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Original Article

Upregulation of PCP4 in human aldosterone-producing adenomas fosters human adrenocortical tumor cell growth via AKT and AMPK pathway

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Abstract: Primary aldosteronism (PA) is characterized by aldosterone hypersecretion and adrenal hyperplasia and ranks as one of the most common causes of secondary hypertension. However, the molecular mechanism involved in adrenal hyperplasia and tumorigenesis is largely unknown. Dysregulation of Purkinji cell protein 4 (PCP4) is involved in the development and progression of neoplasia and aldosterone secretion, but little is known about the effect of PCP4 on human adrenocortical tumorigenesis. We investigated the expression pattern of PCP4 in different adrenal tissues and studied whether PCP4 is involved in cell growth in human adrenal cell lines. The mRNA levels of PCP4 were measured by real-time PCR in tissues from aldosterone-producing adenomas (APAs), idiopathic hyperaldosteronism (IHA) tissues, and normal adrenal (NA) tissues. *In vitro* siRNA knockdown of PCP4 in NCI-H295R and SW13 cell lines was used to determine the effect of PCP4 on cellular growth. Our results show that the mRNA level of PCP4 is upregulated in APAs and IHA compared with that in NA. The PCP4 mRNA expression level was positively correlated with tumor size in APAs. Knockdown of PCP4 decreased cell proliferation. Flow cytometry analysis showed that PCP4 knockdown fosters apoptosis. Finally, PCP4 knockdown inhibited phosphorylation of AKT308 and AMPKThr172. Our data suggest that PCP4 may represent a key player in the development and pathophysiology of PA via targeting the AKT and AMPK signaling pathways and thus may be a promising therapeutic target for PA.

Keywords: Purkinji cell protein 4, primary aldosteronism, aldosterone-producing adenoma, adrenocortical cell proliferation, apoptosis

Introduction

Primary aldosteronism (PA) is one of the common causes of secondary hypertension and greatly contributes to more severe target organ damage (cardiovascular and renal injury, fibrosis, hypertrophy and vascular inflammation) [1-4]. It also increases the morbidity associated with resistant hypertension compared with essential hypertension. PA may affect as many as 13% of all hypertensive patients [5]. In addition to its high risk and high prevalence, PA is also characterized by aldosterone hypersecretion and adrenal hyperplasia due to the high aldosterone secretion by and high proliferation of adrenal cells. There are two main subtypes of PA, including aldosterone-producing adenomas (APAs) and idiopathic hyperaldosteronism (IHA). APA is found in one-third of PA patients, and IHA accounts for nearly two-thirds of PA patients [6].

In recent years, PA caused by human genetic mutations has been increasingly documented. Increasingly more evidence has shown that KCNJ5 [7], ATP1A1, and ATP2B3 [8] as well as CTNNB1 [9], CACNA1D [10], CACNA1H [11], and ARMC5 [12] gene mutations are critical for PA development and progression, contributing to an increase in cell aldosterone secretion. However, these mutations have only occurred in the APA subtype of PA, not in IHA, indicating that they might be used to screen for APAs but not universally applied for the diagnosis and treatment of other subtypes of PA. Although these mutations affect aldosterone biosynthe-

sis, questions regarding more detailed genetic and molecular mechanisms responsible for abnormal adrenal cortex cell proliferation leading to adrenal hyperplasia, adenoma formation and tumorigenesis are currently unanswered. Furthermore, how a normal adrenal (NA) cortex develops into APA or IHA remains to be further elucidated.

Purkinji cell protein 4 (PCP4), also called PEP19, is a calmodulin (CaM)-binding and anti-apoptotic peptide. It is abundantly expressed in the cerebral system and is known to regulate multiple pathophysiological aspects involved in the regulation of cerebellar development [13], proliferation, apoptosis [14, 15], tumorigenesis and cancer migration and invasion [16]. Expression of PCP4 has been found to be higher in human uterine leiomyoma than that in normal myometrium [17]. Altered PCP4 expression may result in tumor initiation and progression. These findings support the hypothesis that PCP4 may also be important for regulation of adrenal cell growth and progression of APAs. PCP4 mRNA and protein expression levels have been reported to be upregulated in APA compared with those in NA. PCP4 has also been described to be an important regulator of aldosterone secretion [18, 19]. However, there is no reported research regarding the functional effect of PCP4 on adrenal cortical hyperplasia and adenoma formation.

Our goal in this study was to further clarify the functional role of PCP4 with regard to dysregulated cell growth in human PA. We examined its expression in different adrenal tissues and studied its effect on human APA prognosis. NCI-H295R and SW13 cells were transfected with negative control siRNA and PCP4-specific siRNA. The influence of PCP4 knockdown on the mRNA and protein expression of PCP4, the cell growth curve, cell viability, and apoptosis was tested. We also explored the underlying mechanism of PCP4-mediated adrenocortical tumor cell growth.

Materials and methods

Human adrenal samples

APA, IHA, and NA tissues were collected from patients at Zhongshan Hospital, Fudan University. We obtained the APA and IHA tissues from patients who underwent adrenalectomies. The ipsilateral NA glands were obtained from renal tumor patients who underwent nephrectomies and were used as NA samples. PA patients were diagnosed based on endocrinal tests, computed tomography scans (CT), and adrenal vein sampling (AVS) of adrenal glands before the removal of adrenal glands. All the APA, IHA, and NA tissues were confirmed with histopathological diagnosis of adrenal tissues. Informed consent of the patients was obtained before their surgeries. All human subject studies were approved by the Zhongshan Hospital Ethics Committee, Fudan University. mRNA expression was analyzed in all the tissues from the patients.

Cell culture

The human adrenocortical carcinoma cell line NCI-H295R was purchased from the National Infrastructure of Cell Line Resources of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). NCI-H295R cells were cultured in DMEM/F12 medium (Gibco) containing 1% penicillin-streptomycin (Invitrogen) supplemented with 2.5% Nu-serum (BD Bioscience) and ITS (Gibco). SW13 cells were obtained from the Cell Resources Center of the Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS) (Shanghai, China). SW13 cells were maintained in DMEM (Gibco) supplemented with 10% FBS and 1% penicillin-streptomycin. Both cell lines were cultured in a 5% CO_2 humidified atmosphere at 37°C.

Transfection

We performed the transfection using Lipofectamine RNAiMAX reagent (Invitrogen) based on the manufacturer's instructions. Two PCP4-specific siRNAs (PCP4-siRNA1 and PCP4-siRNA2) at the concentration of 50 nmol obtained from Sigma (SASI_Hs01_00118358 and SASI_Hs01_00118363) were used, and non-specific siRNA PCP4-NC (Sigma) served as the negative control. After 48 hours of transfection, the cells were lysed, and the RNA and protein were extracted separately. We assessed the knockdown effect by real-time PCR and Western blotting. All experiments were performed in triplicate.

RNA extraction and real-time PCR

Adrenal samples from patients were stored in liquid nitrogen containers until RNA extraction

was performed. Total RNA was isolated from patient adrenal tissues or NCI-H295R and SW13 cell lines using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. To obtain cDNA, reverse transcription was performed using Transcript First Strand Synthesis Supermix (Trans Gene Biotech, Beijing, China, AT301) according to the manufacturer's instructions. Two micrograms of total RNA from each sample was reverse transcribed into cDNA in a total volume of 20 μl. Quantitive real-time reverse transcription (qRT-PCR) reagent was purchased from TAKARA and the 7500 Fast Real-time PCR System (Applied Biosystem, Carlsbad, CA, USA) was used. For each reaction, 1 μl of the RT product was added to 10 μl of 2×SYBR Green Expression PCR Master Mix and 1 μl of predesigned and synthesized forward and reverse primer/probe mix. We analyzed all samples in triplicate. Relative quantification (RQ) was derived from the difference in the cycle threshold (Ct) between the target gene PCP4 and GAPDH (ΔCt) when compared to control cell lines using the equation $RQ=2^{-\Delta\Delta Ct}$. Error bars represent the standard error of the mean (SEM), and statistical significance was calculated using one-tailed, unpaired t-tests. The mRNA levels were quantitatively assessed by SYBR Green-based quantitative real-time PCR with gene-specific primers. GAPDH was used as an internal normalization control. The sequences of the primers used for real-time PCR were as follows: PCP4: F 5'-3' TGACATGGATGCACCAG, R: 5'-3' GTGTGGATTGTGTGTGG; GAPDH: F5'-3' GGGAAACTGTGGCGTGAT, R5'-3'GAGTGGGTGTCGCTGTTGA.

Western blotting

Confluent cells cultured in 6-cm dishes were washed with phosphate buffered saline (PBS), and total cell proteins were extracted using 1× loading lysis buffer. The protein concentration was measured using the Lowry protein assay. Equal amounts of protein were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before being electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore, Milford, MA). The membrane was blocked in 5% nonfat milk for 2 hours at room temperature followed by immunoblotting with primary anti-PCP4 antibodies (Sigma-Aldrich, HPA005792), anti-$pAKT^{308}$ (CST, #13038), anti-$pAKT^{473}$ (CST, #4060), anti-AKT (CST, #4685), anti-$pAMPK^{Thr172}$ (CST, #2535), and anti-AMPK (CST, #2532) and horseradish peroxidase-conjugated secondary antibodies in 5% nonfat milk. Anti-GAPDH monoclonal antibody was purchased from Kangcheng (KC5-G4) and used as a loading control. The proteins on the membrane bound with antibodies were detected by SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Cell growth curve and CCK8 assay

To obtain the cell growth curve, 2×10^4 siRNA-transfected cells were seeded in a 12-well plate and cultured in a 37°C, 5% CO_2 incubator. Cell numbers of each well were counted at 1, 2, 3, and 4 days after seeding. Three wells were seeded and counted for each cell type. Statistical significance was calculated using the one-tailed t-test. For the CCK8 assay, siRNA-transfected cells were seeded at a density of 2×10^3 per well in 96-well plates, and A450 was measured at 1, 2, 3, and 4 days after seeding using the CCK8 Cell Proliferation and Cytotoxicity Assay Kit (DOJINDO LABORATORISE, JAPAN) according to the manufacturer's protocols.

Cell apoptosis assay

Cells were treated with PCP4-siRNA and negative control siRNA for 4 days and then detached using 0.25% trypsin and washed with PE-Annexin V (BD Biosciences) and 7-AAD (BD Biosciences) stains for 15 min at room temperature in the dark before flow cytometry was performed following the manufacturer's instructions. The data were quantified and analyzed with FlowJo 7.6 software (TreeStar Inc.). The negative 7-AAd-stained and positive PE-Annexin V-stained cells were identified as early apoptotic cells, while the PE-Annexin V- and 7-AAD-positive cells were identified as late apoptotic cells.

Statistical analyses

PCP4 expression levels in different adrenal tissues were analyzed by ANOVA. The correlation between the PCP4 mRNA expression level and APA tumor size was analyzed using the Spearman correlation test. The PCP4-siRNA knockdown effects, as shown by the relative mRNA expression level, the cell growth curve,

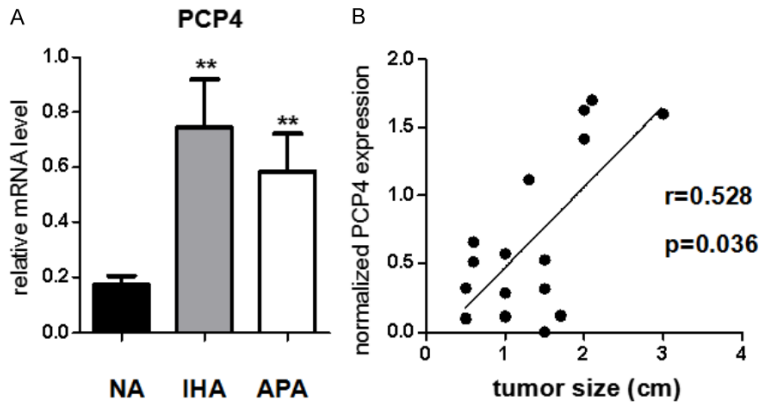

Figure 1. Expression of PCP4 in PA and relevance of PCP4 in APA development and progression. A: Detection of PCP4 levels (mean ± SEM) by real-time PCR in total RNAs derived from NA, IHA and APA. The data represent the mean ± SEM. NA, normal adrenal tissue (n=10); IHA, idiopathic hyperaldosteronism tissue (n=7); APA, aldosterone-producing adenoma tissues (n=16). ***P<0.001, **P<0.01, *P<0.05. B: A correlation between PCP4 mRNA expression level and tumor size (cm) in APA by Spearman's method.

the CCK8 A450 values and the percentages of apoptotic cell numbers, are presented as the mean ± SEM from three different experiments. The data were analyzed by Student's t-test. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). p<0.05 was considered significant.

Results

PCP4 is overexpressed in PA adrenal tissues, and its expression is positively correlated with tumor size in APAs

To identify the relevance of PCP4 to human PA patients and to test whether PCP4 plays a role during APA progression, we examined PCP4 mRNA levels in 10 human NA tissues, 7 IHA tissues and 16 APA tumor tissues by real-time PCR. We found that the mRNA levels of PCP4 were upregulated in APA tissues compared with NA (average 4.0-fold upregulation) (p<0.01) (**Figure 1A**). Interestingly, PCP4 levels in IHA were also upregulated and corresponded closely to those in APA (average 4.3-fold upregulation compared to NA) (p<0.01) (**Figure 1A**). Furthermore, we were interested in whether PCP4 plays a role during APA progression. We performed a univariate correlation analysis with the expression levels of PCP4 in APA patients in this study and their tumor size (**Figure 1B**). In this analysis, a positive correlation between the normalized mRNA levels of PCP4 gene expression and tumor size in APAs (r=0.528, p=0.036) was observed. These data suggest that PCP4 appears to be a feature of both subtypes of PA and is relevant to and important for the progression of human APAs.

RNAi effectively suppresses PCP4 expression in vitro

NCI-H295R and SW13 cell lines were used to evaluate the knockdown effect of PCP4 siRNA. Transfection of NCI-H295R and SW13 cells with negative control siRNA and 2 different PCP4-specific siRNAs (PCP4-siRNA1 and PCP4-siRNA2) was performed to silence endogenous PCP4 expression. After 48 hours of transfection, cellular proteins and RNAs were tested for PCP4 levels by Western blotting and real-time PCR. Compared to the cells treated with negative control siRNA (PCP4-NC), in the NCI-H295R and SW13 (**Figure 2A**) cells transfected with the two specific PCP4-siRNAs, the PCP4 protein levels were significantly reduced. Consistently, the PCP4 mRNA levels in PCP4-siRNA1- and PCP4-siRNA2-transfected cells were decreased by 60% and 92% (p<0.01, **Figure 2B**) in NCI-H295R cells and 87% and 85% in SW13 cells (p<0.01, **Figure 2C**), respectively. Collectively our results showed that both PCP4 siRNAs effectively inhibit PCP4 expression in both cell lines, although PCP4-siRNA2 may be more effective.

PCP4 is critical for cell growth in vitro

To address whether PCP4 plays a critical role in adrenal cell growth, we studied the role of PCP4 in cell proliferation by knocking down PCP4 in two human adrenal cell lines, NCI-H295R and SW13. We transfected cells with PCP4-siRNA and performed the cell growth curve assay for 4 days. We found that PCP4 knockdown resulted in a reduced cell number in both the NCI-H295R (p<0.05) (**Figure 3A**) and SW13 (p< 0.05) (**Figure 3B**) cell lines. The average numbers of control, PCP4-siRNA1- and PCP4-siRNA2-treated NCI-H295R cells at day 4 were 12.13×10^4, 8.64×10^4, and 7.88×10^4, respectively. In the SW13 cell line, the cell numbers

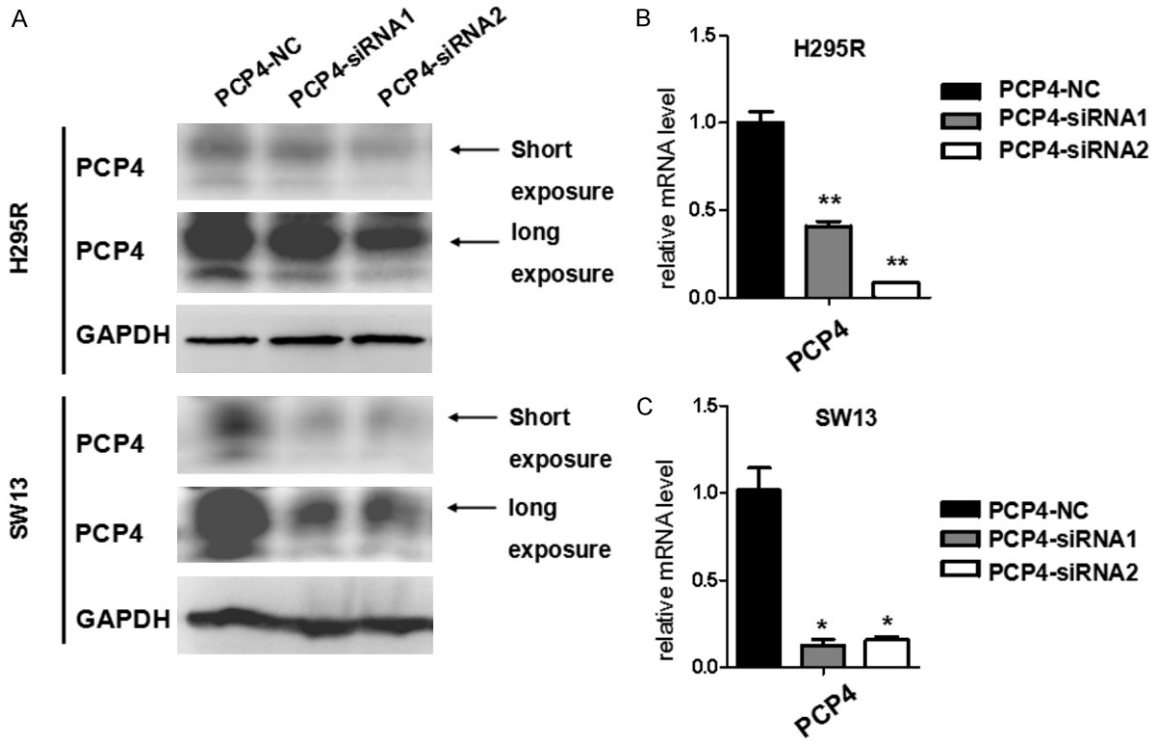

Figure 2. Effect of PCP4-RNAi on PCP4 expression. A: Immunoblotting results of PCP4 in NCI-H295R (upper panels) and SW13 (bottom panels) cells transfected with PCP4-NC or PCP4-siRNA. B: Assessment of PCP4 levels (mean ± SEM) by real-time PCR analysis in NCI-H295R cells transfected with PCP4-NC or PCP4-siRNA. C: Assessment of PCP4 levels (mean ± SEM) by real-time PCR analysis in SW13 cells transfected with PCP4-NC or PCP4-siRNA. ***P<0.001, **P<0.01, *P<0.05.

were 20.67×10^4, 15.58×10^4, and 12.92×10^4 for the control, PCP4-siRNA1- and PCP4-siRNA2-treated NCI-H295R cells, respectively. We further performed the CCK8 cell viability assay. Corresponding to cell growth curve assay results, the viability of NCI-H295R (**Figure 3C**) and SW13 (**Figure 3D**) cells was inhibited in PCP4-knockdown cells relative to control transfected cells from day 2 to day 4 ($p<0.05$). Taken together, our results indicate that knockdown of PCP4 inhibits human adrenal NCI-H295R and SW13 cell proliferation *in vitro*.

PCP4 regulates cell apoptosis in NCI-H295R and SW13 cells

Previous studies have shown that PCP4 plays an important role in cell apoptosis. To investigate how PCP4 regulates cell viability and whether PCP4 is involved in apoptosis in NCI-H295R and SW13 cells, cell apoptosis assays were performed. Because PCP4-siRNA2 knocked down PCP4 mRNA and protein expression more effectively than PCP4-siRNA1 (**Figure 2A** and **2B**), we chose PCP4-siRNA2 for this test. NCI-H295R and SW13 cells were transfected with PCP4-siRNA2 for 96 hours to study the effect on cell apoptosis. NCI-H295R and SW13 cells were cultivated with negative control siRNA and PCP4-specific siRNA2. Apoptosis was tested by flow cytometry after 96 hours of transfection. As shown in **Figure 4A** and **4B**, inhibition of PCP4 led to increased apoptosis. In PCP4-depleted cells, approximately 28.98% of NCI-H295R (**Figure 4A**) cells and 30.37% of SW13 cells (**Figure 4B**) were apoptotic, however in control siRNA-treated cells, only 18.88% of NCI-H295R (**Figure 4A**) cells and 18.2% of SW13 cells (**Figure 4B**) cells were apoptotic. Inhibition of PCP4 induced a nearly 10% increase in the percentage of the total apoptotic cells in both cell lines ($p<0.05$). We further analyzed which phase of apoptosis is mainly regulated by PCP4 depletion. The results show that introduction of PCP4 siRNA2 mainly regulates early apoptosis ($p<0.05$ **Figure 4A** and **4B**). These data suggest that PCP4 plays a dominant role in promoting tumor cell growth and inhibiting apoptosis.

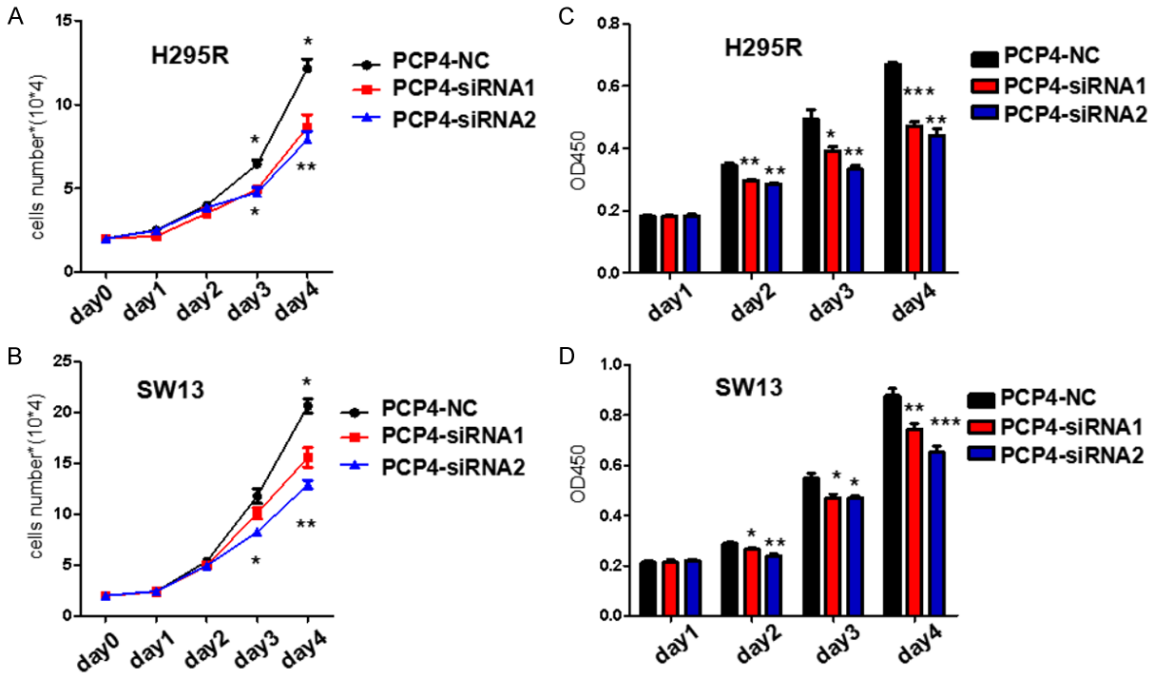

Figure 3. PCP4 knockdown inhibits cell growth *in vitro*. A: Growth curve of NCI-H295R cells treated with NC-siRNA or PCP4-siRNA on the indicated days. B: Growth curve of SW13 cells treated with NC-siRNA or PCP4-siRNA on the indicated days. C: CCK8 results for NCI-H295R cells treated with NC-siRNA or PCP4-siRNA on the indicated days. D: CCK8 results for SW13 cells treated with NC-siRNA or PCP4-siRNA on the indicated days. ***P<0.001, **P<0.01, *P<0.05. (The asterisk above the cell growth curve represents the significance between PCP4-NC and PCP4-siRNA1. The asterisk below the cell growth curve represents the significance between PCP4-NC and PCP4-siRNA2).

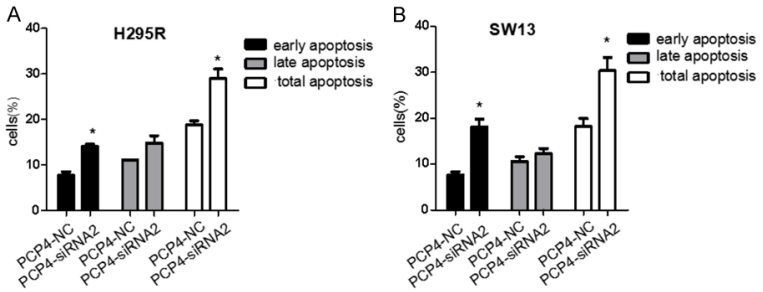

Figure 4. PCP4 alters cell apoptosis *in vitro*. A: Effect of PCP4 knockdown on cell apoptosis in NCI-H295R was measured by flow cytometry analysis. The data are presented as the mean + SEM. ***P<0.001, **P<0.01, *P<0.05. B: The effect of PCP4 inhibition on cell apoptosis in SW13 was measured by flow cytometry analysis. The data are presented as the mean + SEM. ***P<0.001, **P<0.01, *P<0.05.

PCP4 modulates AKT and AMPK signaling pathways in vitro

To explore the mechanism underlying the anti-proliferative and pro-apoptotic effect of PCP4 inhibition, NCI-H295R, and SW13 cells were transfected with PCP4-siRNA for 96 hours. Western blotting was then used to measure AKT and AMPK signaling, which has been demonstrated to be involved in proliferation and apoptosis in tumorigenesis, and the results were compared to those from cells transfected with control siRNA. PCP4-siRNA2-transfected cells showed a moderate decrease in p-AKT^{308} in H295R and SW13 cells after 96 hours (**Figure 5A** and **5B**), while no change was detected in p-AKT^{473} or total AKT levels. Furthermore, significantly decreased phosphorylation of AMPK^{Thr172} was also observed in PCP4-siRNA2-transfected cells versus NC cells (**Figure 5A** and **5B**), which corresponded with the elevated occurrence of apoptosis.

Discussion

PA is the predominant form of secondary hypertension and has been reported to contribute to

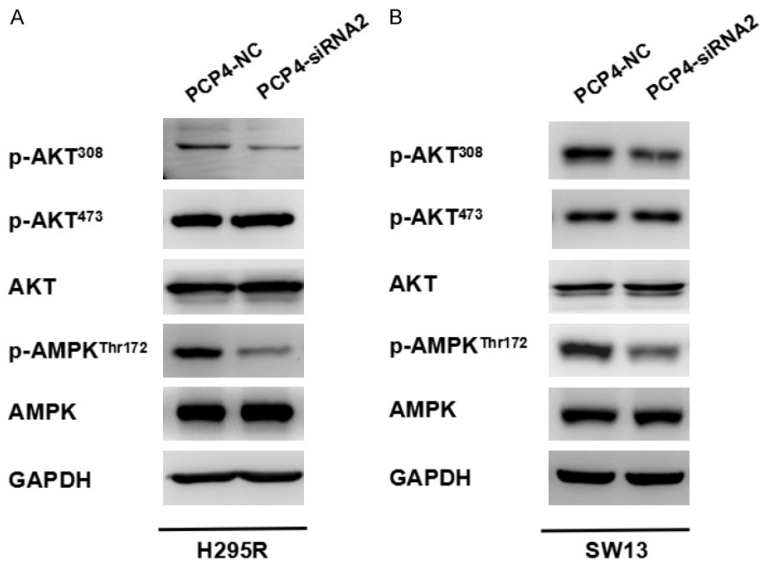

Figure 5. PCP4 inhibition inactivates the AKT and AMPK signaling pathway in H295R and SW13. A: NCI-H295R and B: SW13 cells were transfected with control siRNA and PCP4-siRNA. Western blotting was used to analyze the related proteins. Representative blots are shown from the experiments that were performed 3 times.

adverse cardiovascular consequences, with an estimated prevalence of 10% in all hypertensive patients and 20% in those with resistant hypertension [20]. Due to the development of next-generation sequencing techniques and transcriptomic studies, the underpinning causes of hyperaldosterone secretion have been explained by researchers who have identified several somatic and germline genetic mutations, such as KCNJ5, ATP1A1, ATP2B3, CTNNB1, CACNA1D, CACNA1H, and ARMC5 [7-12, 23, 24], and have developed breakthroughs in understanding the genetic mechanism underlying PA. However, these genetic mutations are reported to be responsible for only 60% of the molecular causation of APA, and the impact of the reported mutations mentioned above on cell proliferation and adenoma formation is still a matter of debate [7-12, 22, 23]. It is still unknown what the other genetic factors are that contribute to IHA and the remaining 40% of APA. Additionally, the underlying genetic mechanism that leads to adrenal hyperplasia and tumor formation is still not elucidated.

Previous studies have suggested that PCP4 plays a role in aldosterone secretion [19]. These studies showed that PCP4 has a zone-specific expression profile in adrenal tissues. PCP4 is most highly expressed in the adrenal zona glomerulosa than in the adrenal cortical or other zone layers. Using siRNA in the NCI-H295R cell model, the disease manifestation marked by the level of aldosterone production in PCP4-blocked cells was reduced under stimulation by angiotensin II (Ang II) by modulating the expression of a key enzyme in human aldosterone biosynthesis, CYP11B2, indicating that PCP4 is a potential target for aldosterone secretion. However, the details about its function in adrenal cortical cell growth are unknown.

This study was undertaken to examine how PCP4 is responsible for adrenocortical cell proliferation and adrenal cortical hyperplasia and tumorigenesis. In the present study, we show that during pathophysiological alterations in PA, PCP4, which is abundantly expressed in the cerebral system [25], plays a key role by controlling cell proliferation and cell apoptosis. In the current study, we found that inhibition of PCP4 by siRNA reduced cell number and cell proliferation and accompanied by increased cell apoptosis, suggesting that PCP4 is involved in PA pathogenesis and is required for tumor cell proliferation and apoptosis.

A previous report [19] demonstrated that PCP4 is upregulated in APA compared with that in NA, and its expression is predominantly restricted to the zona glomerulosa of NA and IHA. PCP4 displays a distinct localization to NA and IHA nuclei, in contrast to its cytoplasmic expression in the surrounding adrenal cortex. This previous study also showed that PCP4 knockdown blocked aldosterone secretion in response to Ang II. However, the role of PCP4 dysregulation in adrenal cortical cell proliferation and tumorigenesis was not studied.

We investigated the comparative mRNA expression levels of PCP4 using different adrenal tissues (NA, APA and IHA). Consistent with previous results, PCP4 in our study, as well as in that by Felizola and colleagues [19], was higher in

the APA group than that in the NA group. Interestingly, PCP4 expression was also higher in IHA compared to that in NA. This important observation in our study indicates that PCP4 is critical for the pathogenetic mechanism of not only APA but also IHA, suggesting that PCP4 may be more broadly useful as a marker in the early diagnosis and treatment of PA. Our findings also demonstrate that PCP4 may be involved in the potentially common mechanism underlying adrenocortical hyperplasia and adrenal adenoma formation and pathogenesis. Next, we also evaluated the correlation between PCP4 expression and APA tumor size. The results of this study revealed that there was a positive correlation between PCP4 expression and tumor size in APA. This supports the hypothesis that PCP4 is a primary feature of PA and may be involved in hyperplasia and tumorigenesis in human adrenal cortical cells.

To prove our hypothesis, we knocked down PCP4 and studied its effect on cell proliferation. As we expected, we found that the loss of PCP4 in both NCI-H295R and SW13 cells reduced cell numbers. A similar result was obtained in the CCK8 cell proliferation assay, where proliferation was reduced upon PCP4 knockdown relative to control cells. Adrenal hyperplasia and tumorigenesis are complicated processes that include elevated cell proliferation and reduced cell apoptosis. Therefore, to determine whether PCP4 affects cell growth by apoptosis, we performed a cell apoptosis assay after treating the NCI-H295R and SW13 cells with PCP4-specific siRNA, and we confirmed that inhibition of PCP4 elevates cell apoptosis. This evidence highlights the potent anti-tumor role of PCP4 inhibition in adrenal cells. To further investigate the mechanism that enables PCP4 to promote cell proliferation and to inhibit cell apoptosis in human adrenal tissues, we tested the AKT and AMPK signaling pathways, which play important roles in cell proliferation, apoptosis, and tumorigenesis. PCP4 knockdown inhibited the activity of AKT and AMPK signaling, which is consistent with previous studies [15]. PCP4 inhibition reduced phosphorylation of AKT^{308} and $AMPK^{Thr172}$. Further investigations should be done to unravel the underlying mechanism.

PCP4, a CaM-binding protein with an IQ motif [25, 26], which was initially identified and described as a nervous system-associated gene, has been found to play functional roles in a wide variety of pathophysiological processes [18, 19]. It has also been reported to contribute to cerebellar development, synaptic plasticity, impaired motor learning and brain function [13, 14]. Specifically, through binding CaM, the role of PCP4 in regulating the association and the dissociation of calcium and CaM, thereby modulating the CaM-mediated signaling pathway [27], has been well demonstrated in different cell lines. By binding CaM, PCP4 also modifies calcium/CaM-dependent protein kinase activity [28, 29]. For example, by binding CaM and further inhibiting CaM signaling, PCP4 mediates synaptic plasticity and regulates locomotor learning [30], abnormal cell growth and inhibition of apoptosis, transition, and metastasis in tumor cells such as breast cancer [15, 16]. In previous studies, PCP4 has been shown to be abundantly expressed in various adenomas and cancer types such as uterine leiomyoma [15, 16] and APA [19]. In addition, PCP4 depletion has been recently demonstrated to reduce cell proliferation and to increase cell apoptosis in a breast cancer cell line. Some investigators have reported that PCP4 knockdown induced apoptosis through the activation of the AKT-mediated pathway with the involvement of CaM/CamKK [15], while others have provided evidence indicating that PCP4 is positively related to neurite outgrowth. Overexpression of PCP4 in PC12 cells promoted neurite outgrowth and reduced apoptosis by activating CaM [14, 31]. In a mouse model of Down syndrome, PCP4 is reportedly responsible for ventriculomegaly [32]. However, additional studies have shown that although PCP4 is upregulated in cardiohypertrophy, overexpression of PCP4 inhibits cardiohypertrophy by inhibiting the phosphorylation of CamKII and the activation of calcineurin triggered by AngII [33].

PCP4 is not always associated with apoptosis. PCP4 mRNA expression is found to be downregulated in human bladder cancer cells compared with that in normal bladder cells [34]. Although these conflicting pieces of evidence are not easily explained, it became evident in our study that PCP4 promotes cell growth and inhibits apoptosis in human adrenal cells via the AKT and AMPK signaling pathways. As Ca^{2+}-CaM signaling regulates cell proliferation and apoptosis [34], and given that PCP4 is a CaM-binding protein, PCP4 regulates cell apoptosis

via modulation of the association between Ca^{2+} and the CaM-dependent enzyme [14, 31, 35]. Activation of CamKK1 by Ca^{2+}/CaM binding can phosphorylate AKT^{308}, which results in Bad phosphorylation and caspase activity suppression, finally leading to apoptosis inhibition [36]. Furthermore, the AKT/mTOR signaling pathway was demonstrated to be over-activated in APA and IHA compared with that in NA, and suppression of this signaling pathway resulted in inhibited H295R cell proliferation and reduced aldosterone secretion [37]. Here, we speculate that the same mechanism of PCP4 may underlie human adrenal cortical cells. PCP4 may exert its hypertrophic, tumorigenesis effect through dysregulation of the association between Ca^{2+} and CaM, which inhibits the CamKK-AKT and CamKK-AMPK signaling cascade, further inhibits Bad phosphorylation, and reduces apoptosis. The possibility of other mechanistic pathways cannot be excluded. Thus, from this study and the previously reported studies, we can draw the conclusion that PCP4 may be sufficient to cause increased aldosterone secretion and cell proliferation and to inhibit apoptosis in the development and progression of PA via the AKT and AMPK pathways.

Our study has some limitations. First, the sample size we used is limited, and our samples may not be representative of the broader PA population. Second, our interpretation of the mechanism underlying the function of PCP4 remains largely speculative and is not fully investigated. Third, all the functional studies were done *in vitro*. To circumvent these limitations, more investigations with large sample sizes are needed. The mechanism should be elucidated in the future, and we also need more studies to clarify the role and mechanism of PCP4 in in vivo situations.

Despite these limitations, the current findings show that PCP4 is associated with tumor cell proliferation and apoptosis, which may then be followed by adrenal cortical hyperplasia and tumorigenesis. The action of PCP4 plays an important role in mediating its oncogenic function in several adrenal cortical diseases.

In summary, our findings are as follows: 1) We detected expression of PCP4 in human APA, IHA, and NA tissues, and PCP4 expression level was positively correlated with APA tumor size. 2) PCP4 is a regulator of cell growth and apoptosis in human adrenocortical cells via AKT and AMPK signaling. Taken together, our results suggest for the first time that altered expression of PCP4 is involved in human adrenal hyperplasia and the formation of APA and IHA. We demonstrate a novel role of PCP4 in upregulation of cell growth and proliferation and in downregulation of cell apoptosis, in addition to its previously described aldosterone-regulating role. Given that increased cell proliferation and decreased apoptosis promote tumorigenesis, these processes make PCP4 a critical modulator of PA in pathophysiological conditions. Upregulation of PCP4 thus not only results in increased aldosterone secretion but also promotes cell proliferation in PA. PCP4 acts as an oncogenic factor, and its depletion sheds light on the understanding and treatment of adrenal-related diseases (adrenal carcinoma cell proliferation, apoptosis, hyperplastic, and neoplastic diseases). We believe that PCP4 upregulation leads not only to APA but also to IHA. This may help explain the hyperplasia that occurs in both subtypes of PA, APA, and IHA.

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Disclosure of conflict of interest

None.

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