Genetics and Molecular Research

# miR-133 inhibits pituitary tumor cell migration and invasion via down-regulating FOXC1 expression

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Genet. Mol. Res. 15 (1): gmr.15017453
Received August 14, 2015
Accepted November 13, 2015
Published March 24, 2016
DOI http://dx.doi.org/10.4238/gmr.15017453

**ABSTRACT.** Many studies have shown that microRNA (miR)-133 functions as a tumor suppressor in a variety of metastatic cancers, including breast cancer, gastric cancer, and liver fibrosis. However, the influence of miR-133 on pituitary tumor malignancy has not yet been reported. The purpose of this study was to explore the role of miR-133 in pituitary tumor cell migration and invasive ability and the molecular mechanisms involved. Our findings suggest that in pituitary adenoma cell lines, through direct targeting and negative control of forkhead box C1 (FOXC1), miR-133 can inhibit pituitary adenoma cell migration and invasion. In addition, epithelial-to-mesenchymal transition can be induced by miR-133. Additionally, a negative correlation was found between FOXC1 and miR-133 expression

 

when comparing their expression levels between cancerous tissue and adjacent normal tissue. This suggests that miR-133 can inhibit cell migration and invasion by directly targeting FOXC1, implying that miR-133 could be a potential therapeutic target for treatment of invasive pituitary adenoma.

**Key words:** Pituitary adenoma; miR-133; FOXC1; Migration; Invasion

## INTRODUCTION

Pituitary adenoma is the third most common primary intracranial tumor of the central nervous system (Nakamura et al., 2011; Ye et al., 2015), as well as the most common saddle area tumor (Qu et al., 2011). Pituitary adenomas are benign tumors, but some have shown the ability to invade areas outside of the central nervous system, such as the intracranial region, which can lead to serious health problems and even death (Trouillas et al., 2003; Norberg et al., 2012; Palumbo et al., 2013). Therefore, it is necessary to explore the molecular mechanism of migration and invasion of pituitary adenomas.

MicroRNAs (miRs) are a class of small non-coding RNAs. One of these miRs, miR-133, has been shown to play a role in tumor suppression in a variety of cancers, including inhibiting breast cancer cell migration and invasion, gastric cancer cell growth and migration, and liver fibrosis (Esquela-Kerscher and Slack, 2006; Cheng et al., 2012; Liu et al., 2014; Mei et al., 2014; Wang et al., 2014; Yongchun et al., 2014; Xie et al., 2015; Zhang et al., 2015). Recent studies have shown that miRs may be a new regulatory mechanism involved in the onset of pituitary adenoma, providing a new method for the diagnosis and treatment of pituitary adenoma (Trivellin et al., 2012; Gadelha et al., 2013; Di Ieva et al., 2014; Leone et al., 2014). However, the precise role of miR-133 in the malignancy of pituitary tumors is unknown.

Forkhead box C1 (FOXC1) is a member of the FOX superfamily of transcription factors that act on gene promoters or interact with other transcription factors to activate transcription (Du et al., 2012; Wang et al., 2012). Furthermore, growing evidence indicates that FOXC1 can also contribute to epithelial-to-mesenchymal transition (EMT), cell signal transduction, and migration of endothelial cells, affecting multiple aspects of the growth cycle and proliferation activities of tumor cells (Chung et al., 2012; Xia et al., 2013; Xu et al., 2014).

Here, we confirmed that FOXC1 can promote cell migration and invasion in tumor cells. Through direct targeting and negative control of FOXC1 in pituitary adenoma cell lines, miR-133 can inhibit cell migration and invasion of tumor cells. This suggests that miR-133 may be a potential therapeutic target for invasive pituitary adenoma and this offers a new strategy for the treatment of pituitary adenoma.

## MATERIAL AND METHODS

### Cell culture

HP75 cells were cultured in DMEM (BioWhittaker, Cambrex Corp., Nottingham, UK) containing 15% horse serum (TCS Cellworks, Buckingham, UK) and 2.5% fetal calf serum (Life Technologies Inc., Invitrogen, Paisley, UK).

## Cell transfection

miR-133a/b mimics and miRNA antisense oligonucleotides (miR-133-ASO) were obtained from OriGene (Beijing, China). miR-133a/b, miR-133-ASO, and negative control with scrambled sequence (NC) were transfected into cells at a concentration of 50 nM according to the manufacturer instructions (Invitrogen).

## RNA isolation and real-time polymerase chain reaction (PCR)

Total RNA from HP75 was extracted using the SV Total RNA Isolation System according to the manufacturer protocol (Promega, Southampton, UK). Briefly, freshly pelleted cells were incubated with lysis buffer. After ethanol washes and deoxyribonuclease treatment, RNA was recovered by centrifugation and eluted with water. Reverse transcription-PCR (RT-PCR) was performed using the Enhanced Avian HS RT-PCR kit (Sigma-Aldrich Corp., USA). The RT reaction was performed using a reaction mixture containing 500 μM each of deoxy (d)-ATP, dTTP, dGTP, and dCTP (Sigma-Aldrich Corp.) and 0.6 μM antisense oligonucleotide primers incubated with 5 μg total RNA at 70°C for 10 min. The reaction was completed by the addition of ribonuclease inhibitor (1 U/μL), enhanced avian myeloblastosis reverse transcriptase (1 U/μL), and PCR buffer to a total volume of 20 μL. Samples were incubated without RT enzyme for negative controls. Five-microliter aliquots from the RT reaction were used for the subsequent PCR in the presence of 0.6 μM each of 3' and 5' primers (Sigma-Genosys, Pampisford, UK), reaction buffer containing 1.5 mM $MgCl_2$, 200 μM each dNTP (dATP, dTTP, dGTP, and dCTP), and JumpStart AccuTaq LA DNA polymerase (2.5 U/μL) (Sigma-Aldrich Corp.). Amplification was performed using the following program: 1) denaturation at 94°C for 5 min; 2) 40 cycles of 94°C, 58°C, and 72°C for 1 min each; and 3) a single-extension step at 72°C for 7 min.

## Luciferase assay

Cells were seeded on a 12-well plate at ~90% confluence and co-transfected with 0.5 μg reporter plasmid, 40 nmol miR-133a/b mimics, or NC by Lipofectamine 2000 (Thermo Fisher Scientific, Rockford, IL, USA). Each sample was also co-transfected with 0.05 μg pRL-CMV plasmid expressing *Renilla* Luciferase (Promega) as an internal control for transfection efficiency. Luciferase assay was performed 48 h after transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each transfected well. Each assay was repeated 3 times.

## Cell migration and invasion assay

The transfected cells (miR-133a/b mimics and NC) growing in the log phase were treated with trypsin and resuspended as a single-cell solution. A total of 1 x $10^5$ cells in 0.2 mL serum-free RPMI-1640 media were seeded on an 8-μm pore polycarbonate membrane Boyden chamber insert in a transwell apparatus (Costar, Cambridge, MA, USA), either coated with or without Matrigel (BD Biosciences, San Jose, CA, USA). RPMI-1640 (600 μL) containing 20% fetal bovine serum was added to the lower chamber. After the cells were incubated for 12-24 h at 37°C in a 5% $CO_2$ incubator, cells on the top surface of the insert were removed by wiping with a cotton swab. Cells that migrated to the bottom surface of the insert were fixed in 100% methanol for 2 min, stained in

0.5% crystal violet for 2 min, rinsed in PBS and then subjected to microscopic inspection (original magnification = 200X). Values for invasion and migration were obtained by counting five fields per membrane and representing the average of three independent experiments.

## Western blotting

Whole cells were washed in PBS and lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). Total protein was quantified using a BCA protein assay kit (Beyotime, Jiangsu, China). The proteins in each sample were resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The blots were blocked in bovine serum albumin (5% w/v in PBS + 0.1% Tween-20) for 30 min at room temperature. The following primary antibodies were used according to the manufacturer instructions: antibodies against FOXC1, and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies against N-cadherin and E-cadherin were purchased from Abcam (Cambridge, MA, USA). The appropriate secondary antibodies (Santa Cruz Biotechnology) were used at 1:1000-1:2000 (v/v) dilutions in PBS + 0.1% Tween-20 for 1 h at room temperature, and the signals were observed using an enhanced chemiluminescence kit (Thermo Fisher Scientific).

## Immunocytochemistry

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded, 40-mm thick tissue sections using the avidin-biotin-peroxidase complex method. The paraffin sections were mounted on SuperFrost glass slides, deparaffinized, and rehydrated in a graded series of ethanol; this was followed by high-pressure antigen retrieval. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide for 15 min. The sections were incubated overnight at 4°C with primary anti-FOXC1 antibody at a dilution of 1:400 (Santa Cruz Biotechnology), and this was followed by conjugation to the secondary antibody (SP-9000) (Santa Cruz) and 3,3'-diaminobenzidine (Dingjie Biological, Shanghai, China) staining. The sections were then counterstained with Mayer's hematoxylin, dehydrated, cleaned, and mounted. In each immunohistochemistry run, negative controls were stained without primary antibody. All slides were examined by two observers who were blinded to clinical data. The final evaluation of ambiguous cases was decided by discussion between the two observers. Immunoreactivity for FOXC1 was evaluated using a combined scoring system based on the sum of the nuclear staining intensity and the percentage of positive cells. Scores from 0 to 3 were given based on the staining intensity and the percentage of positive cells as follows: 0, no staining or staining was observed in <10% of the tumor cells; 1+, weak staining was observed in ≥10% of the tumor cells; 2+, moderate staining was observed in ≥10% of the tumor cells; and 3+, strong staining was observed in ≥10% of the tumor cells. Scores of 0 and 1+ were considered to be negative for FOXC1 expression, and scores of 2+ and 3+ were considered to be positive for FOXC1 expression.

## Statistical analysis

Data are reported as means ± standard deviation (SD) and were compared using the Student *t*-test in Stata 10.0 (College Station, TX, USA). Double-tailed P value <0.05 was considered to be statistically significant.

## RESULTS

### Target gene prediction for miR-133

The miRBase and TargetScan target gene prediction software were used to predict the candidate target genes of miR-133 (Figure 1A). Thus, our results indicated that FOXC1 was a direct target of miR-133. In order to confirm this result, we performed a Dual Luciferase Reporter Assay for fluorescence intensity detection of overexpressed or inhibited miR-133. The assays showed that upregulated miR-133 notably decreased FOXC1 mRNA levels, whereas inhibition of miR-133 increased FOXC1 mRNA levels (Figure 1B). The results demonstrate that miR-133 negatively regulates FOXC1 expression. Next, we analyzed the endogenous FOXC1 mRNA and protein levels by overexpression of miR-133 or miR-133-ASO (Figure 1C and D). The results showed an inverse correlation between miR-133 and FOXC1 expression. In conclusion, these data imply that FOXC1 is a direct target of miR-133 and miR-133 negatively regulates FOXC1.

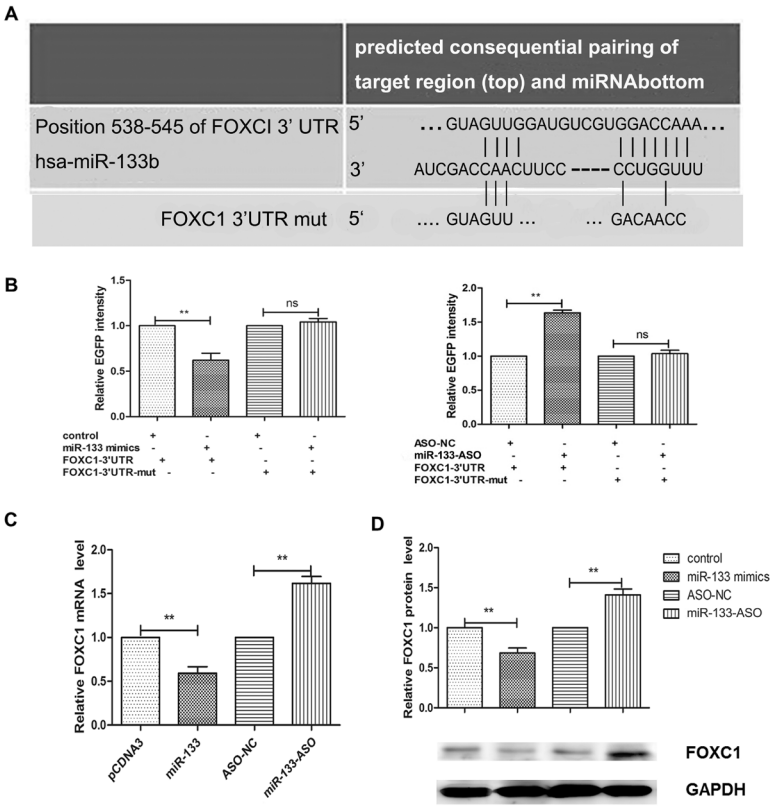


**Figure 1.** Prediction of target genes of miR-133. **A.** To investigate the underlying mechanisms, we predicted the potential targeted genes of miR-133 by bioinformatic algorithms. Among all predicted genes, FOXC1 was found to be a potential target for miR-133. **B.** Intensity for EGFP was significantly decreased in the miR-133 mimic + FOCX1-3'-UTR group, but this effect was suppressed by the addition of ASO, an inhibitor for miR-133, indicating that miR-133 could negatively regulate the expression of FOXC1. **C. D.** FOXC1 mRNA and protein levels after transfection with pcDNA3, miR-133 mimics, ASO-negative control (NC), and miR-133-ASO. **P < 0.01.

## Effects of FOXC1 knockdown on cell migration and invasion

We further explored the biological functions of miR-133 and FOXC1 as well as their influences on the malignant behavior of pituitary adenoma cells and found that overexpression of miR-133 inhibited cell growth and invasion, while depletion of miR-133 increased cell growth and invasion (Figure 2). The results showed that the tumor cell migration and invasion ability were suppressed by the overexpressed miR-133, but this kind of effect was reversed by the application of miR-133 inhibitor of ASO (Figure 2A and B). Besides, the overexpressed FOXC1 significantly increased the tumor cell migration and invasion ability compared to the control, while this kind of effect was reversed by the silencing (Figure 2C and D). Remarkably, the knockdown of FOXC1 weakened the enhancement of migration and invasive ability caused by miR-133-ASO (Figure 2E and F).

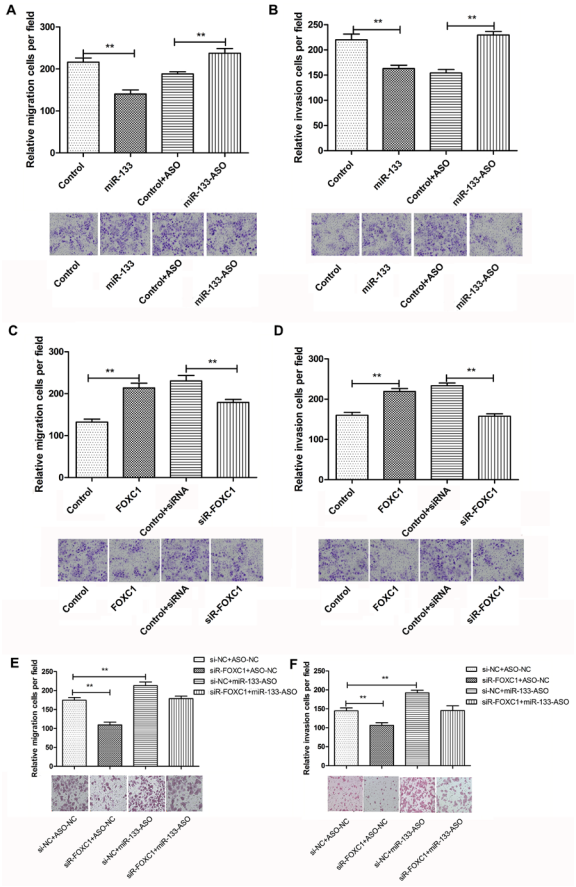


**Figure 2.** Migration and invasion of cells. **A. B.** The overexpressed miR-133 significantly decreased the migrated and invaded tumor cells compared to the control cells, which is opposite to that in cells transfected with the miR-133 inhibitor of ASO. **C. D.** The migrated or invaded tumor cells were significantly increased by the overexpressed FOXC1 compared to the control; however, this kind of effect was reversed by the silencing FOXC1. NC: control cells transfected with the pcDNA3.0; ASO: the inhibitor for miR-133; si-NC: control cells transfected with the scramble siRNA; siR-FOXC1: cells transfected with the silenced vector with FOXC1 sequence; ns: no significant difference between two groups; **P < 0.01.

## miR-133 inhibits EMT and FOXC1 promotes EMT

Based on previous research, we confirmed that miR-133 can inhibit the migratory and invasive ability of pituitary tumor cells, while FOXC1 can promote the migratory and invasive ability of pituitary tumor cells. We next explored the molecular mechanism by which this occurs. As is known, the migration and invasion of tumor cells are mainly attributed to the loss of polarity of epithelial cells and the loss of linkage and adhesion between cells, giving them the ability to invade and migrate, which results in the metastasis from pituitary tumor cells to other sites. Thus, using Western blot analysis, we detected the molecular changes associated with EMT. As shown in Figure 3A and B, overexpression of miR-133 increased the related molecular expression of EMT, whereas inhibition of miR-133 decreased the related molecular expression of EMT. Inversely, overexpression of FOXC1 suppressed the related molecular expression of EMT, whereas knockdown of FOXC1 promoted the related molecular expression of EMT (Figure 3C and D). Most importantly, FOXC1 knockdown significantly weakened the enhancement of EMT caused by miR-133-ASO (Figure 3E).

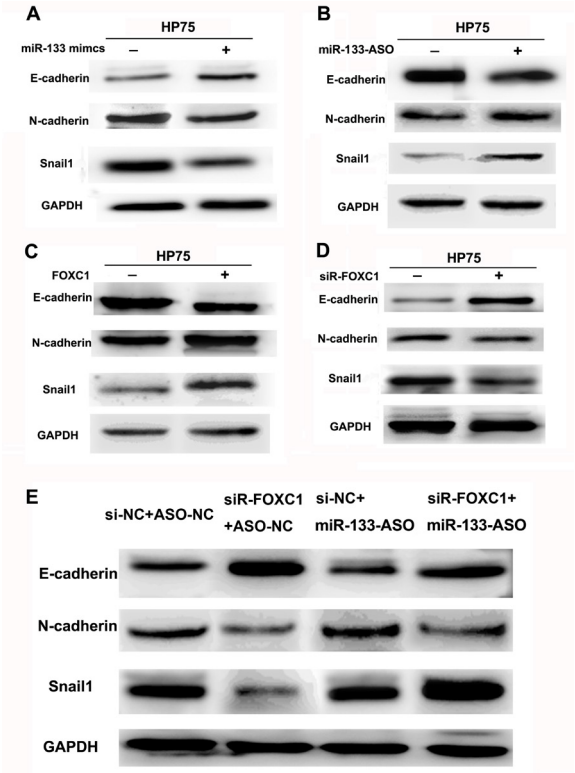


**Figure 3.** Effects of miR-133 or FOXC1 expression on the cell signal pathway-related protein expression. **A. B.** E-cadherin expression was increased while N-cadherin and Snail1 expression were decreased by the overexpressed miR-133, which was suppressed by the inhibitor of miR-133, ASO. **C. D.** E-cadherin expression was lower while N-cadherin and Snail1 expression were higher in the FOXC1 overexpressed group than the control, which was opposite to that in cells transfected with the silencing FOXC1. **E.** E-cadherin expression was increased while N-cadherin and Snail1 were decreased by the silencing FOXC1, which was opposite to that in cells with ASO. NC: control cells transfected with the pcDNA3.0; ASO: the inhibitor for miR-133; si-NC: control cells transfected with the scramble siRNA; siR-FOXC1: cells transfected with the silenced vector with FOXC1 sequence.

## Correlation between miR-133 and FOXC1 in pituitary tumor tissues

In order to further validate our conclusions, we analyzed pituitary tumor tissue samples. We extracted the RNA from six pairs of pituitary tumor tissues and matched tumor adjacent normal tissues and then examined the miR-133 and FOXC1 mRNA levels using quantitative RT-PCR. The results revealed that the relative expression of miR-133 was upregulated, while the expression of FOCX1 was downregulated in tumor tissues compared with the corresponding adjacent normal samples (Figure 4A and B). At the same time, we performed immunohistochemistry of pituitary tumor tissues and matched tumor adjacent normal tissues, and the results are shown in Figure 4C and D. According to the analysis, there is a strong negative correlation between miR-133 and FOCX1 expression (Figure 4E).

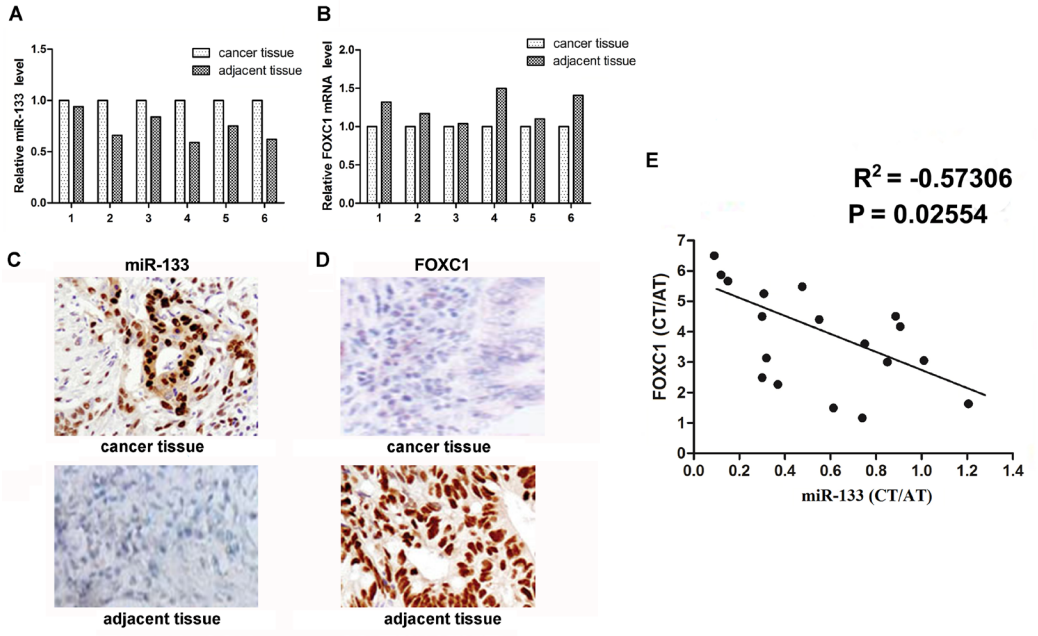


**Figure 4.** Expression of miR-133 and FOXC1 in tumor tissues. **A.** qRT-PCR analysis showed that miR-133 expression in cancer tissues was higher than that in adjacent tissues. **B.** The mRNA level of FOXC1 in cancer tissues was lower than that in the adjacent tissues. **C. D.** Immunohistochemical staining showed that the expression for miR-133 was more than that in adjacent tissues, while FOXC1 in cancer tissues was less than that in its adjacent tissues. **E.** Correlation analysis showed that there was a negative regulation between miR-133 and FOXC1 in tumor samples ($R^2$ = -0.57306; P = 0.02554). CT refers to cancer tissues and AT refers to corresponding adjacent tissues.

## DISCUSSION

Tumor metastasis is a critical cause of cancer-related death and the high mortality of pituitary tumors is closely related to recurrent metastasis. Tumor metastasis involves multiple processes, including five stages at the molecular level. First, adhesion is reduced between tumor cells; second, extracellular matrix is degraded; third, tumor cell motility is enhanced, allowing them to pass through the basement membrane and into circulation in the process of loss of cell-

cell adhesion; fourth, tumor cells escaped the recognition from the immune system; and fifth, the establishment of angiogenesis is the premise of metastasis (Carmeliet and Jain, 2011; Valastyan and Weinberg, 2011). One of the most critical steps in this process is the one involving tumor cell migration and invasion enhancement, and extraordinary complex molecular mechanisms are involved in these processes, including miRs. For example, miR-200c can inhibit the transformation of epithelial mesenchymal cells and the EMT process in pancreatic cancer cells (Hur et al., 2013) and miR-300 can promote cell migration and invasion of osteosarcomas (Xue et al., 2015). It has been reported that miR-132, miR-15a, and miR-16 can suppress the migration and invasion of pituitary tumor cells during its metastasis (Bottoni et al., 2007; Palumbo et al., 2013). miR-133 is relatively less studied but, recently, a potential role for miR-133 in gastric cancer and its molecular mechanisms have been reported (Zhang et al., 2015).

As is known, target genes play an important role in miR expression, as miRs directly regulate target genes by binding to their 3'-UTRs. In this study, we have explored the role of miR-133 and its underlying mechanisms in pituitary adenoma. We verified that miR-133 targets FOXC1 through direct targeting and negative control of FOXC1 expression in pituitary adenoma cell lines. In this way, miR-133 can inhibit cell migration and invasion of tumor cells. To further understand the mechanisms, we performed Western blotting and monitored the EMT process. The Western blotting experiment showed that upregulation of miR-133 increased the related molecular expression of EMT, whereas inhibition of miR-133 decreased the related molecular expression of EMT. Inversely, overexpression of FOXC1 suppressed the related molecular expression of EMT. In addition, we showed that FOXC1 knockdown partially abolished the enhanced migration and invasive ability caused by miR-133 inhibition in pituitary adenoma.

In conclusion, our study revealed previously unrecognized roles and correlation between miR-133 and FOXC1 in pituitary adenoma. Our data suggest that miR-133 may be a potential therapeutic target for the treatment of invasive pituitary adenoma.

## Conflicts of interest

The authors declare no conflict of interest.

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