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CTNNB1 Knockdown Inhibits Cell Proliferation and Aldosterone Secretion Through Inhibiting Wnt/β-Catenin Signaling in H295R Cells

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Abstract

Aldosterone-producing adenomas (APA) is one of the causative factors of primary aldosteronism. Previous studies have suggested that there are somatic *CTNNB1* mutations in APA, but the specific mechanism of *CTNNB1* mutation in APA tumorigenesis and aldosterone secretion remains unclear. In the present study, human adrenocortical carcinoma cell line H295 R was used to establish stable *CTNNB1* knockdown cell lines. Cell proliferation and aldosterone secretion of H295 R cells in response to angiotensin II (Agn II) were analyzed. We found that *CTNNB1* knockdown reduced β-catenin expression and inhibited proliferation of H295 R cells. *CTNNB1* knockdown inhibited Wnt/β-catenin signaling pathway and downregulated expression of downstream genes including axin 2, lymphoid enhancer binding factor 1 (*LEF1*), and cyclin D1. In addition, *CTNNB1* knockdown decreased responses of H295 R cells to Agn II and decreased aldosterone secretion. Our findings suggest that *CTNNB1* knockdown can inhibit H295 R cell proliferation and decrease aldosterone secretion in the responses of H295 R cells to Ang II through inhibiting Wnt/β-catenin signaling pathway, indicating that targeting Wnt/β-catenin signaling pathway may be an important approach to decrease aldosterone secretion in the treatment of aldoster-producing adenomas.

Keywords

aldosterone-producing adenomas, *CTNNB*, Wnt/β-catenin signaling pathway, aldosterone secretion, H295 R cell

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Introduction

Primary aldosteronism (PA), which is characterized by hyperaldosteronism, affects 20% of patients with refractory hypertension.[1] The excessive production of aldosterone leads to hypertension, low plasma renin activity, varying degrees of hypokalemia, and metabolic alkalosis.[2] Now, PA is being recognized as the most common cause of secondary hypertension with a prevalence of 5-10% in hypertensive individuals and up to 20% in therapy-resistant hypertension.[3,4] The pathogenesis of PA is caused by adrenal aldosterone-producing adenomas (APA) or bilateral hyperplasia in 95% of patients.[5] Clinical therapy of PA relies on surgical resection and/or treatment with mineralocorticoids antagonists to minimize hypertension.[6,7] However, the medication time is still inconclusive and serious side effects are still not resolved. Therefore, existing treatment strategies are limited, which warrants exploring the molecular mechanisms of PA to improve therapeutic outcomes.

Wnt/β-catenin signaling pathway has extensive biological effects and is relatively conservative in evolution, which has important effects on ontogeny, cell differentiation, apoptosis and necrosis.[8] Previous studies indicate that the abnormal activated

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Table 1. Sequences of the Primers Used in This Study.

Target gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
CTNNB1	CCCAGAATGCAGTTCGCCTT	TAGTCGTGGAATGGCACCCT
AXIN2	CACCACCACCACCACCATTCG	ACATGCTTCGTCGTCTGCTTGG
LEF1	AGATTCTTGGCAGAAGGTGGCATG	GGACCTGTACCTGATGCAGATTCC
CYP11B1	GAACTGTCGCCAGATGCCAT	CGCTCCAGAATCAGACCCAC
CYP11B2	GTGACCGCAGGTTGCTTTCC	AGCCATCTCTGAGGTCTGTGC
Cyclin D1	GCTGCGAAGTGGAAACCATC	CCTCCTTCTGCACACATTTGAA
β-actin	CCTGGCACCCAGCACAAT	GGGCCGGACTCGTCATAC

Wnt/β-catenin pathway has been involved in tumor progression, such as breast cancer, colorectal cancer, and ovarian cancer.[9-11] β-catenin is the core molecule of Wnt/β-catenin signaling pathway, which has transcriptional regulatory activity and is encoded by *CTNNB1* gene, which located on human chromosome 3p21～22, with a total length of 23.2 kb and 16 exons.[11,12] Abnormal Wnt/β-catenin activation caused by somatic *CTNNB1* mutations has been found in APA,[13] and silencing *CTNNB1* has been shown to inhibit cell proliferation and stimulates apoptosis in APA cell line H295 R through decreasing Wnt/β-catenin-LEF/TCF dependent transcription.[14] Transgenic mice with specific constitutive activation of β-catenin in the adrenal cortex present with increased aldosterone production and even a risk of adrenocortical tumors.[15,16] These researches suggest that Wnt/β-catenin signaling pathway triggers off APA tumorigenesis and may be involved in aldosterone secretion.

APA patients with *CTNNB1* mutation are older and have more obvious hypertension symptoms. After adrenalectomy, *CTNNB1* mutation carriers have a higher likelihood of residual hypertension than other APA patients.[17] Akerstrom et al reported that APA tissue containing the *CTNNB1* mutant gene has a high expression of CYP11B2.[13] As the last rate-limiting enzyme for aldosterone synthesis, CYP11B2 expression is upregulated by angiotonin II (Ang II) through activating calcium signaling to induce aldosterone secretion.[18] The specific mechanism of *CTNNB1* mutation in APA-induced hyperaldosteronism remains unclear.

In order to explore the effect of Wnt/β-catenin signaling pathway on the development of APA and to better understand the role of *CTNNB1* gene in APA cell proliferation and aldosterone secretion, we knocked down *CTNNB1* gene expression in vitro and investigated the effect of Wnt/β-catenin signaling pathway on cell proliferation and aldosterone secretion of human adrenocortical carcinoma cell line H295 R. Our study suggests that Wnt/β-catenin signaling pathway is a vital role in mediating the secretion of aldosterone in the responses of H295 R cells to Ang II.

Materials and Methods

Cell Culture

Human adrenocortical carcinoma H295 R cell line was cultivated in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 medium complemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 50 U/mL penicillin and 100 mg/mL streptomycin, at 37°C with 5% CO_2. All media and supplements in this study were purchased from Invitrogen (Carlsbad, CA, USA).

CTNNB1 Knockdown

CTNNB1 was knocked down in H295 R cells with siRNA (RiboBio Co., Ltd., Guangzhou, China) in accordance with the manufacturer's instructions. There were 3 target sequences: si-CTNNB1_001, 5′-TGGTTGCCTTGCTCAACAA-3′; si-CTNNB1_002, 5′-GCTTGGAATGAGACTGCTG-3′; si-CTNNB1_003, 5′-AGCTGATATTGATGGACAG-3′. The corresponding negative control sequence for the target gene was purchased from RiboBio Co., Ltd. To stably and efficiently knockdown *CTNNB1* in H295 R cells, siRNA targeting the si-CTNNB1_003 coding sequence 5′-AGCTGATATTGATGGACAG-3′ was designed and inserted into a pGMLV-SC5RNAi lentiviral vector (Genomeditech Co., Ltd, Shanghai, China), and an interference-free siRNA was used as a negative control. Quantitative reverse transcription PCR (qRT-PCR) and Western blot analysis were used to determine the knockdown efficiencies.

Total RNA Extraction and qRT-PCR

Total RNA extraction kit (Solarbio, Beijing, China) was used to extract total RNA from H295 R cells, and was in accordance with the manufacturer's instructions. were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to determine the RNA concentrations. The iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA, USA) was used to reverse-transcribe 1 microgram of total RNA to synthesize cDNA. CFX96 real-time system (Bio-Rad) and SYBR Green Supermix (Bio-Rad) were used to perform PCR and the procedures were performed according to the manufacturer's instructions. The sequences of the primers used in this study are listed in Table 1.

Western Blot Analysis

The prepared protein samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 12% gel. Subsequently, in Tris buffered saline containing 5% skim milk and 0.1% Tween-20, the separated protein samples were transferred to the nitrocellulose membranes after blocking at room

temperature for 1 h. The nitrocellulose membranes were placed in a suitable concentration of primary antibody and incubated for 18 h at 4 °C. Then, membranes were placed in an incubator and stained with horseradish peroxidase-conjugated secondary antibodies for 1 hat room temperature. Finally, membranes were visualized with enhanced chemiluminescence (Super-Signal, Pierce, Rockford, IL) or ECL Plus (Amersham Pharmacia Biotech, Buckinghamshire, UK) substrates in accordance with the manufacturers' instructions.

Cell Viability Assays

Cell viability was assessed by cell counting kit-8 (CCK-8) and colony formation assays. Briefly, cells were inoculated in a 6-well plate (Corning, Corning, NY, USA) at 500 cells per well after being treated with or without *CTNNB1* knockdown. The cells were cultured for 10 days, and the medium was changed at least every 3 days depending on the growth of the cells. The colonies were washed with phosphate-buffered saline (PBS), fixed in methanol, and stained with crystal violet. The number of colonies was counted under a microscope. CCK-8 assays were performed in accordance with the manufacturer's instructions.

Quantification of Aldosterone Using ELISA Kit

After incubation of H295 R cells with the respective control medium, aldosterone in the supernatant of the cells was measured using a commercial human aldosterone ELISA Kit (ALD ELISA kit) in accordance with the manufacturer's protocol (AmyJet Scientific, Wuhan, China).

Statistical Analysis

Each experiment was performed at least 3 times independently. The GraphPad Prism 7.0 statistical program (GraphPad, San Diego, CA, USA) was used for data analysis. The results were presented as the mean $\pm$ standard deviation (SD) unless otherwise indicated. The significance of the difference between the 2 groups was analyzed by 2-tailed Student's t-tests. The differences between the 2 groups of count data were analyzed by chi-square test. $P < 0.05$ was considered statistically significant.

Results

CTNNB1 *Knockdown Reduced β-Catenin Expression and Inhibited Proliferation of H295 R Cells*

Stable *CTNNB1* knockdown in H295 R cell lines were established to actively explore the function of endogenous *CTNNB1*. Based on qRT-PCR and western blot assays, si-h-CTNNB1_003 significantly downregulated the mRNA level and protein expression level of β-catenin (Figure 1A). We utilized this lentivirus-mediated siRNA to induce *CTNNB1* knockdown in H295 R cells (Figure 1B). CCK-8 assays showed that *CTNNB1* knockdown markedly reduced cell viability, compared with the control group (Figure 1C). Similarly, colony formation assays showed that cell proliferation was significantly decreased with *CTNNB1* knockdown in H295 R cells (Figure 1D).

Wnt/β-Catenin Signaling Pathway Was Inhibited by CTNNB1 *Knockdown*

To explore the effects of *CTNNB1* knockdown on Wnt/β-catenin signaling pathway in H295 R cells, we detected the downstream target genes of Wnt/β-catenin signaling pathway including *AXIN2* and lymphoid enhancer binding factor 1 (*LEF1*). Compared with the control group, the mRNA expression levels of AXIN2 (Figure 2A) and LEF1 (Figure 2B) in *CTNNB1* knockdown group were significantly decreased. Western blot analysis revealed that the protein expression levels of AXIN2 and LEF1 were downregulated in *CTNNB1* knockdown group (Figure 2C). After adding angiotensin II (Ang II) to the cells for 6 hours, the results were not affected (Figure 1B and Figure 2A-C). These results suggest that *CTNNB1* knockdown inhibited Wnt/β-catenin signaling pathway.

CTNNB1 *Knockdown Reduced Expression of Cycling D1*

Since cycling D1 is known to play an important role in cell proliferation, qRT-PCR and Western blot analysis were used to detect expression of cycling D1 in H295 R cells. In *CTNNB1* knockdown group, the mRNA levels of cycling D1 were significantly decreased (Figure 3A) and the protein expression levels of cycling D1 were also downregulated (Figure 3B), compared to the control group. In addition, the expression levels of cycling D1 were not affected by adding Ang II. These results suggest that *CTNNB1* knockdown inhibits cell proliferation through reducing expression of cycling D1.

CTNNB1 *Knockdown Decreased Aldosterone Secretion in H295 R Cells*

Cytochrome P450 family 11 subfamily B member 2 (CYP11B2) is one of the rate-limiting enzymes for aldosterone synthesis and is also the terminal enzyme. In order to explore the effect of *CTNNB1* on aldosterone secretion, we detected the basal and Ang II-treated CYP11B2 expression levels. *CTNNB1* knockdown inhibited the mRNA and protein expression levels of *CYP11B2* (Figure 4A and C), but not the expression levels of *CYP11B1* (Figure 4B and C). In addition, Ang II significantly increased the expression levels of *CYP11B2* in H295 R cells, which was reversed by *CTNNB1* knockdown (Figure 4A and C). On the contrary, Ang II also increased the expression levels of *CYP11B1*, which was not influenced by *CTNNB1* knockdown (Figure 4B and C). Moreover, aldosterone ELISA Kit was used to detect the concentration of aldosterone in the supernatant of H295 R cells. We found that Ang II-induced upregulation of aldosterone secretion was reversed by *CTNNB1*

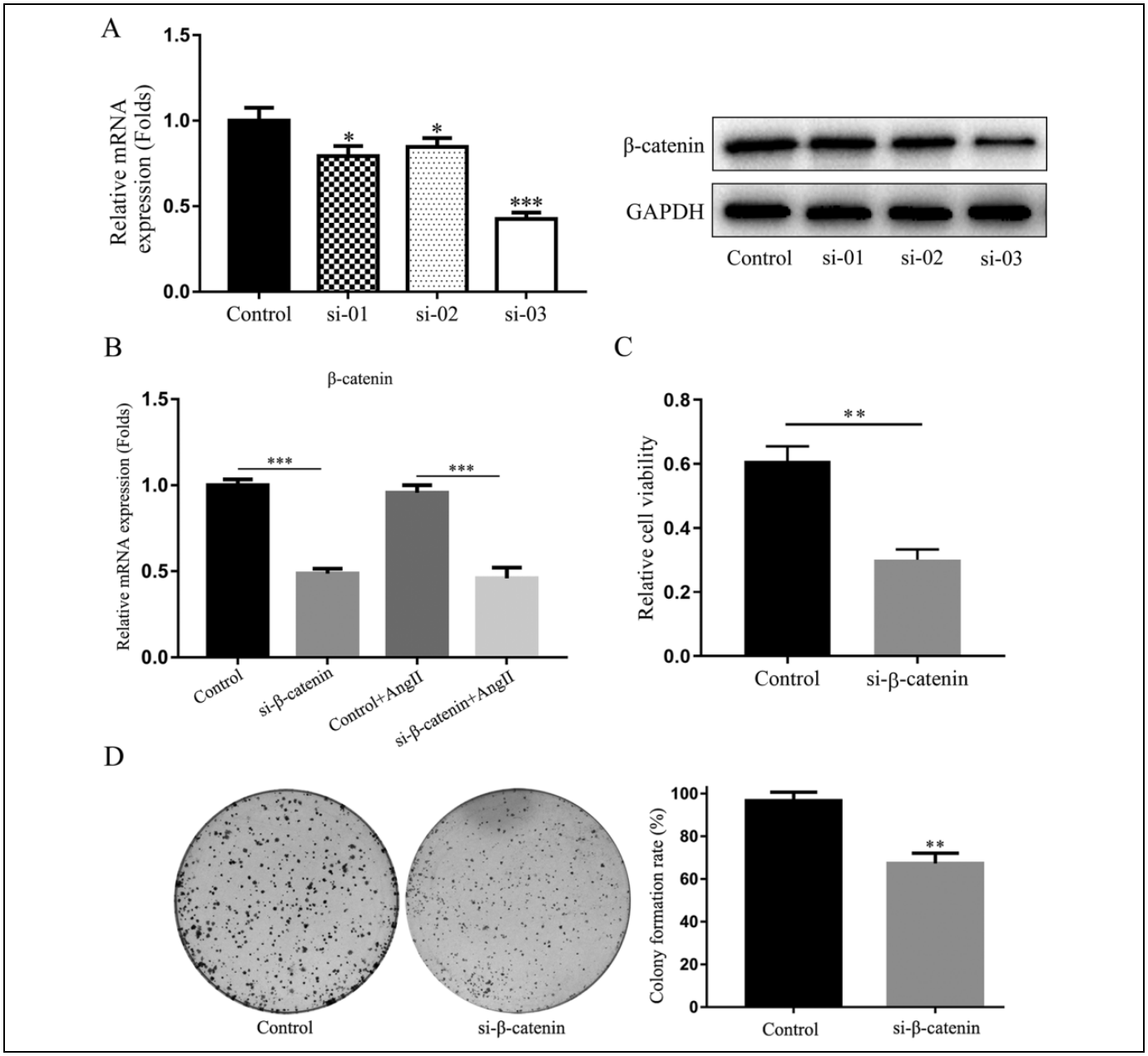

Figure 1. CTNNB1 knockdown reduces β-catenin expression and inhibits proliferation of H295 R cells. (A) Efficiency of β-catenin knockdown was detected by qRT-PCR and Western blot analysis. (B) Transfection efficiency of CTNNB1 knockdown lentiviruses in H295 R cells. (C) Cell viability was detected using CCK-8 assays. (D) Cell proliferation was detected using colony formation assays. Error bars indicate SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

knockdown (Figure 4D). These results suggest that *CTNNB1* knockdown reduces the responses of H295 R cells to Ang II and decreases secretion of aldosterone.

Discussion

Hypertension is one of the most common cardiovascular diseases in China.[19] In recent years, with the clinical application of plasma aldosterone/renin activity ratio (ARR) and the extensive application of computed tomography, magnetic resonance imaging and other imaging technologies, the detection rate of PA in hypertensive population has been significantly improved. PA has become the common cause of intractable hypertension.[1] In addition, the tissues in the heart, brain and kidneys in PA patients were more seriously damaged than those in PH patients, which results in more grievous consequences.[3] Therefore, exploring novel therapeutic targets is an urgent and promising undertaking for the clinical treatment of PA.

Aldosterone production is stimulated by Ang II or extracellular K^+ and is mediated mainly by Ca_2^+ influx into

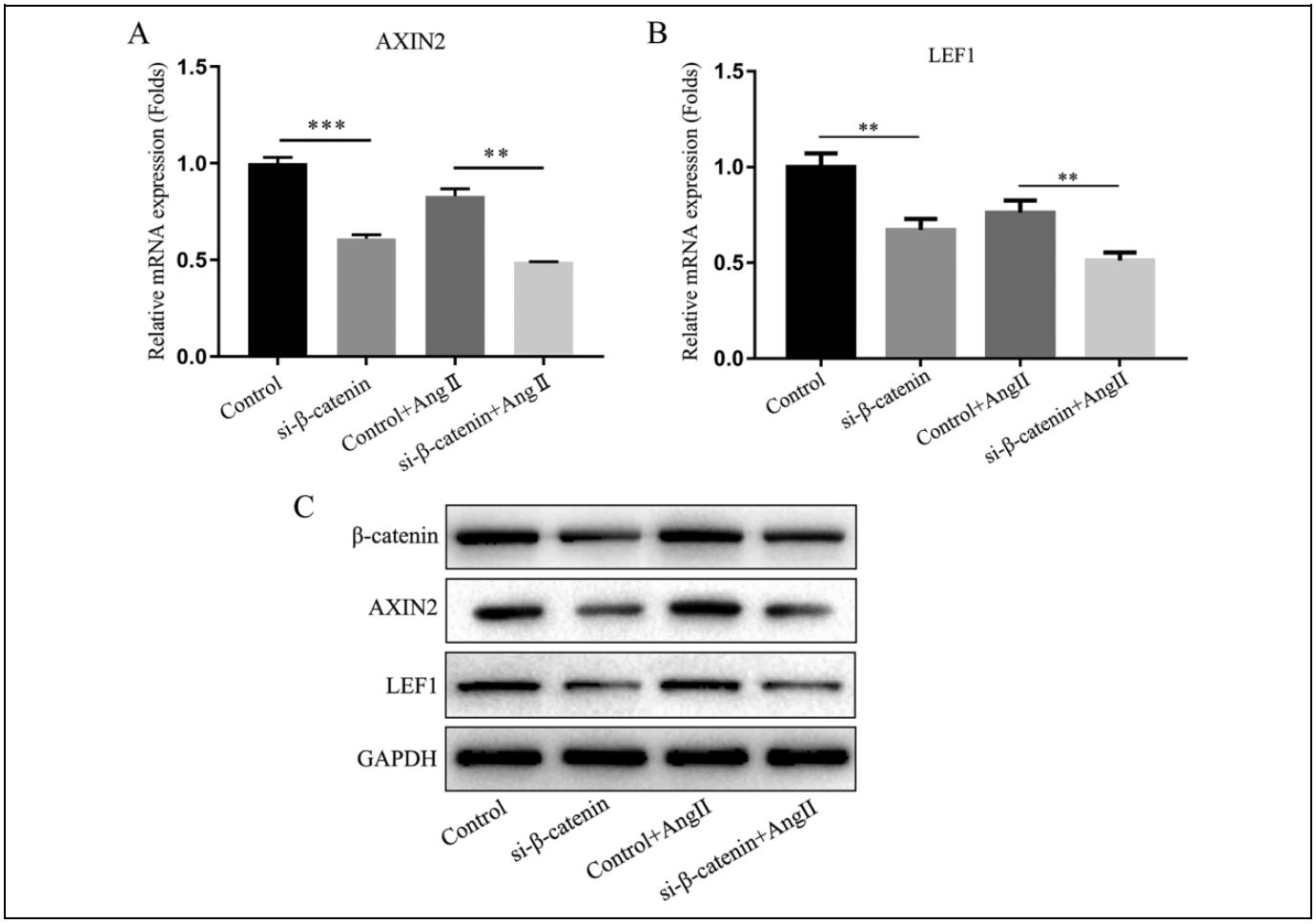

Figure 2. Wnt/β-catenin signaling pathway was inhibited by CTNNB1 knockdown. (A) mRNA levels of AXIN2 were detected by qRT-PCR. (B) mRNA levels of LEF1 were detected by qRT-PCR. (C) Levels of protein expression of AXIN2 and LEF1 were detected using Western blot analysis. Error bars indicate SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

adrenal glomerulosa cells through calcium signaling pathway.[18,20] During the past years, significant progress has been made in the knowledge of the genetic basis of APA development. The mutation site of APA is related to the specific biochemical and clinical features of PA. Over 50% of APA patients have been identified to have multiple gene mutation sites, such as *KCNJ5*, *CACNA1D*, *ATP1A1*, and *ATP2B3* mutations, which belong to cell membrane ion channel protein encoding gene.[21] These mutations may increase intracellular calcium concentrations through various ways, opening voltage-dependent calcium channels, thereby activating calcium signaling and aldosterone secretion.[22] However, there are no obvious association between these mutations and adrenal tumorigenesis.

Most majorities of APA patients have been detected to possess activation of Wnt/β-catenin signal pathway.[23] In adrenal tumors, the main cause for activation of Wnt/β-catenin signal pathway is *CTNNB1* mutation.[13] Patients with *CTNNB1* mutation have larger adenomas size, but not higher level of aldosterone, compared to the patients with other mutations.[17,24] H295 R cells have been shown to be an Ang II-responsive steroid-producing adrenocortical cell line.[25] Gaujoux et al has reported that silencing *CTNNB1* can inhibit cell proliferation and stimulate apoptosis of H295 R through decreasing Wnt/β-catenin-LEF/TCF dependent transcription,[14] but the relationship between Wnt/β-catenin signaling and Ang II-induced aldosterone secretion is unclear. In the present study, the secretion of aldosterone and the expression of rate-limiting enzyme gene *CYP11B1* and *CYP11B2* were increased by Ang II, and the silencing *CTNNB1* inhibited aldosterone secretion and the expression of *CYP11B2* but not *CYP11B1* in H295 R cells. Furthermore, the expression of *CYP11B2* and aldosterone secretion of H295 R cells in response to Ang II were both decreased by *CTNNB1* knockdown, but *CTNNB1* knockdown had no effect on expression of *CYP11B1*. These results suggested that silencing *CTNNB1* genes reduced aldosterone secretion and responsiveness to Ang II of H295 R cells by inhibiting the expression of *CYP11B2*.

In addition, the relationship between *CTNNB1* knockdown and H295 R cell proliferation in response to Ang II was also

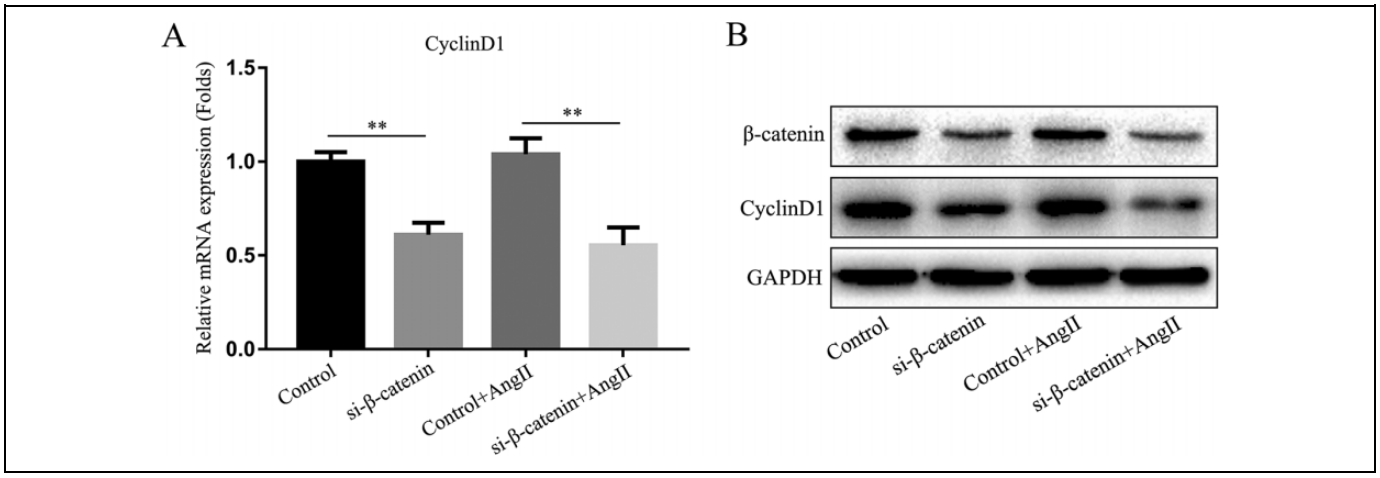

Figure 3. CTNNB1 knockdown reduces expression of cycling D1. (A) mRNA levels of cyclin D1 were detected by qRT-PCR. (B) Levels of cyclin D1 protein expression was detected using Western blot analysis. Error bars indicate SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

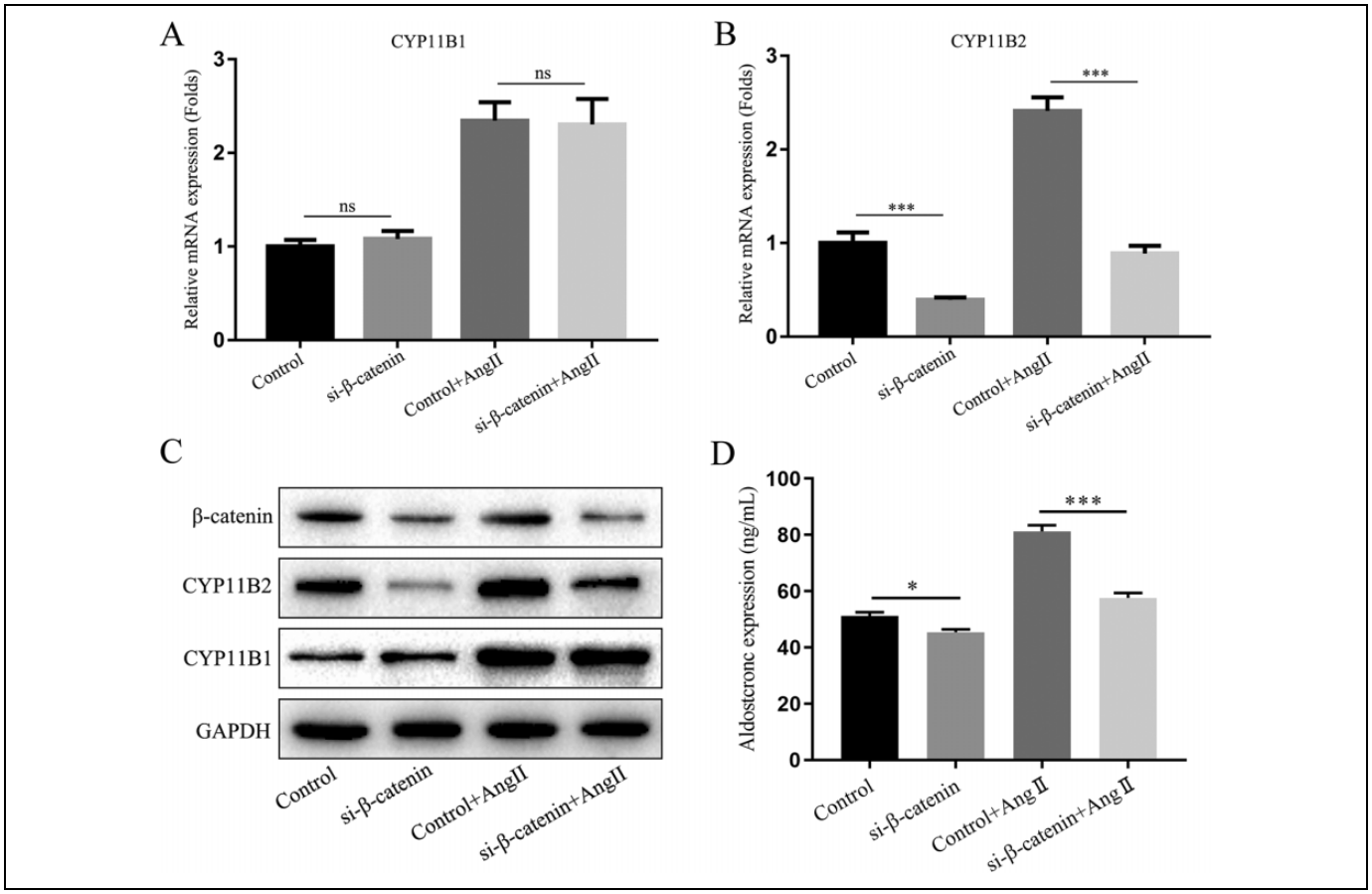

Figure 4. CTNNB1 knockdown decreases aldosterone secretion in H295 R cells. (A) mRNA levels of CYP11B2 were detected by qRT-PCR. (B) mRNA levels of CYP11B1 were detected by qRT-PCR. (C) Levels of CYP11B2 and CYP11B1 protein expression were detected using Western blot analysis. (D) Secretion of aldosterone was detected by ELISA kits. Error bars indicate SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

investigated. Our results showed that *CTNNB1* knockdown decreased expression of β-catenin and inhibited proliferation of H295 R cells. Moreover, the downstream target genes of Wnt/β-catenin signaling pathway, *AXIN2* and *LEF1*, were downregulated by *CTNNB1* knockdown in H295 R cells, which were consistent with Gaujoux et al.[14] However, there was no difference in the Wnt/β-catenin signaling pathway of H295 R cells after Ang II treatment. These results indicate that the activation of Wnt/β-catenin signal pathway promoted APA tumorigenesis, which was independent of Ang II.

Uncontrolled cell proliferation is the most important hallmarks of cancer cells. Abnormal progression of cell cycle is the leading reason for dysregulation of cell proliferation. Cell cycle is regulated by a coordination of several cyclins and cell cycle inhibitors.[26] Different cyclins are involved in different cell cycle stages. Cyclin D1 is a key protein that drives G1/S transition of the cell cycle.[27] In our study, expression of cyclin D1 was investigated in H295 R cells. The protein and mRNA levels of cyclin D1 were significantly reduced by *CTNNB1* knockdown. These results suggest that *CTNNB1* knockdown downregulates cyclin D1 expression to inhibit H295 R cell proliferation.

In conclusion, our findings suggest that *CTNNB1* knockdown can inhibit H295 R cell proliferation and decrease aldosterone secretion in the responses of H295 R cells to Ang II through inhibiting Wnt/β-catenin signaling pathway, indicating that targeting Wnt/β-catenin signaling pathway may be an important approach to decrease aldosterone secretion in the treatment of aldoster-producing adenomas.

Authors' Contributions

P.L and T.Z conducted the study design; P.L and L.W carried out experiments, data analysis and wrote the manuscript; S.Y, J.L, S.Q and Z.W provided the technical or material support. All authors read and approved the final manuscript.

Tingting Zhou, MM Pengwei Luo, MM are authors contributed equally to this work.

Availability of Data and Material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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