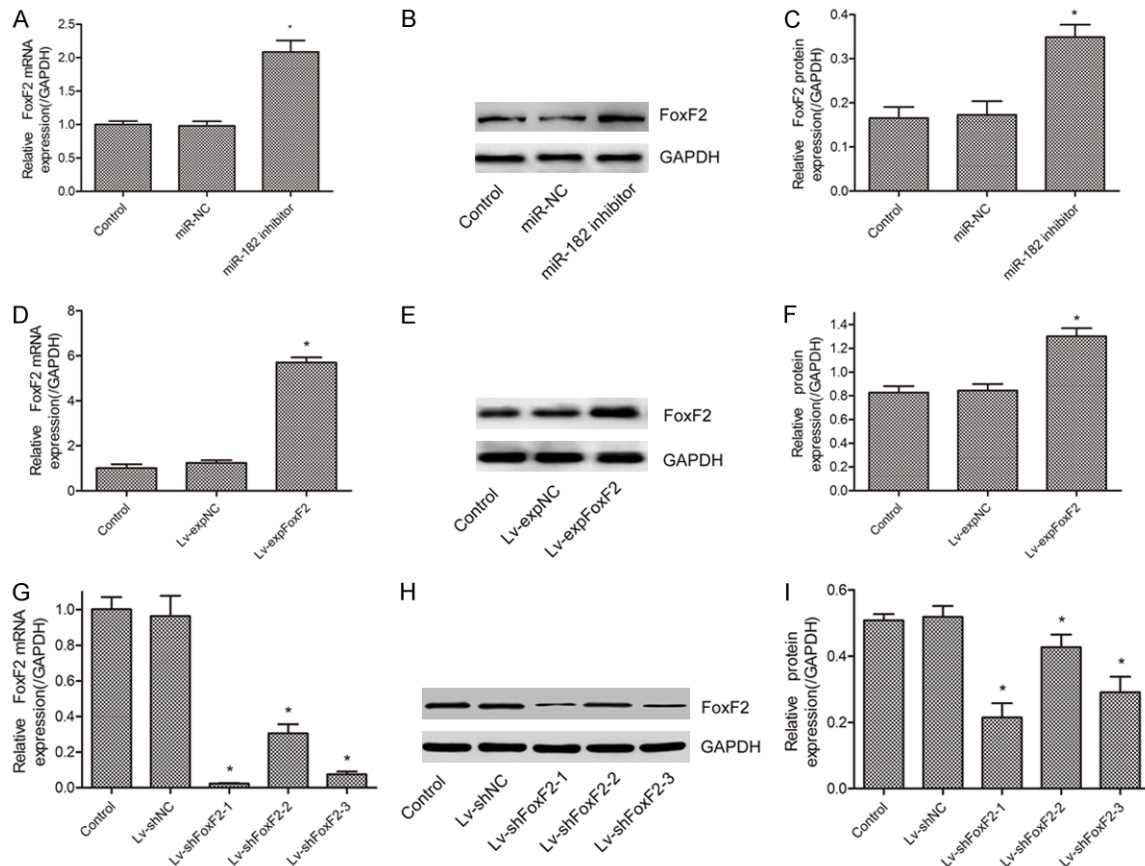
Effects of miR-182 by targeting FoxF2 in EC

A

B

C

Figure 1. Expression of miR-182 and its effects on migration, invasion, colony formation and cell cycle progress. The result of qRT-PCR (A) showed a significant decrease of miR-182 expression. The capacities of migration (B), invasion (C), and colony formation (D) were detected and quantitatively analyzed, showing a decrease of these capacities after miR-182 inhibition. The cell cycle progress analysis (E) was performed by flow cytometry and displayed the increased percentage of RL95-2 cells in G1 phase. The data are expressed as mean ± SD for three independent experiments. Compared with the control group or the percentage of control cells in G1 phase, *P<0.05. Compared with the percentage of control cells in S phase, #P<0.05.

Figure 2. Expression of FoxF2 after different treatments. The mRNA and protein expression of miR-182 were measured by qRT-PCR (A) and western blot (B) and the protein expression was quantitatively analyzed (C) after the treatment with miR-182 inhibitor. The expression of FoxF2 was assessed (D and E) and quantitatively analyzed (F) after transfection with lentiviral-mediated overexpression of FoxF2 vector (D-F) or interfering RNA vectors (G-I). The data are expressed as mean ± sd for three independent experiments. Compared with control group, *$P<0.05$.

cells was obviously sparse when RL95-2 cells were transfected with miR-182 inhibitor or Lv-expFoxF2 vector alone, while it was intense when the cells were transfected with Lv-shFoxF2-1 vector. The quantitative analysis of colony number also verified these significant differences (**Figure 3B**). Furthermore, compared to the miR-182 inhibitor group, the density and number of colony cells were restored and even increased after the combined utilization of miR-182 inhibitor and Lv-shFoxF2-1 vector. The results of cell cycle also demonstrated that miR-182 inhibition could suppress cell cycle progress in RL95-2 cells, and this effect was mimicked by lentiviral-mediated FoxF2 over-expression, which caused a significant increase of the percentage of cells in G1 phase compared to the control group (**Figure 3C** and **3D**). However, the inhibitory effect on cell proliferation induced by miR-182 inhibitor in RL95-2

cells could be reversed in co-transfected cells with Lv-shFoxF2-1 vector. Taken together, these results disclosed that miR-182 motivated colony formation and cell cycle progress by targeting FoxF2.

The effects of miR-182 on cell migration, invasion, and EMT in RL95-2 cells are mediated by targeting FoxF2

The effects of miR-182 on cell migration and invasion were examined after miR-182 inhibition and FoxF2 over-expression or down-regulation. The results displayed that the numbers of both migratory and invasive cells sharply decreased after FoxF2 over-expression and it was the opposite after FoxF2 inhibition, which indicated that FoxF2 exerted an important role in cell migration and invasion (**Figure 4A-D**). What's more, compared to the miR-182 inhibi-
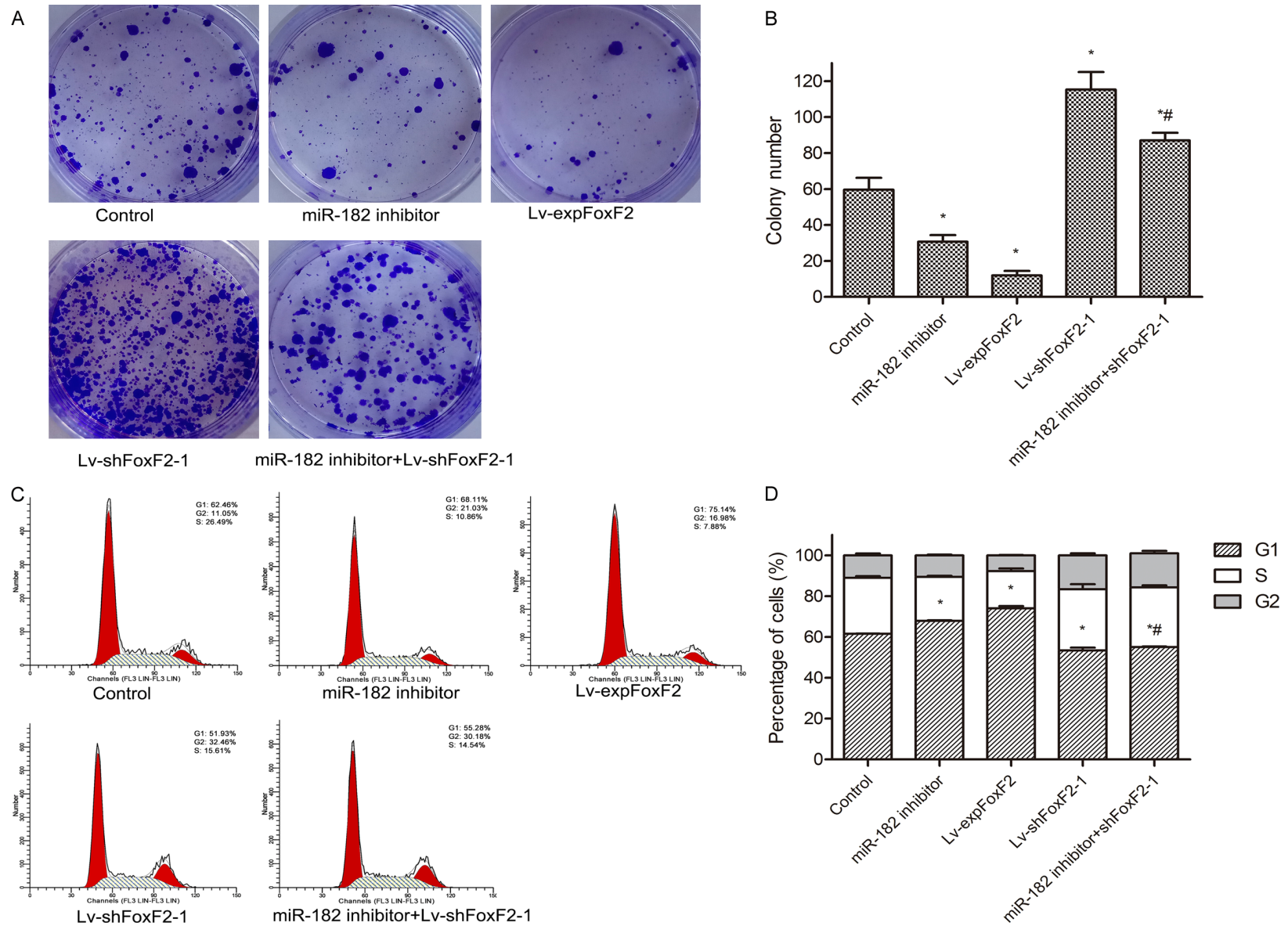
Figure 3. miR-182 promoted the colony formation and cell cycle progress in RL95-2 cells by targeting FoxF2. The colony formation capacity analysis (A and B) showed that down-regulation of miR-182 and the overexpression of FoxF2 inhibited colony formation, and the inhibition of FoxF2 increased colony formation. The cell cycle analysis (C and D) displayed cell cycle arrest after the inhibition of miR-182 or overexpression of FoxF2. The data are expressed as mean ± sd for three independent experiments. Compared with control group, *$P<0.05$. Compared with miR-182 inhibitor group, #$P<0.05$.
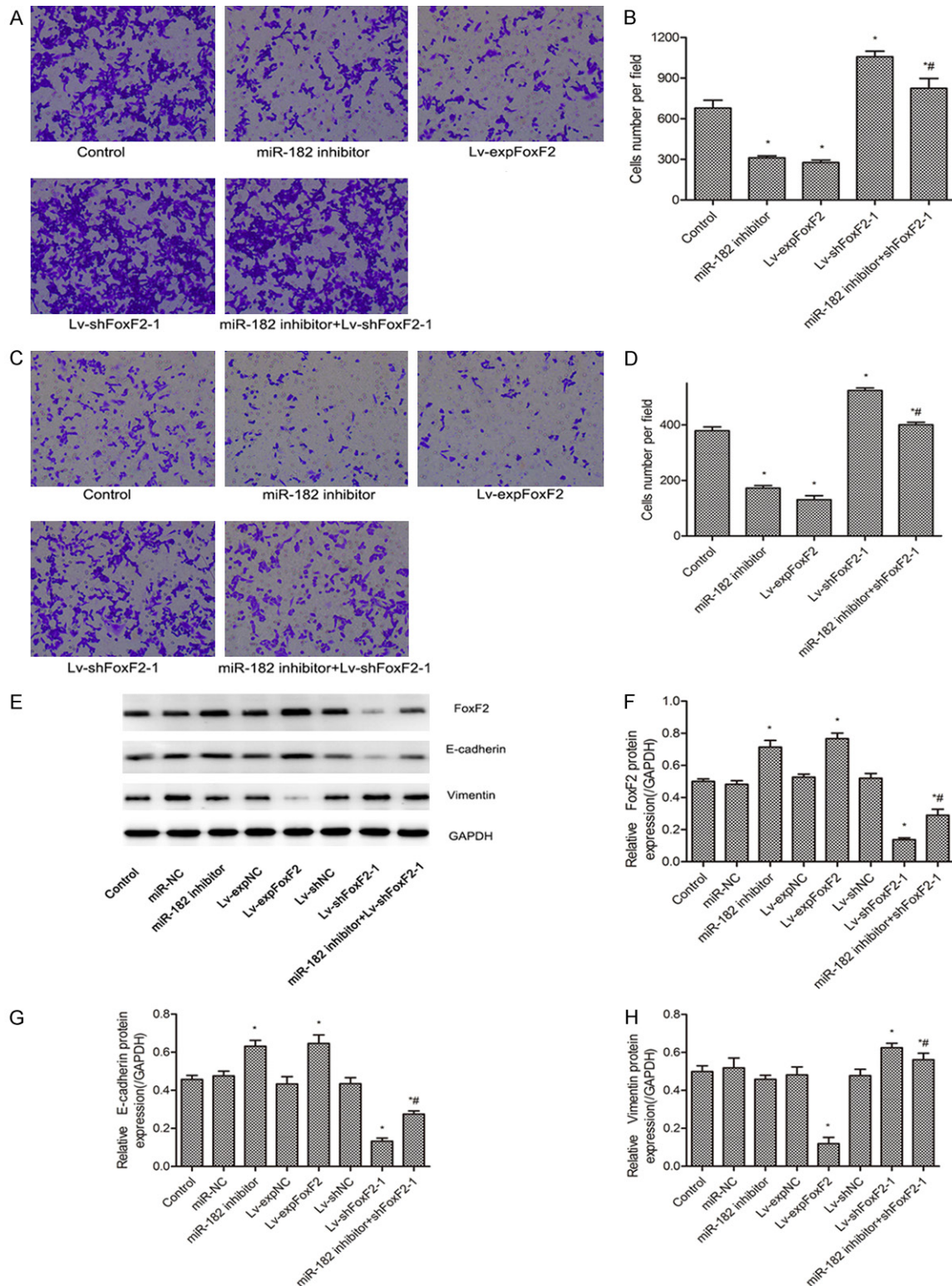
Figure 4. miR-182 promoted migration, invasion, and EMT in RL95-2 cells by targeting FoxF2. Cell migration (A and B) and invasion (C and D) were detected and quantitatively analyzed, showing that miR-182 increased the cell migration and invasion capacities. The expressions of proteins involved in EMT process were detected and quantitatively analyzed (E-H). The data are expressed as mean ± sd for three independent experiments. Compared with control group, *P<0.05. Compared with miR-182 inhibitor group, #P<0.05.

tor group, the density and number of migratory and invasive cells significantly increased when the expression of both miR-182 and FoxF2 were inhibited in RL95-2 cells ($P<0.05$).

In addition, we detected the protein expression levels of FoxF2, E-cadherin and vimentin, which are involved in the EMT and exert significant effects in tumor metastasis. Expectedly, the results showed that the FoxF2 expression increased in the RL95-2 cells transfected with miR-182 inhibition or lentiviral-mediated FoxF2 over-expression vector, whereas FoxF2 expression significantly decreased in cells transfected with Lv-shFoxF2-1 vector (**Figure 4E** and **4F**). Compared to that in control group, the protein level of E-cadherin in cells transfected with miR-182 inhibitor was up-regulated and the expression of E-cadherin was partly restored when the FoxF2 expression were down-regulated by Lv-shFoxF2-1 vector. But the expression of vimentin showed an opposite trend to the expression of E-cadherin (**Figure 4E-H**). These results indicated that miR-182 promoted cell migration, invasion, and EMT by targeting FoxF2.

Discussion

As a malignant tumor in the female reproductive system, endometrial cancer (EC) causes much mortality for women in the world. It usually has a poor prognosis in patients with advanced EC. At present, the potential treatment strategy for EC is focused on targeting EC cells by suppressing key signaling pathways or progress which is involved in tumorigenesis and tumor development [3]. Some studies found that the abnormal expression of miRNAs is closely related with tumorigenesis and tumor development [7, 17-19], including EC. The functions of miRNAs are diverse, including participating in the tumor cell proliferation, migration, invasion and EMT. Thus, the regulation of dysregulated miRNAs could be a good strategy to treat EC.

Dysregulation of cell cycle progression or excessive cell proliferation is one of major hallmarks of tumors. A study reported that miR-182 exerted the oncogenic miRNA properties in EC, including accelerating cell proliferation and colony formation ability [12]. Similarly, in the present study inhibition of miR-182 induced RL95-2 cell cycle arrest into G1 phase; mean-

while, decreasing the migration, invasion and colony formation capacities, suggesting that miR-182 could promote the development of EC. Tumor cell migration and invasion lead to tumor metastasis, which is one of the most serious challenges for the treatment of human cancers [7]. But in epithelial tumors, such as EC, one of the mechanisms of the acquisition of metastatic ability is epithelial-mesenchymal transition (EMT) and during the EMT program, epithelial tumor cells switch into mesenchymal cells due to the loss of epithelial cell polarity and intercellular adhesion, resulting in tumor cell migration and invasion [20, 21].

The transcription factor Forkhead box (Fox) superfamily, has a highly conserved Forkhead/winged helix DNA binding domain and acts in a wide range of biological processes, including proliferation, migration and apoptosis. FoxF2, one member of Fox superfamily, regulates the switch of epithelium and mesenchyme to maintain the epithelial properties [22]. However, FoxF2 has a dual effect in regulation of cell cycle progress and the EMT in breast cancer progression. FoxF2 can act as a tumor suppressor to inhibit EMT process in basal-like breast cancer [22]. Another study showed that FoxF2 could exert its dual effect in breast tumorigenesis by acting as an oncogenic miRNA that increased the EMT process and migration and invasion ability or as a tumor suppressor that negatively regulated DNA replication [23]. Additionally, FoxF2 and FoxF1 can synergistically induce tumor cell proliferation by promoting cell cycle progress, leading to rhabdomyosarcoma carcinogenesis. Thus the role of FoxF2 in EC remains uncertain. In this study, we found that lentiviral-mediated over-expression of FoxF2 in RL95-2 cells could significant suppress the migration and invasion capacities and colony formation ability, induce cell cycle arrest into G1 phase, whereas these effects were opposite after the lentiviral-mediated inhibition of FoxF2. These data suggested FoxF2 acts as a tumor suppressor in EC.

FoxF2 has been verified to be a target of miR-182. miR-182 can exert its oncogenic miRNA function by down-regulation of FoxF2 in multiple cancers, such as triple-negative breast cancer and prostate cancer [8, 9]. But the effects and the cellular and molecular mechanisms of miR-182 and FoxF2 in the development of EC

were not fully understood so far. In this study, the results showed that the inhibition of miR-182 or over-expression of FoxF2 significantly suppressed the migration and invasion capacities, induced cell cycle arrest, and inhibited colony formation of RL95-2 cells. The effect induced by miR-182 inhibitor could be partially reversed when the expression of both miR-182 and FoxF2 were suppressed in RL95-2 cells.

In conclusion, our findings indicate that miR-182 promotes cell proliferation, migration, and invasion in EC by targeting down-regulation of FoxF2. Additionally, the inhibition of miR-182 may be beneficial for increasing tumor suppressor FoxF2 expression in EC, suggesting that miR-182 may serve as a target in the therapy of EC. Our findings will provide a theoretical basis for the development of a novel therapeutic regimen.

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Disclosure of conflict of interest

None.

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