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ORIGINAL ARTICLE

MiR-193a-3p functions as a tumour suppressor in human aldosterone-producing adrenocortical adenoma by down-regulating CYP11B2

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SUMMARY

The mechanism of aldosterone-producing adrenocortical adenoma (APA) pathogenesis and the role of microRNAs (miRNAs) in APA pathogenesis have not been completely clarified. We examined the expression and function of miR-140-3p, miR-193a-3p and miR-22-3p, which have binding sites in *CYP11B2*. Expression of miRNAs and *CYP11B2* mRNA was measured by quantitative reverse transcription PCR (qRT-PCR). Cell proliferation was monitored by colorimetric analysis, and cell apoptosis and cell cycle progression were analysed by flow cytometry. ELISA was carried out to detect aldosterone levels in cell culture supernatants. Luciferase reporter assays, qRT-PCR and Western blotting were performed to identify *CYP11B2* as a target of miR-193a-3p. Of the three miRNAs examined, miR-193a-3p exhibited a significant decrease and *CYP11B2* mRNA exhibited a significant increase in expression in APA compared with adjacent normal adrenal gland tissue. Transfection of miR-193a-3p mimic into the human adrenocortical cell line H295R showed that elevated miR-193a-3p expression inhibits proliferation and aldosterone secretion, induces G1-phase arrest and promotes apoptosis in H295R cells. Furthermore, in luciferase reporter assays, overexpression of miR-193a-3p in H295R cells significantly reduced the luciferase activity of the wild-type *CYP11B2* 3′-UTR construct, which could be reversed by mutation of the miR-193a-3p-binding site. Moreover, miR-193a-3p overexpression downregulated *CYP11B2* mRNA and protein expression. Finally, overexpression of *CYP11B2* diminished the effects of miR-193a-3p on H295R cells. Taken together, our results suggest that *CYP11B2* levels may be modulated by miR-193a-3p in APA, which could explain, at least partially, why downregulation of miR-193a-3p during APA formation may promote cell growth and suppress apoptosis.

Keywords
aldosterone secretion, CYP11B2 aldosterone-producing adrenocortical adenoma, miR-193a-3p, target gene

Primary aldosteronism, caused by excessive secretion of aldosterone from pathological adrenal tissue, occurs in more than 11% of hypertensive patients (Rossi 2011). Aldosterone-producing adrenocortical adenoma (APA) is one of the two most common subtypes of primary aldosteronism (Rossi *et al.* 2008; Funder *et al.* 2016). The genetic and molecular basis of APA has been investigated extensively. Somatic mutation in KCNJ5, ATP1A1, ATP2B3 and CACNA1D is responsible for autonomous aldosterone production in around 50% of sporadic APA (Williams *et al.* 2014), but the mechanism leading to cell proliferation and APA formation remains poorly elucidated.

The capacity of the adrenal gland to produce aldosterone is controlled, in large part, by strict regulation of the expression of aldosterone synthase, encoded by cytochrome P450 family 11 subfamily B member 2 (*CYP11B2*) (Bassett *et al.* 2004). CYP11B2 converts deoxycorticosterone to

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corticosterone, corticosterone to 18-hydroxycorticosterone and, finally, 18-hydroxycorticosterone to aldosterone (Curnow *et al.* 1991). Elevation of *CYP11B2* expression may be one of the mechanisms that causes excessive production and secretion of aldosterone in APA. Although *CYP11B2* mRNA and protein levels are not significantly higher in all APA cases compared to normal adrenals (Saner-Amigh *et al.* 2006; Sakuma *et al.* 2013; Nakamura *et al.* 2016), an increasing number of studies have demonstrated that *CYP11B2* overexpression is involved in the pathogenesis of APA (Nakamura *et al.* 2014, 2016; Nanba *et al.* 2016). Therefore, it is critical to identify the regulatory mechanism and other critical factors governing *CYP11B2* overexpression. Recent studies have demonstrated that various factors and regulatory mechanisms, such as hypomethylation, regulate the expression of *CYP11B2* in APA at the transcriptional level, controlling its function (Yoshii *et al.* 2016).

MicroRNAs (miRNAs) are small non-coding RNA molecules about 22 nucleotides (Choudhuri 2010; Taft *et al.* 2010). miRNAs function in RNA silencing and post-transcriptional regulation of gene expression by binding the 3′-untranslated region (UTR) of a target gene (Choudhuri 2010). Although there are many potential miRNA-binding sites in the 3′-UTR of *CYP11B2*, only a small number of miRNAs, such as miR-24 and miR-10b, have been demonstrated to regulate *CYP11B2*, including miR-24 and miR-10b (Robertson *et al.* 2013; Nusrin *et al.* 2014). In addition, He *et al.* (2015) assessed miRNA expression in APA, unilateral adrenal hyperplasia (UAH) and normal adrenal cortex tissue by microarray profiling and found that 31 miRNAs, including miR-140-3p, miR-193a-3p and miR-22-3p, were differentially expressed significantly in these three groups. Interestingly, these three miRNAs have binding sites in the 3′-UTR of *CYP11B2* (Lewis *et al.* 2005); however, their roles in APA pathogenesis are largely obscure. In addition, it is uncertain whether miR-140-3p, miR-193a-3p and miR-22-3p can regulate *CYP11B2* expression.

Here, we measured the expression levels of miR-140-3p, miR-193a-3p, miR-22-3p and *CYP1B2* mRNA in APA and adjacent normal adrenal gland tissues. In addition, we investigated the role of miR-193a-3p on cell proliferation, cell cycle progression, apoptosis and aldosterone secretion in the human adrenocortical cell line H295R. Our results indicate that miR-193a-3p acts as a suppressor of APA formation, and *CYP11B2* is a direct target of miR-193a-3p.

Materials and methods

Patients and samples

Fifteen paired samples of APA and adjacent normal adrenal gland tissue were obtained from patients attending the Department of Urology, First Affiliated Hospital of Gannan Medical University, China. The criteria adopted for APA diagnosis were as described previously (Mulatero *et al.* 1998). Briefly, (i) all patients had refractory hypertension, elevated plasma aldosterone concentration (>15 ng/dl) and an increased ratio of plasma aldosterone concentration to plasma renin activity (>50 ng/dl/ng/ml per hour); (ii) all had positive results for intravenous saline loading (Holland *et al.* 1984; Moneva & Gomez-Sanchez 2001); and (iii) all patients underwent a computed tomography scan, as well as adrenal venous sampling for differential diagnosis of IHA. Ethical approval was obtained from the local research ethics committee, and all patients gave written informed consent for the use of these samples in this study. The study was conducted in accordance with the Declaration of Helsinki.

RNA extraction and quantitative reverse transcription PCR analysis

Total RNA was extracted from frozen samples or harvested cells, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). To analyse miR-140-3p, miR-193a-3p and miR-22-3p expression, reverse transcription was performed using specific stem-loop primers, using the ImProm-IITM Reverse Transcription System (Promega, Madison, WI, USA). The ImProm-IITM Reverse Transcription System was also used to quantify the mRNA level of *CYP11B2*. Fluorescence-based quantitative reverse transcription PCR (qRT-PCR) was performed using SYBR GREEN qPCR Super Mix (Invitrogen) on an Applied Biosystems 7500 system. U6 was used as an internal control for normalization of miRNA expression. 18S rRNA was used as an internal control for normalization of *CYP11B2* expression. The primer sequences used for qRT-PCR are shown in Table 1. Gene expression was measured in triplicate, quantified using the $2^{-\Delta\Delta CT}$ method and normalized using the relevant control.

Cell culture

The human adrenocortical cell line H295R was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). This cell line was cultured in ATCC-formulated DMEM: F12 Medium (Catalogue No. 30-2006) with the following components added to the base medium: 0.00625 mg/ml insulin, 0.00625 mg/ml transferrin, 6.25 ng/ml selenium, 1.25 mg/ml bovine serum albumin, 0.00535 mg/ml linoleic acid and 2.5% (v/v) Nu-Serum I. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C.

miRNA mimic and CYP11B2 *overexpression plasmid transfection*

miR-193a-3p mimic and negative control (NC) miRNA mimic were purchased from RiboBio Co. (Guangzhou, China). H295R cells were plated at 50% confluency and transfected with 100 nM miR-193a-3p mimic or NC mimic, using Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's protocol. Full-length *CYP11B2* (NM_000498) was cloned into the expression plasmid pcDNA3.0. Transfection of the plasmid was conducted using Lipofectamine® 2000 (Invitrogen) according to the manufacturer's instructions.

Cell proliferation assay

Cell proliferation was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega) according to the manufacturer's instructions. Briefly, H295R cells (1×10^4) were seeded onto 96-well plates after transfection for 12 h. After transfection for 24, 48 or 72 h, 10 μl of CellTiter 96 AQueous One Solution reagent was added to each well, and the cells were incubated for 3 h at 37°C. The optical density (OD) at 490 nm was measured using a microplate reader (Multiskan MK3; Thermo Fisher Scientific, Waltham, MA, USA).

Flow cytometric analysis

A cell cycle assay kit was used for cell cycle analysis. Briefly, cells were harvested, washed with PBS and fixed with 70% ethanol at 4°C. After overnight fixation, the cells were washed with PBS again and stained with propidium iodide (PI) for 30 min. Cell cycle analysis was performed using the BD FACSCalibur Cytometer System (BD LSRII; San Jose, CA, USA). An annexin V-FITC apoptosis detection kit (Nanjing KGI Biological Technology Development, Nanjing, China) was used to detect apoptotic cells. Briefly, the cells were harvested, washed twice with PBS and resuspended in 500 μl binding buffer. Five microlitres of annexin V-FITC and then 5 μl PI were added and mixed at room temperature in the dark for 15 min. The cells were analysed by flow cytometry within 1 h. Each experiment was repeated three times.

Western blotting

After transfection, each group of cells was lysed using radioimmunoprecipitation assay (RIPA) buffer. Total protein concentration was determined using the BCA Protein Assay Kit (Beyotime, Nantong, China). Equal amounts of total protein were separated using 10% SDS polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Pall Corporation, Port Washington, NY, USA). Transferred membranes were blocked with 5% non-fat milk in TRIS-buffered saline containing 0.05% Tween-20 (TBST) for 1 h at room temperature, and then incubated with rabbit monoclonal anti-CYP11B2 antibody (1:2000; ab167413; Abcam, Cambridge, MA, USA), rabbit monoclonal anti-Bax antibody (1:3000; ab32503; Abcam) or rabbit polyclonal anti-Bcl2 antibody (1:5000; ab59348; Abcam) for 1 h. After being washed with TBST, PVDF membranes were incubated with Goat Anti-Rabbit IgG(H + L) secondary antibody (1:5000; Southern Biotech, Birmingham, AL, USA) and visualized using Thermo Scientific Pierce ECL Plus Substrate.

Aldosterone measurement

H295R cells were plated at a concentration of 5×10^5cells/ml in 24-well plates and incubated for 24 h. After transfection, each group of cells was cultured with complete media and allowed to recover for 16 h. Following, cell culture media were removed and replaced with media with or without Ang II (10 nM) and cells cultured for an additional 24 h. At the end of the incubation period, cell culture supernatants were saved for aldosterone measurement using a human aldosterone ELISA kit (Cayman Chemical Company, Ann Arbor, MI, USA). Cells were lysed with M-PER lysis buffer (Pierce, Rockford, IL) and protein concentration measured by the bicinchoninic acid method (Thermo Scientific Pierce, Rockford, IL, USA) using BSA as standard.

Reporter vector construction and luciferase reporter assay

The full-length wild-type 3′-untranslated region (UTR) of *CYP11B2* and the mutant 3′-UTR of *CYP11B2* were amplified and cloned into the psiCHECK-2 vector (Promega), yielding the wild 3′-UTR and mutant 3′-UTR constructs respectively. The primer sequences used for construction of the luciferase reporter vectors are shown in Table 2. Sequences of all plasmids were confirmed by DNA sequencing. 293T cells plated on 24-well plates were co-transfected with 100 ng plasmid and 200 nmol/l of miR-193a-3p mimic or NC miRNA mimic. Cells were harvested and lysed 48 h after transfection, and firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Three independent experiments were performed.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 software (SPSS Inc., USA). All data are expressed as the mean ± standard deviation (SD). Differences between two groups were assessed by Student's *t*-test. Differences between more than two groups were analysed by one-way ANOVA followed by a post hoc least significant difference test. $P < 0.05$ was considered statistically significant.

Results

Expression levels of miR-140-3p, miR-193a-3p and miR-22-3p in APA tissue

The expression levels of miR-140-3p, miR-193a-3p and miR-22-3p in 15 paired samples of APA and adjacent normal adrenal gland tissue were measured by qRT-PCR. miR-193a-3p showed a statistically significant decrease in expression in APA compared with that in normal adrenal gland tissue ($P < 0.05$) (Figure 1a); there was no significant difference in the expression level of miR-140-3p or miR-22-3p between the two groups (Figure 1a). In addition, CYP11B2 mRNA expression showed a statistically significant increase in APA compared with that in normal adrenal gland tissue ($P < 0.05$)

Table 1 Primers for quantitative real-time RT-PCR

Primer name	Sequence (5′- 3′)
miR-140-3p-F	ACACTCCAGCTGGGTACCACAGGGTAGAACC
miR-193a-3p-F	ACACTCCAGCTGGGAACTGGCCTACAAAGTCC
miR-22-3p -F	ACACTCCAGCTGGGAAGCTGCCAGTTGAAGAA
miRNA-R	CTCAACTGGTGTCGTGGA
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
CYP11B2-F	AAAGGCCCCTGTGGTCACTTA
CYP11B2-R	GACCTGGTCCATGAAAGACGA
18S rRNA-F	CCTGGATACCGCAGCTAGGA
18S rRNA-R	GCGGCGCAATACGAATGCCCC

F, Forward primer; R, reverse primer.

Table 2 Primers for luciferase reporter construction

Primer name	Sequence (5′-3′)
CYP11B2-XhoI-F	CCGCTCGAGCAGGATTGGGCCAACAAGGACTC
CYP11B2-NotI-R	ATAAGAATGCGGCCGCGGATCCCATGATCTAGTGCTG
Mutant-F	GTAGTTCCAGGACTCAGGGACTCACGCGTCTCACCCTGTGACCGCAGGTTGC
Mutant-R	GCAACCTGCGGTCACAGGGTGAGACGCGTGAGTCCCTGAGTCCTGGAACTAC

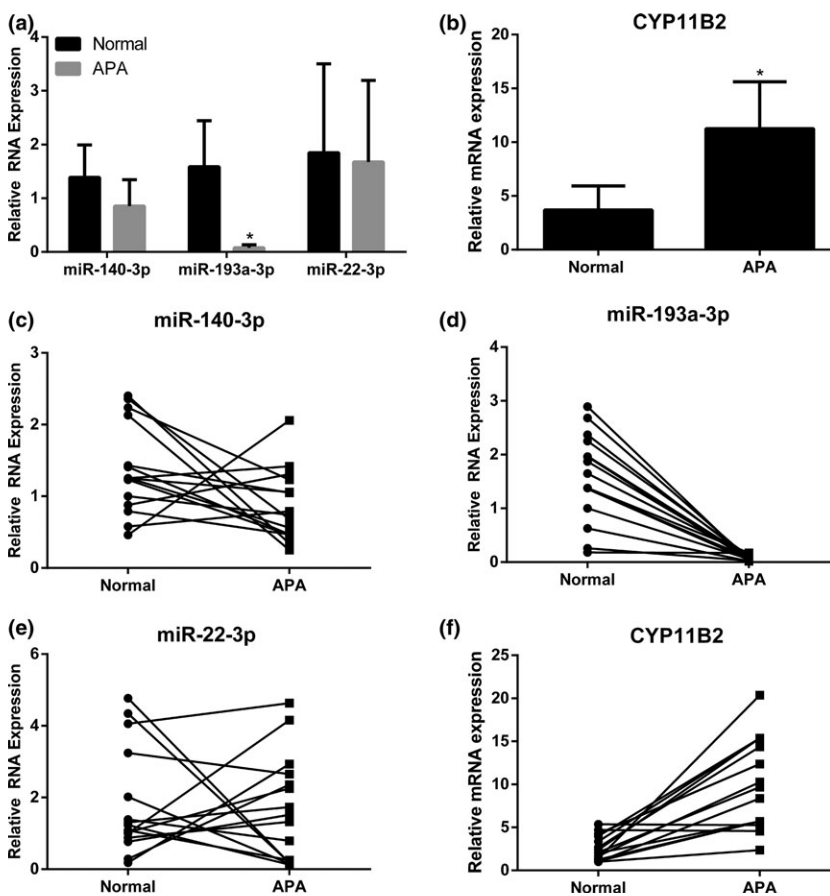

Figure 1 Expression levels of miR-140-3p, miR-193a-3p and miR-22-3p in aldosterone-producing adrenocortical adenoma (APA) and adjacent normal adrenal gland tissue (normal), measured by qRT-PCR. (a, b) The statistical results of miR-140-3p, miR-193a-3p, miR-22-3p and CYP11B2 presented as bar graph. *$P < 0.05$. (c–f) Expression levels of miR-140-3p, miR-193a-3p, miR-22-3p and CYP11B2 in 15 paired samples of APA and adjacent normal adrenal gland tissue.

(Figure 1b). Following, the comparison of individual samples was made (Figure 1c–f). The results indicated that only miR-140-3p and miR-22-3p expression levels have wide disparities between 15 paired samples of APA and adjacent normal adrenal gland tissue (Figure 1c,e). Therefore, miR-193a-3p was investigated further in the following assays.

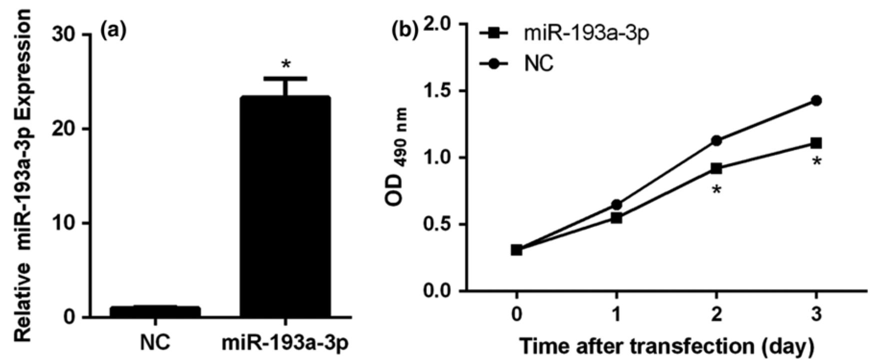

Figure 2 miR-193a-3p overexpression inhibits cell proliferation in the human adrenocortical cell line H295R. (a) miR-193a-3p expression levels after transfection with miR-193a-3p mimic or negative control (NC) miRNA mimic. (b) OD_{490} of H295R cell cultures immediately after transfection with miR-193a-3p mimic or NC miRNA mimic, and one, two and three days after transfection. $*P < 0.05$.

Effect of miR-193a-3p overexpression on cell proliferation in the human adrenocortical cell line H295R

The effect of miR-193a-3p on the regulation of adrenocortical cell proliferation was assessed by transfecting H295R cells with miR-193a-3p mimic or NC miRNA mimic. A significant increase in miR-193a-3p expression after transfection for 48 h was confirmed by qRT-PCR ($P < 0.05$) (Figure 2a). The OD_{490} of H295R cells 2 or 3 days after transfection with miR-193a-3p mimic was significantly reduced compared with that of cells transfected with NC miRNA mimic (Figure 2b), indicating that miR-193a-3p overexpression can inhibit cell proliferation in the human adrenocortical cell line H295R.

To uncover the underlying mechanism for suppression of cell proliferation by miR-193a-3p overexpression, we performed flow cytometry to observe the distribution of cell cycle stages in H295R cells after transfection with miR-193a-3p mimic or NC miRNA mimic. The results showed that overexpression of miR-193a-3p induced a significant G1-phase arrest in H295R cells and a significant decrease in the percentage of cells in S phase (Figure 3a,b).

Effect of miR-193a-3p overexpression on cell apoptosis in the human adrenocortical cell line H295R

The effect of miR-193a-3p overexpression on cell apoptosis was assessed by flow cytometry analysis. Analysis of cell

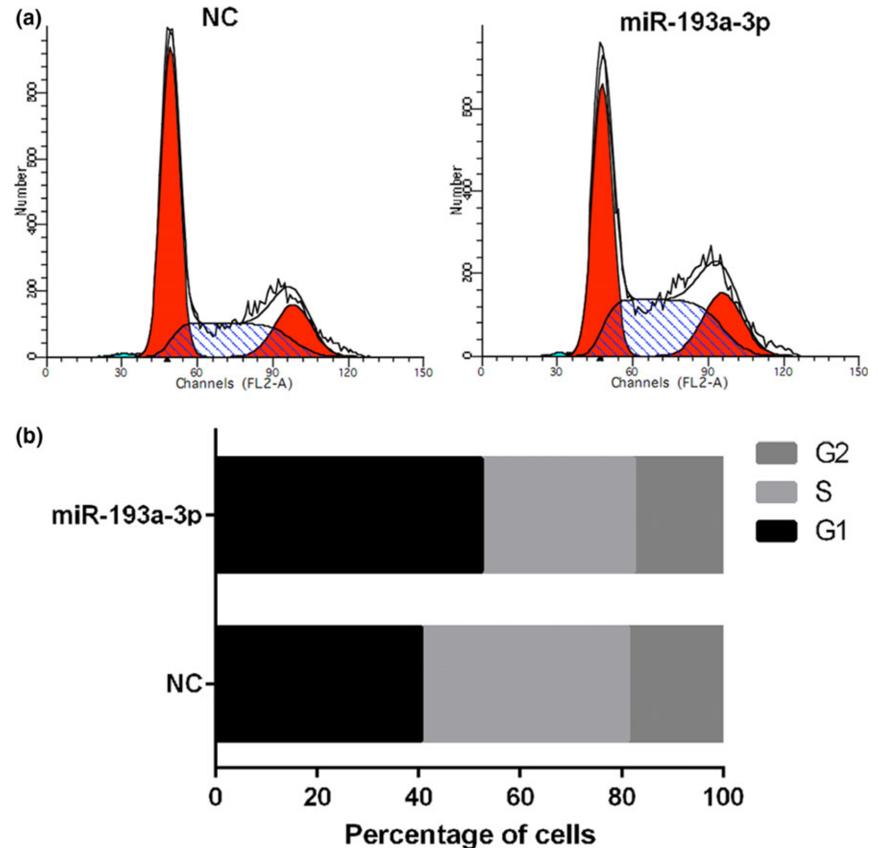

Figure 3 Effect of miR-193a-3p overexpression on cell cycle progression in the human adrenocortical cell line H295R. After transfection with miR-193a-3p mimic or negative control (NC) miRNA mimic, the distribution of cell cycle stages in H295R cells was analysed by flow cytometry. (a) Representative graphs; (b) distribution of cell cycle stages. [Colour figure can be viewed at wileyonlinelibrary.com]

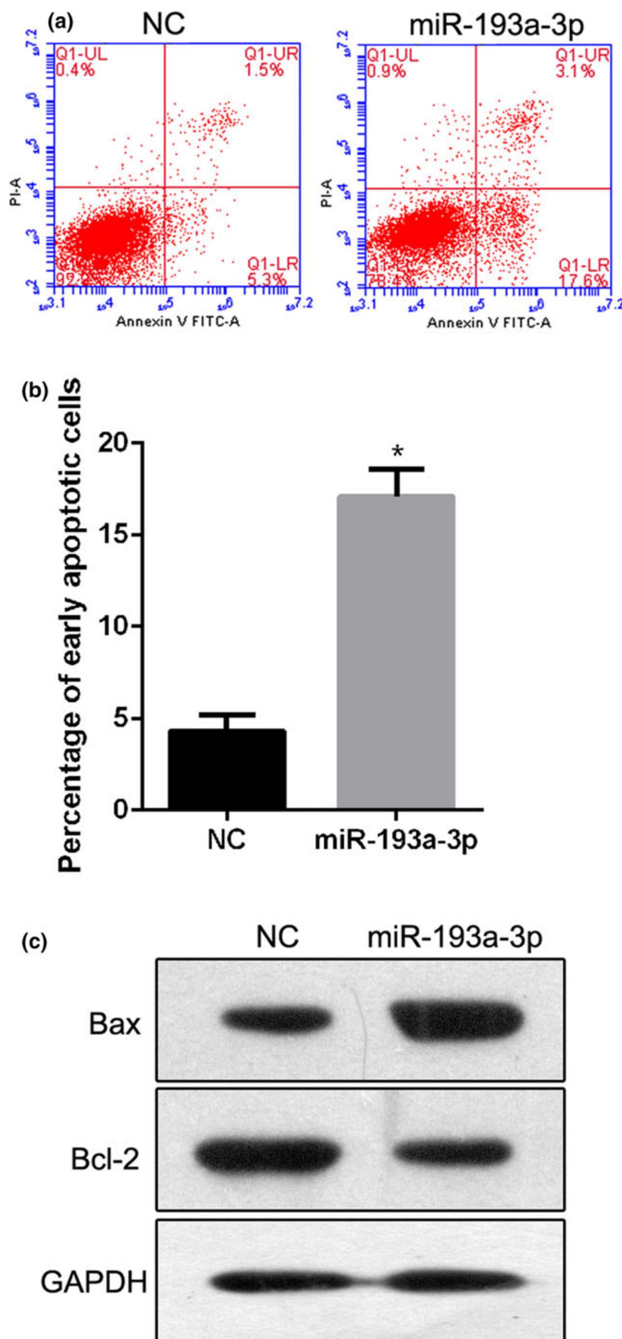

Figure 4 Effect of miR-193a-3p overexpression on apoptosis in the human adrenocortical cell line H295R. H295R cells were transfected with miR-193a-3p mimic or negative control (NC) miRNA mimic. (a) Representative results from flow cytometry analysis. (b) Percentage of early apoptotic cells determined by flow cytometry analysis. (c) Protein expression of Bax and Bcl-2 measured by Western blotting. *$P < 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com]

apoptosis showed that overexpression of miR-193a-3p markedly increased the percentage of early apoptotic H295R cells (Figure 4a,b). In addition, we measured the expression levels of the apoptosis-related proteins Bax and

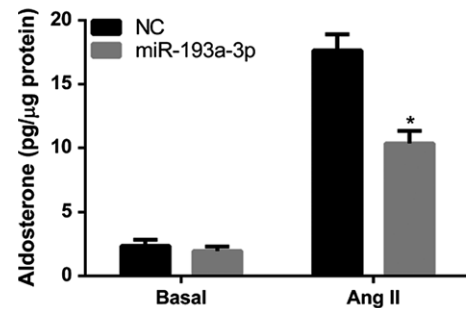

Figure 5 Effect of miR-193a-3p overexpression on aldosterone secretion by H295R cells incubated in the presence or absence of a submaximal Ang II concentration (10 nM). Aldosterone level in cell culture supernatants was detected using ELISA. *$P < 0.05$.

Bcl-2 by Western blot analysis, which showed that miR-193a-3p overexpression upregulated the protein expression of Bax and downregulated the protein expression of Bcl2. Together, these results indicate that miR-193a-3p can promote apoptosis of H295R cells.

Effect of miR-193a-3p overexpression on aldosterone secretion by H295R cells

To study the role of miR-193a-3p overexpression on aldosterone secretion, H295R cells were incubated in the presence or absence of a submaximal Ang II concentration (10 nM) and ELISA was carried out to detect aldosterone level in cell culture supernatants. The results showed that miR-193a-3p overexpression caused a 40% decrease in aldosterone secretion under Ang II submaximal stimulatory conditions and did not cause any significant effect under basal conditions (Figure 5).

CYP11B2 *is a direct target of miR-193a-3p*

To determine whether miR-193a-3p downregulates *CYP11B2* expression by binding to the 3′-UTR of *CYP11B2* mRNA, luciferase reporter assays were performed. A 573-bp fragment of the 3′-UTR of the *CYP11B2* gene, which contains the wild-type miR-193a-3p-binding site (Figure 6a), was cloned into the psiCHECK-2 vector. Additionally, mutant construct with mutation that disrupts the putative miR-193a-3p binding site in the 3′-UTR of *CYP11B2* (Figure 6b) was also prepared. The constructs were co-transfected with miR-193a-3p mimic or NC miRNA mimic in H295R cells, and the luciferase activity associated with each construct was measured. Overexpression of miR-193a-3p in H295R cells significantly reduced the luciferase activity of the wild-type *CYP11B2* 3′-UTR construct (WT 3′-UTR), and this reduction in luciferase activity was abolished when the miR-193a-3p-binding site was removed, as in the mutant *CYP11B2* 3′-UTR construct (mutant 3′-UTR) (Figure 6c). Next, we examined the effect of miR-193a-3p overexpression on *CYP11B2* mRNA and protein levels. The results showed that miR-193a-3p overexpression caused degradation of

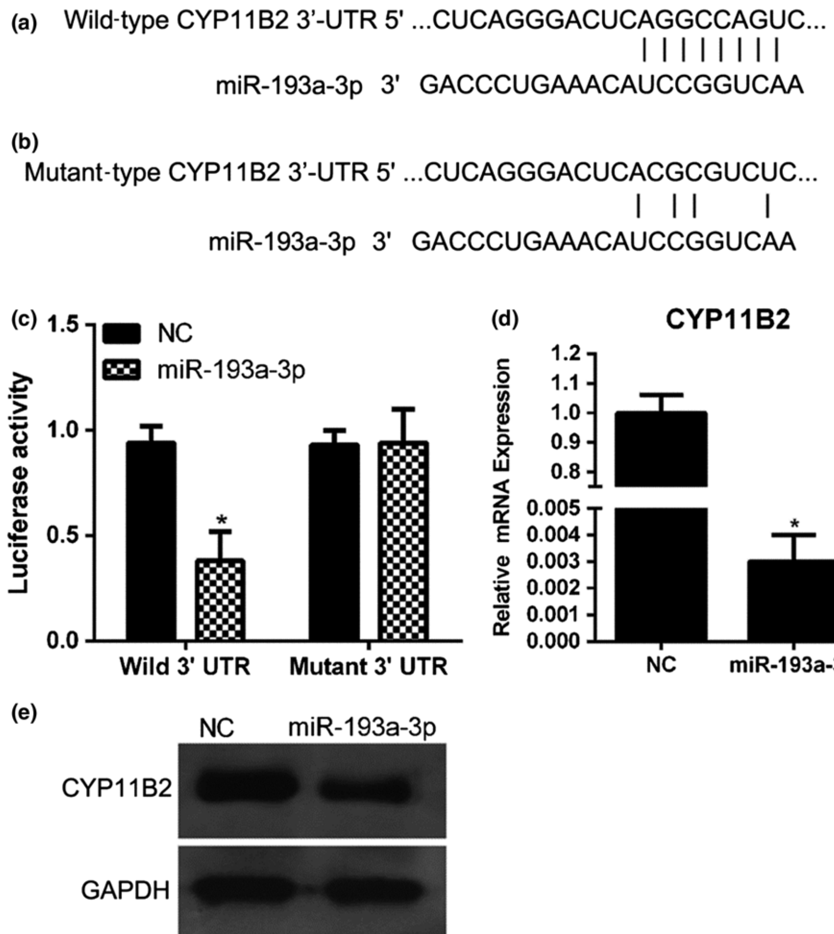

Figure 6 *CYP11B2* is a direct target of miR-193a-3p. (a) Predicted duplex formation between the wild-type *CYP11B2* 3′-UTR and miR-193a-3p. (b) Duplex formation between the mutant *CYP11B2* 3′-UTR and miR-193a-3p. (c) Luciferase activity of wild-type (WT 3′-UTR) or mutant (mutant 3′-UTR) *CYP11B2* 3′-UTR reporters in H295R cells transfected with miR-193a-3p mimic or negative control (NC) miRNA mimic. (d) qRT-PCR data for *CYP11B2* mRNA in H295R cells transfected with miR-193a-3p mimic or NC miRNA mimic. Data were normalized relative to that for 18S rRNA. (e) Western blotting of CYP11B2 in H295R cells transfected with miR-193a-3p mimic or NC miRNA mimic. Data are expressed as mean ± SD, *$P < 0.05$.

CYP11B2 mRNA (Figure 6d). In addition, a clear reduction in the level of endogenous CYP11B2 protein was observed (Figure 6e). However, the inhibition ratio of miR-193a-3p overexpression on *CYP11B2* mRNA is far greater than that on CYP11B2 protein (Figure 6c–e).

CYP11B2 *overexpression diminishes the effect of miR-193a-3p overexpression on cell proliferation, cell cycle progression and cell apoptosis*

To further examine whether miR-193a-3p affects proliferation, cell cycle progression and apoptosis of H295R cells through a *CYP11B2*-dependent mechanism, a *CYP11B2* overexpression vector was constructed and transfected into H295R cells. As shown in Figure 7a, CYP11B2 protein levels increased in cells transfected with the *CYP11B2* overexpression vector pcDNA-CYP11B2, compared with that in cells transfected with the empty pcDNA vector. Transfection of H295R cells with both miR-193a-3p and the *CYP11B2* overexpression vector resulted in a decrease in OD_{490} compared with that in cells transfected with both miR-193a-3p and empty vector (Figure 7b). In addition, transfection of H295R cells with the *CYP11B2* overexpression vector resulted in an increased proportion of cells in G1 phase and a decreased proportion of cells in S phase, compared with that for cells transfected with empty vector (Figure 7c,d). Finally, the percentage of early apoptotic H295R cells and the protein expression level of Bax were decreased, and the protein expression level of Bcl-2 was increased in cells transfected with the *CYP11B2* overexpression vector, compared with cells transfected with empty vector (Figure 7e). Together, these results indicate that *CYP11B2* overexpression diminishes the effect of miR-193a-3p overexpression on proliferation, cell cycle progression and apoptosis in H295R cells.

CYP11B2 *overexpression diminishes the effect of miR-193a-3p overexpression on aldosterone secretion by H295R cells*

To further examine whether miR-193a-3p affects aldosterone secretion of H295R cells through a *CYP11B2*-dependent mechanism, a *CYP11B2* overexpression vector was constructed and transfected into H295R cells. After transfection, H295R cells were incubated in the presence or absence of a submaximal Ang II concentration (10 nM) and ELISA was carried out to detect aldosterone level in cell culture supernatants. The results showed that transfection of H295R cells with both miR-193a-3p and the *CYP11B2*

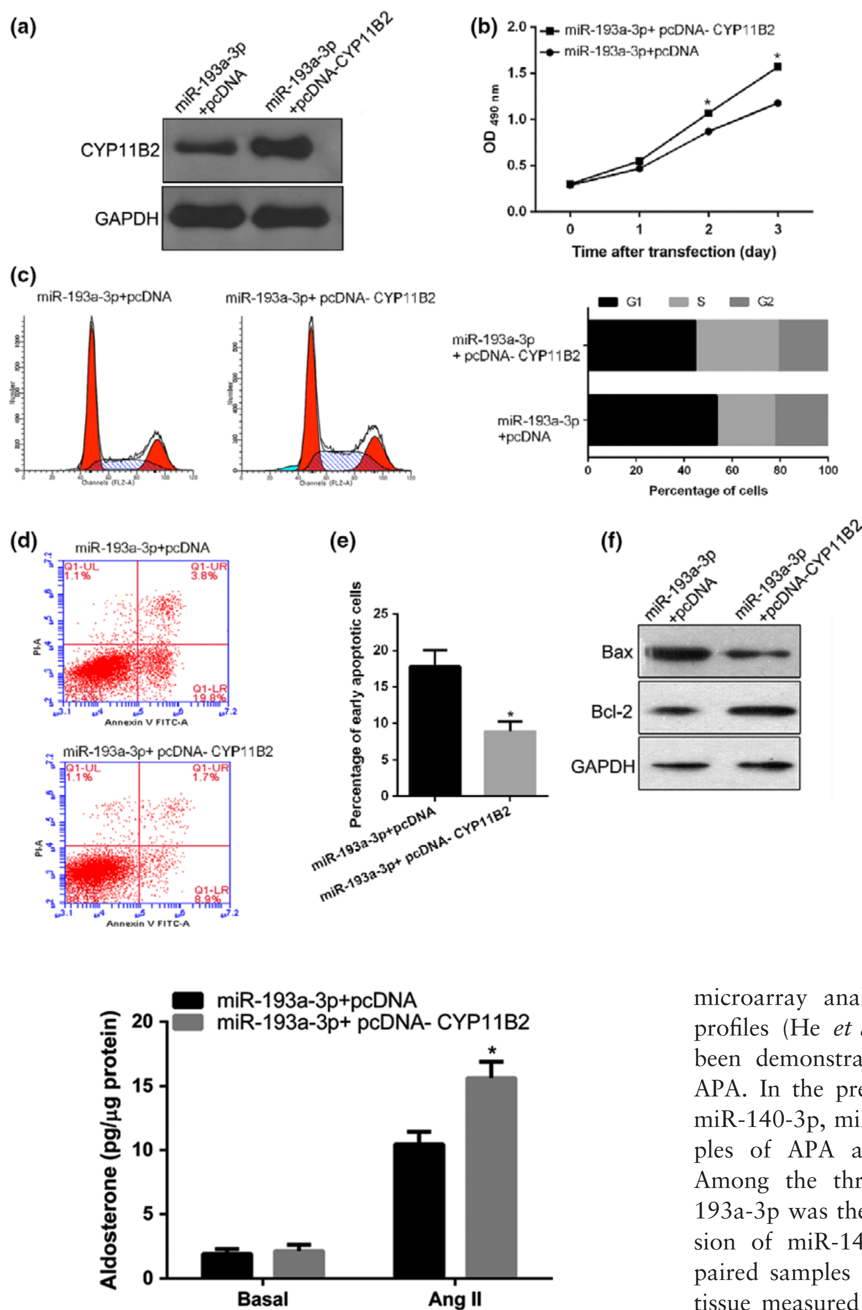

Figure 7 CYP11B2 overexpression diminishes the effect of miR-193a-3p overexpression on proliferation, cell cycle progression and apoptosis in H295R cells. For each assay, cells were harvested after transfection with miR-193a-3p plus the *CYP11B2* overexpression vector pcDNA-*CYP11B2* or miR-193a-3p plus the empty pcDNA vector. (a) CYP11B2 protein expression detected by Western blotting. (b) The OD_{490} of H295R cells immediately after transfection and 1, 2 and 3 days after transfection. (c) Distribution of cell cycle stages in transfected H295R cells. The left panels show representative results from flow cytometry analysis of cell cycle distributions. The right panel summarizes the distribution of cell cycle stages. (d) Representative results of flow cytometry analysis of cell apoptosis. (e) Percentage of early apoptotic cells measured by flow cytometry analysis. (f) Bax and Bcl-2 protein expression detected by Western blotting. $*P < 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com]

Figure 8 *CYP11B2* overexpression diminishes the effect of miR-193a-3p overexpression on aldosterone secretion of H295R cells.

overexpression vector resulted in an increase in aldosterone secretion compared with that for cells transfected with both miR-193a-3p and empty vector under Ang II submaximal stimulatory conditions and did not cause any significant effect under basal conditions (Figure 8).

Discussion

Aldosterone-producing adrenocortical adenoma and normal adrenal gland tissue have been demonstrated by miRNA microarray analysis to have different miRNA expression profiles (He *et al.* 2015). Nevertheless, few miRNAs have been demonstrated to play a role in the pathogenesis of APA. In the present study, we measured the expression of miR-140-3p, miR-193a-3p and miR-22-3p in 15 paired samples of APA and adjacent normal adrenal gland tissue. Among the three differentially expressed miRNAs, miR-193a-3p was the most downregulated. However, the expression of miR-140-3p, miR-193a-3p and miR-22-3p in 15 paired samples of APA and adjacent normal adrenal gland tissue measured by qRT-PCR in this study is not in accordance with those in previous studies; He *et al.* (2015) showed by miRNA microarray analysis that miR-140-3p, miR-193a-3p and miR-22-3p are all upregulated in nine APA tissue compared with that in four normal human adrenal cortex tissue. This difference may be a result of the difference in methods used for miRNA detection, the difference in patients' sample number and the difference references used to compare miRNA expression. However, patients' sample number used both in our present study and the study of He *et al.* is all small. So large samples are needed to further confirm the expression levels of miR-140-3p, miR-193a-3p and miR-22-3p in APA.

In previous investigations, miR-193a-3p has been reported to suppress cell proliferation and promote apoptosis

(Nakano *et al.* 2013; Wang *et al.* 2013). However, its role in APA has not been clarified. In the present study, we investigated the effects of miR-193a-3p on aldosterone secretion, cell proliferation, cell cycle progression and cell apoptosis in the human adrenocortical cell line H295R. H295R is the most commonly used *in vitro* model of hyperaldosteronism in preclinical research, because this cell line possesses a steroid secretion pattern and regulation similar to those of primary adrenal cell cultures (Lichtenauer *et al.* 2013). Our results show that miR-193a-3p overexpression can markedly inhibit aldosterone secretion, cell proliferation, promote cell apoptosis and induce a significant G1-phase arrest. The results related to cell proliferation and apoptosis are in accordance those of with previous studies; for example, miR-193a-3p has been shown to suppress cell proliferation in human endothelial cell (Khoo *et al.* 2017) and promote apoptosis in lung cancer (Liang *et al.* 2015). Based on the results that miR-193a-3p overexpression can markedly inhibit aldosterone secretion, we predicted that miR-193a-3p may be involved in the pathogenesis of APA.

It is known that miRNAs exert their function by binding to the 3′ UTR of target genes, resulting in degradation of mRNA or inhibition of mRNA translation (Choudhuri 2010). In the present work, miR-193a-3p overexpression reduced the luciferase activity of the wild-type *CYP11B2* 3′-UTR construct, and this reduction in luciferase activity was abolished when the miR-193a-3p-binding site was mutated, indicating that miR-193a-3p can bind to the 3′-UTR of *CYP11B2*. Moreover, miR-193a-3p overexpression reduced both *CYP11B2* mRNA and protein levels. Considering the effect of miR-193a-3p overexpression on the mRNA level of *CYP11B2*, we predict that miR-193a-3p regulates *CYP11B2* expression by degrading *CYP11B2* mRNA. However, the inhibition ratio of miR-193a-3p overexpression on CYP11B2 mRNA is far greater than that on CYP11B2 protein. This yawning disconnect between the decreased mRNA and protein levels induced by miR-193a-3p overexpression indicated that there may be other factors that can regulate CYP11B2 mRNA translation to upregulate the CYP11B2 protein. And these factors may be regulated by miR-193a-3p. It is well known that a single miRNA can target multiple genes and that multiple miRNAs can target a single gene. Thus, miR-193a-3p may also have mRNA targets other than *CYP11B2*; these additional targets may also play important roles in APA formation and may regulate CYP11B2 mRNA translation.

Interestingly, we observed that restoration of *CYP11B2* expression can successfully attenuate the effect of miR-193a-3p overexpression on aldosterone secretion, cell proliferation, cell cycle progression and cell apoptosis in H295R cells, even though miR-193a-3p has many other targets. These results suggest that targeting of *CYP11B2* is a major mechanism by which miR-193a-3p exerts its function in the pathogenesis of APA. Therefore, the modulation of *CYP11B2* by miR-193a-3p might explain, at least in part, why the downregulation of miR-193a-3p during APA formation may promote aldosterone secretion, cell growth and suppress cell apoptosis. However, the function of miR-193a-3p in APA needs to be further demonstrated *in vivo*. Because CYP11B2 expression can successfully attenuate the effect of miR-193a-3p overexpression on aldosterone secretion, cell proliferation, cell cycle progression and cell apoptosis in H295R cells, these results may raise the possibility that the effects of miR-193a-3p may be self-limiting *in vivo* and that some other driver is involved to disturb such a possible homeostatic mechanism, for example a somatic mutation leading to a disordered intracellular milieu (Williams *et al.* 2014).

In conclusion, the expression level of miR-193a-3p is decreased in APA tissue. Additionally, elevated miR-193a-3p expression can suppress proliferation and promote apoptosis in H295R cells through downregulation of its target gene, *CYP11B2*. However, studies *in vivo* are needed to confirm the role of miR-193a-3p in the pathogenesis of APA.

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Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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