miR-34c plays a role of tumor suppressor in HEC-1-B cells by targeting E2F3 protein

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Abstract. Endometrial carcinoma (EC) is a common malignancy of the female genital tract with a poor prognosis. It has been reported that miR-34c is significantly reduced in EC, but research concerning its function in EC is rare. In the present study, the expression of miR-34c was upregulated in the EC cell line, HEC-1-B, by transfecting the cells with hsa-miR-34c-5p mimics. Then, after determining the transfection efficiency by RT-qPCR, we analyzed the effects of miR-34c on the HEC-1-B cells. We found that overexpression of miR-34c significantly inhibited cell proliferation, colony formation, migration and invasion and induced cell cycle arrest and apoptosis. Finally, western blot analysis demonstrated that the expression of E2F3 was reduced after upregulation of the expression of miR-34c in the HEC-1-B cells, and the effects of miR-34c are likely associated with the reduction in E2F3 protein. In conclusion, our study demonstrated that miR-34c plays a role of tumor suppressor in HEC-1-B cells, and E2F3 protein may be a target of miR-34c.

Introduction

Endometrial carcinoma (EC) is one of the most common malignancies of the female genital tract. Mortality rates from EC are increasing in many countries and this may be attributed to an increased rate of advanced stage cancers and high-risk histologies (1). Surgery is currently the major treatment for EC, yet it is critically important to develop new therapeutic strategies for EC. Research of the molecular mechanisms of EC may help to identify new diagnostic and therapeutic targets.

MicroRNAs (miRNAs) are 18-24 nucleotide non-coding RNAs which play an important role in regulating gene expression by cleaving or translational repression of mRNAs (2).

Recent studies have demonstrated the dysregulation of specific miRNAs in different types of tumors, including stomach, lung, breast, ovarian and cervical cancer and leukemia (3-5). These miRNAs may act as tumor suppressors or oncogenes by regulating processes such as cell proliferation, apoptosis, metastasis and invasion (5-8), and our previous research demonstrated that miR-449a is a suppressor of EC (9).

The miR-34 family consists of miR-34a (5'-UGGCAG UGUCUUAGCUGGUUGU-3'), miR-34b (5'-CAAUCACUA ACUCCACUGCCAU-3') and miR-34c (5'-AGGCAGUGU AGUUAGCUGAUUGC-3'). Numerous studies have shown the dysregulation of miR-34 in various types of cancers, including hepatocellular, mesothelial and colon cancer, melanomas, neuroblastomas and leukemia (10-12). Yet, little research has been conducted in endometrial cancer.

E2F3 is a member of the E2F family, and is essential for tumor growth by regulating cell proliferation and apoptosis (13,14). Whether E2F3 acts on endometrial cancer cells remains unclear.

In the present study, we investigated the relationship between miR-34c and EC. It has been reported that miR-34c is significantly reduced in EC (15,16), and numerous studies have shown that decreased expression of miR-34c in cancer cells correlates with aberrant DNA methylation (17,18). To further study the function of miR-34c in EC, we upregulated the expression of miR-34c in the EC cell line, HEC-1-B, and then sought to identify the effect of miR-34c on cell proliferation, colony formation, cell cycle arrest, apoptosis, migration and invasion. Finally, we determined the expression of E2F3 in the HEC-1-B cells using western blot analysis.

Materials and methods

Cell line and culture. EC cell line (HEC-1-B) was obtained from the Shanghai Institute of Cell Biology (Shanghai, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from Gibco), and incubated under a condition of a humidified atmosphere of 5% CO_2 and 95% air at 37˚C.

Cell transfection. We purchased hsa-miR-34c-5p mimics and a negative control (NC) from GenePharma (Shanghai, China). The sequence of hsa-miR-34c-5p mimics was 5'-AGGCAGU

GUAGUUAGCUGAUUGC-3' and the sequence of NC was 5'-UUCUCCGAACGUGUCACGUTT-3'. One day before transfection, HEC-1-B cells were seeded in 96-well plates at $2.5x10^3$ or $2x10^5$ cells/well in 6-well plates in growth medium (DMEM supplemented with 10% FBS) without antibiotics, such that they were 30-50% confluent at the time of transfection. On the following day, the cells were transfected with hsa-miR-34c-5p mimics or NC at a final concentration of 50 nmol/l using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. There were three groups: the miR-34c group, the NC group, and the untransfected group.

RNA isolation and RT-qPCR. Reverse transcription quantitative PCR (RT-qPCR) was applied to ensure that the HEC-1-B cells were successfully transfected. Forty-eight hours after transfection, total RNA was isolated from the HEC-1-B cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription PCR (RT-PCR) and real-time PCR (qPCR) were performed according to the manufacturer's instructions. Briefly, miRNAs (contained in 500 ng total RNA each group) were polyadenylated and further reverse transcribed into cDNA using miRNA First-Strand cDNA Synthesis and qRT-PCR kits (Invitrogen). Next, we diluted the cDNA 1:10 in DEPC-treated water and the diluent cDNA served as the template for qPCR.

We used Applied Biosystems 7500 detection system to perform the qPCR. U6 snRNA was used as an endogenous control for normalization. The forward primers of hsa-miR-34c-5p and U6 used in our study were synthesized by Invitrogen. The primer sequence of hsa-miR-34c-5p was 5'-AGGCAGTGTAGTTAGCTGATTGC-3' and the primer sequence of U6 was 5'-CGCAAGGATGACACGCAAA TTC-3'. The universal reverse primer was offered in the kit. After mixing 10 μl of the PCR system, which included 5 μl of SYBR-Green Master Mix (Toyobo, Japan), 1 μl of the specific forward primer (1 μmol/l), and 1 μl of the reverse primer (1 μmol/l), 1 μl plus solution, 1 μl template and 1 μl DEPC-treated water, we placed the reactions in a real-time instrument at 95˚C for 3 min, and 40 cycles (95˚C, 15 sec and 60˚C, 1 min for each cycle). Then, we obtained Ct values for each sample, and the relative expression of miR-34c was calculated by the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation assay. Cells were transfected 24 h after seeding in 96-well plates at 2,500 cells/well. Then we evaluated cell proliferation using CCK-8 reagent (Beyotime Institute of Biotechnology, China) at 24, 48, 72, and 96 h after transfection, respectively. Briefly, we replaced the DMEM in the well, then added 10 μl of CCK-8 solution into each well and incubation was carried out at 37˚C for an additional 2 h. We then determined the absorbance at a wavelength of 450 nm.

Colony formation assay. Seventy-two hours after transfection, the cells were collected and resuspended in DMEM with 10% FBS. Then the cells were seeded in 6-well plates at an amount of 800 cells/well, and incubated at 37˚C in a humidified incubator with 5% CO_2 for 14 days until visible colonies (containing 40 or more cells) formed. The cell colonies were

stained with crystal violet for 30 min. We counted the numbers of colonies in each group.

Cell apoptosis assay. The cell apoptosis assay was performed using the Alexa Fluor 488 Annexin V apoptosis kit (Invitrogen). Briefly, the cells were collected 72 h after transfection, washed twice with cold PBS and resuspended in Annexin-binding buffer supplied in the kit. Subsequently, the cells were stained using Annexin V and PI according to the manufacturer's instructions. Cell apoptosis was analyzed by flow cytometry.

Cell cycle assay. Approximately $2x10^5$-$1x10^6$ cells were collected 48 h after transfection, and washed once with PBS. Then the cells were resuspended in 1 ml DNA staining solution (MultiSciences Biotech Co., Ltd.), incubated for 30 min at room temperature and analyzed by flow cytometry in the presence of the dye.

Transwell migration and invasion assays. For the Transwell migration assay, the cells were harvested 72 h after transfection and resuspended in serum-free medium at a concentration of $1.5x10^5$ cells/ml. Two hundred microliters of the cell suspension was transferred into the top chamber with a non-coated membrane (8-μm pore; BD Biosciences), and the chamber was inserted in a 24-well plate. For the Transwell invasion assay, 200 μl of the cell suspension at a concentration of $3.0x10^5$ cells/ml was transferred into the top chamber with a Matrigel-coated membrane (8-μm pore; BD Biosciences). In both assays, 500 μl DMEM containing 10% FBS was added to the bottom chamber in a 24-well plate. The plate was placed in an incubator for 24 h. After that, we wiped away the cells on the upper surface of the membrane with a cotton swab, then fixed the cells on the lower surface of the membrane in 4% paraformaldehyde for 30 min, stained them with crystal violet for 30 min, and evaluated the migration or invasive ability by counting the numbers of stained cells under a microscope.

Western blot analysis. Total proteins in each group of cells were extracted 72 h after transfection, and resolved by 10% SDS-PAGE. The proteins were transferred onto PVDF membranes (Solarbio) and blocked in TBST containing 5% skim milk for 1 h. Next, the membranes were incubated with primary antibodies for E2F3 (rabbit anti-human polyclonal antibody, diluted as 1:1,000, ab50917; Abcam) and β-actin (1:10,000, AP0060; BioWorld) for 1 h, respectively. After washing 3 times with TBST (each time 15 min), the membranes were incubated with secondary antibody (peroxidase conjugated goat anti-rabbit IgG, 1:5,000, GAR007; MultiSciences Biotech Co., Ltd.) for 1 h. After washing the membranes 3 times with TBST (each time 15 min), we detected the blots using SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Fisher Scientific) and protein bands were exposed to Kodak film (Sigma-Aldrich). Relative expression of E2F3 protein was normalized to β-actin.

Statistical analysis. Statistical analysis of all data was performed using SPSS 19.0 software package. Each experiment was performed at least 3 times, and differences between each group were evaluated by one-way ANOVA. The results
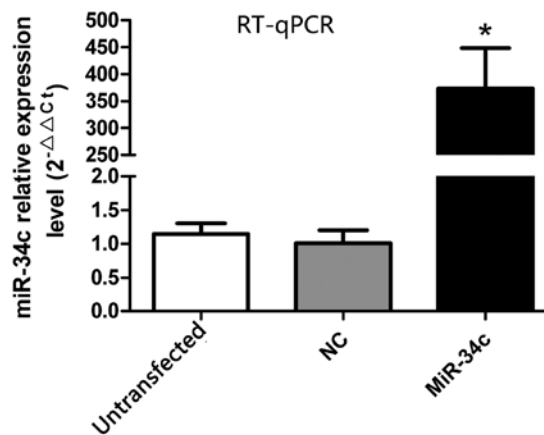
Figure 1. Expression levels of miR-34c as detected by RT-qPCR 48 h after transfection. Bars show the mean ± SD of triplicate experiments, *P<0.01.

are shown as the mean ± SD. P<0.05 was considered to indicate a statistically significant result.

Results

Expression of miR-34c after transfection. The expression of miR-34c was detected by RT-qPCR 48 h after transfection (Fig. 1). There was increased expression of miR-34c in the miR-34c group compared with the NC and untransfected group (miR-34c group, 373.43±74.95; NC group, 1.01±0.19; untransfected group, 1.14±0.16, P<0.01).

miR-34c inhibits the growth of HEC-1-B cells. To confirm a proliferation-suppressive effect of miR-34c in the HEC-1-B cells, the absorbance at the wavelength of 450 nm was measured 2 h after adding CCK-8 to reflect the amount of cells indi-

rectly. The absorbance in the miR-34c group was significantly decreased by ~28.0% (P<0.01) at 72 h and 54.1% (P<0.01) at 96 h compared with the NC and untransfected group (Fig. 2A), indicating that the cell proliferation was suppressed followed by increased expression of miR-34c.

To further prove the function of miR-34c on growth inhibition of the HEC-1-B cells, we examined the role of miR-34c on cell colony formation. As shown in Fig. 2B, the colony formation capacity was markedly decreased in the miR-34c group. We counted the numbers of colonies in each group, and the numbers of colonies in the NC and untransfected group reached 149.7±4.0 and 143.0±2.6, respectively, while it was only 49.0±2.5 in the miR-34c group (P<0.01).

miR-34c promotes the apoptosis of HEC-1-B cells. Seventy-two hours after transfection, the cells in the miR-34c group were in an obviously poorer status when compared to the NC and untransfected group (Fig. 3A). Following analysis by flow cytometry (Fig. 3B), the percentages of early and late apoptotic cells were significantly increased in the miR-34c group (16.76% in total) when compared with these percentages in the other 2 groups (4.32 and 5.33% in total, respectively).

miR-34c induces cell cycle arrest. Cell cycle distribution was evaluated by flow cytometry. Overexpression of miR-34c led to the accumulation of cells in the G_1 phase and the reduction of cells in the S and G_2 phase (Fig. 4). These results suggest that miR-34c influenced cell cycle transition from G_1 to the S phase and induced cell cycle arrest at the G_1 phase.

miR-34c inhibits the migration and invasion of HEC-1-B cells. We identified the effect of miR-34c on the migration (Fig. 5A) and invasion (Fig. 5C) of the HEC-1-B cells by Transwell assay. In the Transwell migration assay, a 70.5%
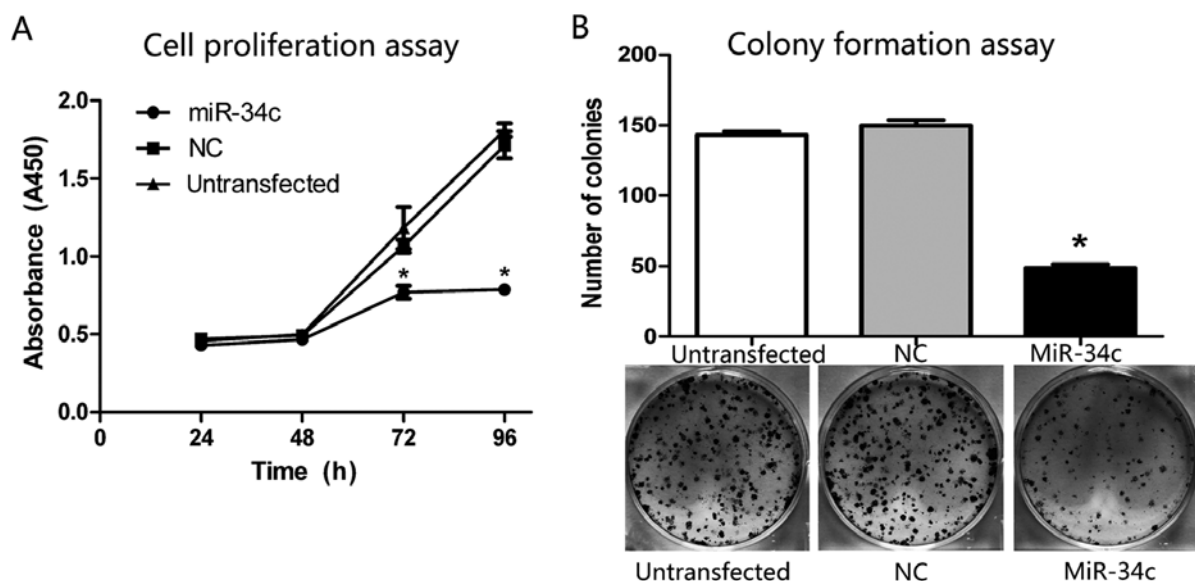

Figure 2. (A) The amounts of active cells in each group were assessed at the indicated times after transfection by CCK-8 assay. The data represent the mean ± SD of triplicate experiments, *P<0.01. (B) Colony formation assay. The numbers of colonies are presented as the mean ± SD (bars) of three separate experiments, *P<0.01.
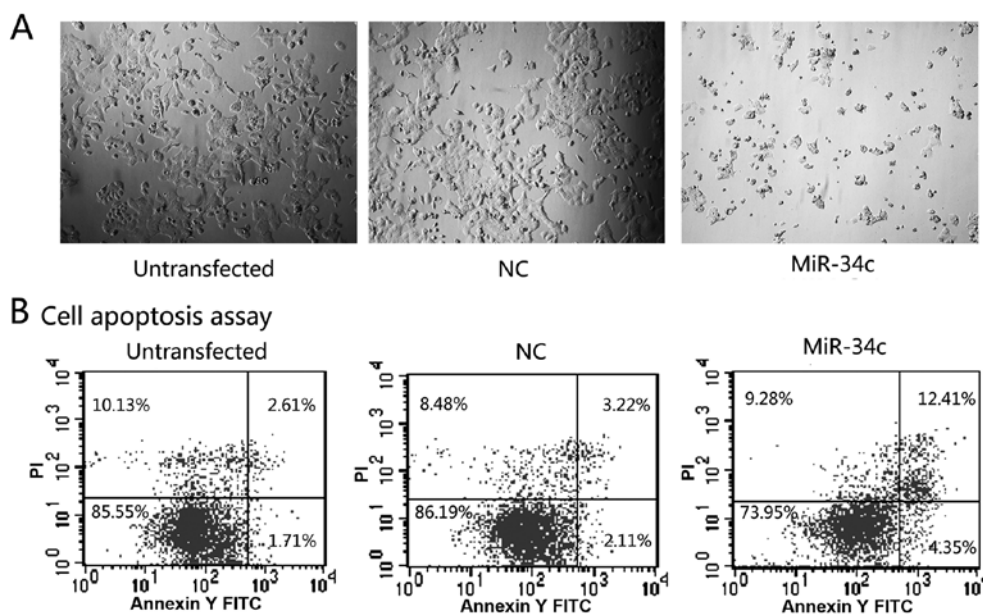
Figure 3. (A) State of the cells in each group observed under a microscope 72 h after transfection. (B) Cell apoptosis assay. Cell apoptosis was detected by flow cytometry. In each plot, the right lower quadrant represents early apoptotic cells, and the right upper quadrant represents late apoptotic cells. The assay was repeated 3 times and similar results were observed.
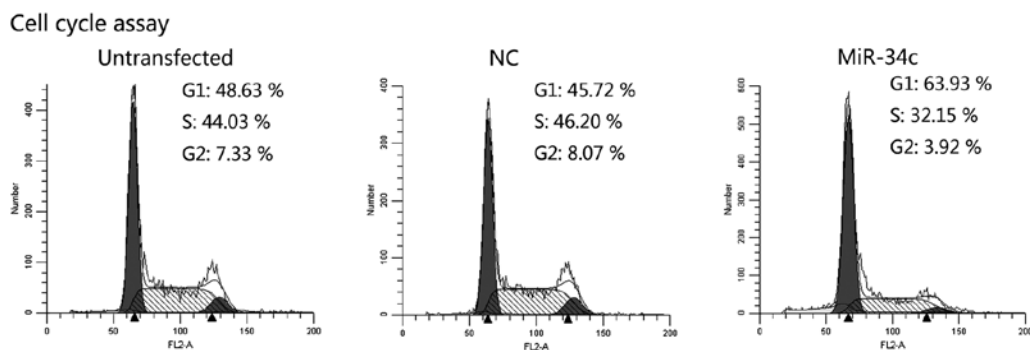

Figure 4. Cell cycle assay. The data show the percentage of each phase of cell cycle arrest in each group. This experiment was repeated 3 times and similar results were observed.

decrease in HEC-1-B cells (P<0.01) was detected 72 h after transfection of the hsa-miR-34c-5p mimics (Fig. 5B). In the Transwell invasion assay, a 75.8% decrease in HEC-1-B cells was noted after transfection of the hsa-miR-34c-5p mimics (P<0.01, Fig. 5D).

miR-34c suppresses the expression of E2F3 protein. To determine whether miR-34c regulates the expression of E2F3 protein, the expression of E2F3 was detected by western blot analysis 72 h after transfection. As expected, the expression of E2F3 protein was apparently inhibited after the transfection of the hsa-miR-34c-5p mimics (Fig. 6A and B).

Discussion

Our research together with a study conducted by Hiroki *et al* (15) showed that miR-34c is downregulated in EC, and this transformation may be an important factor in the poor prognosis of EC. To further study the function of miR-34c, we upregulated the expression of miR-34c in HEC-1-B cells by

transfection with the hsa-miR-34c-5p mimics, and we found that the overexpression of miR-34c inhibited cell proliferation, colony formation, migration and invasion, promoted apoptosis, and induced cell cycle arrest, demonstrating that miR-34c is a suppressor of HEC-1-B cells, and western blot analysis demonstrated that E2F3 protein may be a target of miR-34c. To our knowledge, the present study is the first to confirm that miR-34c suppresses HEC-1-B cells by targeting E2F3.

In the present study, we found that the decrease in the number of cells may be partly related to cell apoptosis. Inhibition of cell proliferation together with apoptosis ultimately inhibited the formation of cell colonies. A previous study showed that inhibition of cell proliferation and apoptosis may be partly caused by cell cycle arrest after transfection with miR-34s (19). Notably, our results found that both inhibition of cell proliferation and apoptosis appeared 72 h after transfection with the hsa-miR-34c-5p mimics, later than cell cycle arrest (48 h after transfection). Cell cycle arrest at the G_1 phase is likely to be the key function of miR-34c in HEC-1-B cells. We also found that miR-34c significantly inhibited the
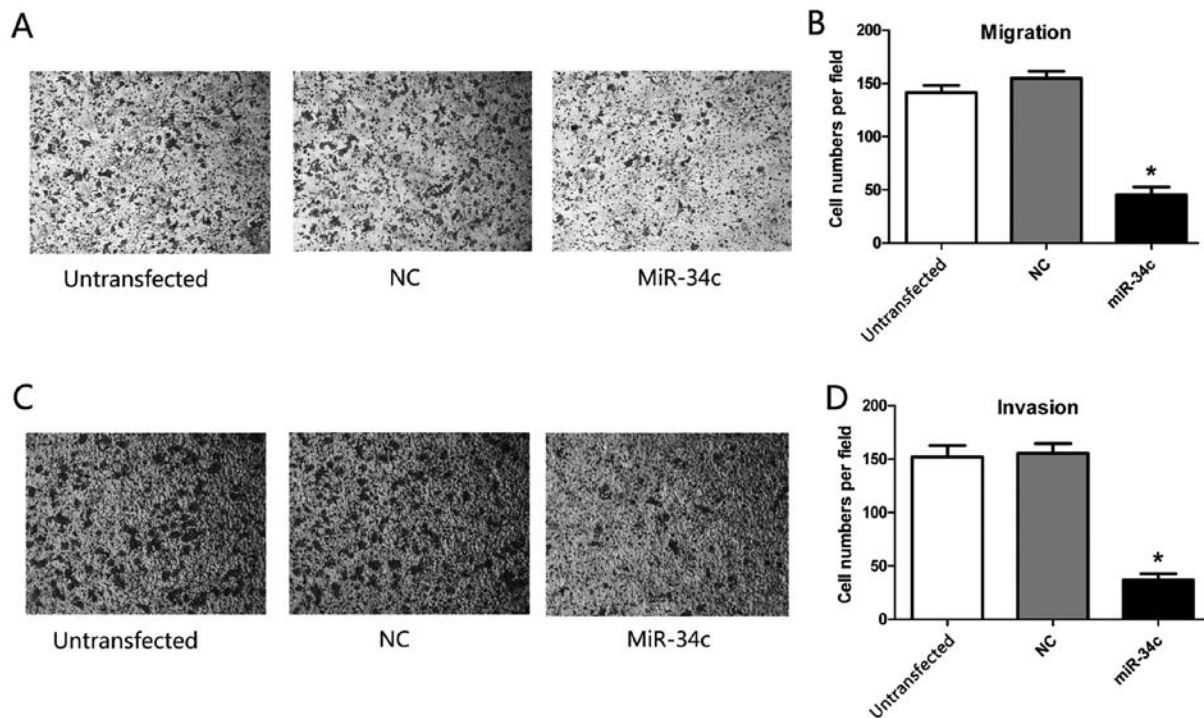
Figure 5. (A) Migration assay observed under a microscope. (B) Bars show the cell numbers in each group after staining in the migration assay. The data represent the mean ± SD of triplicate experiments, *P<0.01. (C) Invasion assay observed under a microscope. (D) Bars show the cell numbers in each group in the invasion assay. The data represent the mean ± SD of triplicate experiments, *P<0.01.
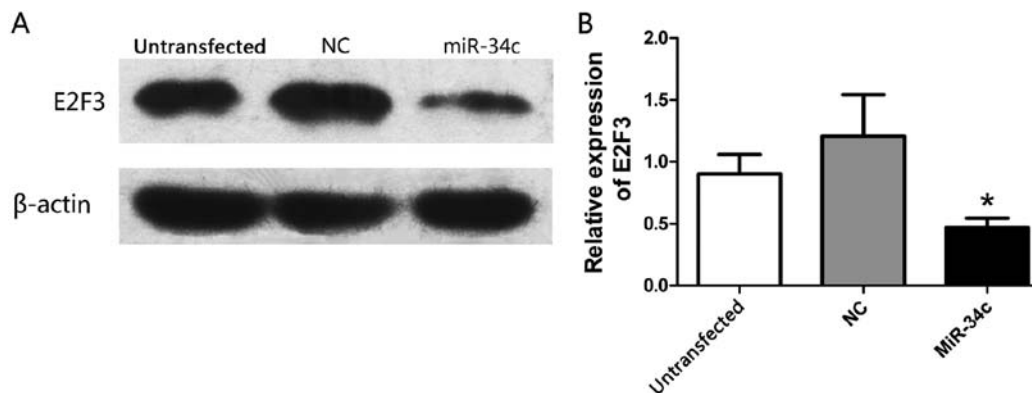

Figure 6. (A) The levels of E2F3 and β-actin proteins detected in the HCE-1-B cells 72 h after transfection by western blot analysis. (B) Bars show the expression levels of E2F3 relative to β-actin. The expression of E2F3 in the miR-34c group was significantly inhibited. Mean ± SD of triplicate experiments, *P<0.05.

migration and invasion of the HEC-1-B cells, which may also be clinically significant.

TargetScan analysis indicates that E2F3 may be a target of miR-34c. E2F3 is a member of the E2F family, whose potential oncogenic capacity was reported (20), and studies show that E2F3 regulates E2F-mediated DNA replication and cell proliferation by a p53-dependent negative feedback loop (21). Lax et al (22) reported that p53 mutations were found in 93% of uterine serous carcinoma and 17% of uterine endometrioid carcinoma, and that this may be responsible for their morphology and biologic behavior. In addition, it has been demonstrated that members of the miR-34 family are direct transcriptional targets of p53, playing a role as tumor suppressors (19,23). Based on the reports mentioned above, we proposed that the effects of miR-34c on HEC-1-B cells operate through E2F3

protein. To test this hypothesis, we used western blot analysis to demonstrate that E2F3 was markedly downregulated after transfection with the hsa-miR-34c-5p mimics. These studies, as well as our results, suggest that E2F3 protein is a target protein of miR-34c. It has been reported that overexpression of E2F3 protein is an important marker for poor clinical outcome in prostate cancer (24). These results suggest that E2F3 may exert a significant influence on tumor progression.

Leone et al (25) demonstrated that E2F3 influences cell cycle arrest in the induction of S phase during the transition from quiescence to proliferation by regulating Cdc6, cyclin E, Cdk2 and Mcm proteins. Similarly, our data showed reduced E2F3, and cell cycle arrest at G_1 phase accompanied by reduction in the percentage of cells in the S phase after transfection of hsa-miR-34c-5p mimics, suggesting that miR-34c acts as a

tumor suppressor by inducing cell cycle arrest at the G_1 phase via E2F3.

Invasive growth in many human tumors is associated with overexpression of MET (26), and recent studies show that knockdown of cyclophilin A leads to a significant inhibition of cell migration and invasion in HEC-1-B cells by down-regulating focal adhesion signaling (27). Our data revealed that miR-34c significantly inhibited migration and invasion of HEC-1-B cells; however, futher studies are necessary to confirm the effects of miR-34c on the migration and invasion of HEC-1-B cells and whether this is also associated with MET and cyclophilin A.

In conclusion, miR-34c acts as a direct target of p53, its overexpression suppresses the expression of E2F3 protein, and a low level of E2F3 causes cell cycle arrest at the G_1 phase by acting on a series of cell cycle arrest-related proteins. Then, cell cycle arrest induces cell proliferation inhibition and apoptosis partly and further inhibits cell colony formation. miR-34c can also inhibit cell migration and invasion.

Although therapeutic strategies for EC have improved greatly in the last few decades, EC still has a significantly poor prognosis. Understanding the pathogenesis at the molecular level is essential for the development of new targeted therapies. In the present study, we successfully identified miR-34c as a suppressor of the EC cell line (HEC-1-B), and demonstrated E2F3 as a target of miR-34c in HEC-1-B cells. Further studies to ascertain whether overexpression of miR-34c has a thera-peutic role in EC are necessary. We believe that miR-34c has potential value in the early diagnosis and molecular therapy of EC.

Acknowledgements

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