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YPEL4 modulates HAC15 adrenal cell proliferation and is associated with tumor diameter

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Abstract

Yippee-like (YPEL) proteins are thought to be related to cell proliferation because of their structure and location in the cell. The aim of this study was to clarify the effects of YPEL4 on aldosterone production and cell proliferation in the human adrenocortical cell line (HAC15) and aldosterone producing adenoma (APA). Basal aldosterone levels in HAC15 cells over-expressing YPEL4 was higher than those of control HAC15 cells. The positive effects of YPEL4 on cell proliferation were detected by XTT assay and crystal violet staining. YPEL4 levels in 39 human APA were 2.4-fold higher compared to those in 12 nonfunctional adrenocortical adenomas, and there was a positive relationship between YPEL4 levels and APA diameter ($r = 0.316$, $P < 0.05$). In summary, we have demonstrated that YPEL4 stimulates human adrenal cortical cell proliferation, increasing aldosterone production as a consequence. These results in human adrenocortical cells are consistent with the clinical observations with APA in humans.

Keywords

Aldosterone production; Aldosterone producing adenoma; YPEL4; Tumor progression

1. Introduction

Aldosterone plays a significant pathophysiological role in hypertension and cardiovascular diseases. It is synthesized in the zona glomerulosa of the adrenal and is primarily under the regulation of the renin-angiotensin system (Hattangady et al., 2012). A major site of action

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of aldosterone is in the distal tubules of the kidney, where it promotes sodium absorption and potassium (K^+) excretion. Angiotensin II (A-II) binds to the angiotensin receptor (AT1R) in the adrenal cortex to stimulate the production of aldosterone; K^+ ions directly stimulate aldosterone production independently of the RAA system (Pralong et al., 1992).

The binding of A-II to the AT1R triggers several intracellular signaling cascades including protein kinase C, calcium/calmodulin-dependent kinases, and mitogen-activated protein kinase (MAPK) (Côté et al., 1998; Hattangady et al., 2012; Hunyady et al., 2004; Otis et al., 2005). The concentration of extracellular K regulates calcium influx into zona glomerulosa cells and activates calcium/calmodulin-dependent kinases (Pralong et al., 1992). Importantly, some of the cascades stimulated by A-II may cause adrenal cell growth or proliferation as well as aldosterone production. Although we and other researchers have investigated mechanisms of adrenal cell growth and aldosterone production (Côté et al., 1998; Hunyady et al., 2004; Otis et al., 2005; Romero et al., 2010; Romero et al., 2007a, 2007b), the pathways have not been fully elucidated.

The human yippee-like (YPEL) gene family consists of 5 genes, YPEL1 to YPEL5 cloned in 2004 (Hosono et al., 2004). These proteins are located at the centrosome, adjacent to nucleolus and mitotic apparatus, and are ubiquitously expressed in all eukaryotes (Hosono et al., 2010; Hosono et al., 2004). Therefore, the YPEL family proteins are likely to have role in cell life cycle. We previously suggested that YPEL4 is one of the most up-regulated mRNA after A-II or K^+ stimulation in rat adrenal cells (Romero et al., 2007a, 2007b), however the roles of YPEL4 in the adrenal gland has not been studied.

We hypothesized that YPEL4 has a role in regulating aldosterone production and/or adrenal cell proliferation. Thus, the aim of our study was to investigate the effects of YPEL4 on aldosterone production and cell proliferation in the human adrenal cortical cell line, HAC15. Herein, we report that YPEL4 potentiates aldosterone production by increasing cell proliferation. We also found that YPEL4 expression in aldosterone-producing adenomas (APA) from patients was 2.4-fold higher compared with its expression in nonfunctioning adrenocortical adenomas (NF) and there was a positive relationship between YPEL4 expression levels and tumor diameter in APA.

2. Methods

2.1. Cell culture and materials

The HAC15 human adrenocortical carcinoma cell line, a subclone of the H295R (Parmar et al., 2008), was provided by W. E. Rainey (University of Michigan). The HAC15 cells were cultured in Dulbecco's modified Eagle's medium (DMEM):F12 (1:1) supplemented with 10% Cosmic Calf serum (HyClone, Logan, UT) at 37C under a humid atmosphere of 95% air and 5% CO2. A-II and potassium chloride were purchased from Sigma Aldrich Co. Ltd. (St. Louis, MO).

2.2. Plasmids

The full length cDNA of YPEL4 was ligated downstream of the cytomegalovirus promoter of a feline immunodeficiency virus based lentivector (System Biosciences, Mountain View,

CA), resulting in pCDF-YPEL4. Control plasmid was prepared as pCDF without YPEL4. pCMV-VSV-G [pCMV-VSV-G was a gift from Bob Weinberg (Addgene plasmid # 8454)], and pCPRDEenv [pCPRDEnv was a gift from Garry Nolan (Addgene plasmid # 1732)] for virus production were obtained from Addgene.org. (Cambridge, MA).

2.3. Lentiviral production and infection

293 TN cell line (System Biosciences, Mountain View, CA) were cultured in DMEM supplemented with FetalClone II serum (HyClone, Logan, UT) until 60–70% confluent, then transfected with lentiviral vector, pCMV-VSV-G, and pCPRDEev (0.28 (μg/cm^2, 10:8:5 M ratio) using linear PEI25000 transfection reagent (4.8 μl/μg DNA, Polysciences, Inc Warrington, PA). Medium was replaced after overnight incubation, and then cells were cultured for an additional 48 h when the supernatant from cell culture was collected and centrifuged at × 1500g and 4C for 30 min. This supernatant was mixed with 80 μg/ml of polybrene and 10 μg/ml of chondroitin, and after 30 min centrifuged at 6000 × g and 4C for 45 min (Le Doux et al., 2001). After aspiration of the supernatant, the virus was reconstituted with DMEM/F12 without phenol red (Invitrogen, Carlsbad, CA).

Transduction with YPEL4 or control lentivirus were performed as described previously at 24 h after seeding the HAC15 cells, and the cells were cultured for 72 h after transduction (Romero et al., 2007a, 2007b). followed by serum deprivation with DMEM:F12 supplemented with 0.1% Cosmic Calf serum for 24 h to assess the effect of YPEL4 overexpression on aldosterone production and mRNA expression. The medium was replaced with fresh DMEM:F12 containing 0.1% Cosmic Calf serum with or without various combination of A-II or K^+ at indicated concentrations for another 24 h. Second, to investigate the effect of YPEL4 on cell proliferation and aldosterone production, the cells were selected with 2.0 μg/ml of puromycin. The cells were cultured for at least 4 weeks in the absence of selecting agent before performing the experiments to avoid any confounding effect due to the antibiotic selection.

2.4. Patients and tissue collection

The diagnosis of PA, subtype diagnosis and non-functional adenomas (NF) were performed as previously reported (Matsumoto et al., 2015; Oki et al., 2012a, 2012b), based on the guidelines from the Japan Endocrine Society (Nishikawa et al., 2011). We enrolled 51 consecutive patients (12 NF and 39 APA) for the pathological study. Written informed consent was obtained from all subjects. Our study was approved by the ethics committee of Hiroshima University. Tissue samples were immediately preserved in RNAlater (Life Technology, Delhi, India) and stored at –80 °C until assayed, or fixed in formalin and embedded in paraffin for immunohistochemical analysis. APA was confirmed by detecting the expression of *CYP*11*B2* by immunohistochemistry and/or quantitative polymerase chain reaction (qPCR) assays as previously reported (Gomez-Sanchez et al., 2014).

2.5. RNA extraction and RT-PCR

Total RNA was extracted with the RNAzol-RT Reagent (Molecular Research Center, Inc., Cincinnati, OH). For reverse transcription, 2.5 μg of total RNA was incubated with

SUPERase-In (Applied Bio-systems/Ambion, Austin, TX) and SuperScript III (Invitrogen, Carlsbad, CA) following the manufacture's protocol.

Table 1 shows real-time PCR primers designed to generate amplicons of approximately 100-bp as we previously reported (Oki et al., 2012a, 2012b). Aldosterone synthase (CYP11B2) and GAPDH mRNA expression were determined by the Taqman Gene expression assay as previously reported (Romero et al., 2010). mRNA expression of steroidogenic acute regulatory protein (STAR), cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1), 3β-hydroxysteroid dehydrogenase (HSD3B2), cytochrome P450, family 21, subfamily A, polypeptide 2 (CYP21A2), YPEL1-4, and glyceral-dehyde-3-phosphate dehydrogenase (GAPDH) were quantified in 1 μl RT product, 1 μl Titanium Taq DNA polymerase (Clontech, Mountain View, CA), 1:20 000 dilution SYBR Green I (Molecular probes, Carlsbad, CA), 10 nM Fluorescein (Bio-Rad, Hercules, CA), 0.2 mM dNTPs, and 0.1 μM of each primer. Real time data were obtained during the extension phase and critical threshold cycle values were calculated on the log phase of each gene amplification curve. Gene expression levels were analyzed as arbitrary units normalized against GAPDH mRNA expression.

2.6. Cell proliferation and viability assay

The XTT cell proliferation assay kit (American Type Culture Collection, Manassas, VA) was used to evaluate the effect of YPEL4 on cell proliferation as previously described (Berridge et al., 2005). Briefly, the cells stably transduced with control and YPEL4 vectors were seeded separately on 96 well plates at a concentration of 5×10^5 cells per well. The cells were incubated with 50 μl of activated-XTT solution for 4 h, following the manufacture's protocol. The absorbance of the wells was measured at a wavelength of 475 nm and 660 nm.

For Crystal violet staining, the stably transduced cells were plated at 1×10^6 cells per well in 48-well plates and the supernatants were collected for aldosterone concentration at indicated times and the cells were fixed for 20 min in 10% paraformaldehyde. Fixed cells were stained for 30 min with a 0.05% solution of crystal violet, washed extensively, solubilized in 1% SDS solution for 1 h, and the absorbance of the supernatant read at 590 nm wavelength.

2.7. Aldosterone and protein assays

Aldosterone was measured in cell culture supernatants by a time resolved fluorescence method developed in our laboratory (Manolopoulou et al., 2008). Protein levels were detected by ELISA using a Micro BCA Protein Assay kit (Thermo Scientific, Rockford, IL).

2.8. Statistical analysis

Results from the *in vitro* study were expressed as mean ± S.E. of at least three separate experiments in which each sample was assayed in triplicate or quadruplicate, and clinical results were expressed as mean ± S.D. Differences between two groups were analyzed for statistical significance by *t*-test and multiple groups were analyzed by one-way ANOVA followed by Bonferroni comparisons. Time-response effects were tested by two-way ANOVA. The associations between clinical results and YPEL4 mRNA levels were analyzed by Pearson's correlation analysis. The differences were considered to be significant at $P <$

0.05. Analyses were performed using SPSS for Windows (release 12.0; SPSS Inc., Chicago, IL).

3. Results

3.1. Effect of A-II or K^+ on YPEL1-5 mRNA expression

First, we investigated the effect of A-II or K^+ stimulation on the mRNA expression of YPEL family members. As we previously reported (Romero et al., 2007a, 2007b), A-II and K^+ increased YPEL4 mRNA expression levels by 2.3 ($P < 0.05$) and 3.8-fold ($P < 0.05$), respectively (Fig. 1). In contrast, YPEL3 mRNA levels were significantly suppressed by A-II (0.30-fold, $P < 0.05$) and K^+ (0.60-fold, $P < 0.05$). YPEL2 mRNA levels were also decreased by A-II (0.57-fold, $P < 0.05$).

3.2. Effect of YPEL4 on aldosterone production

HAC15 have very low levels of YPEL4 expression as 33 to 34 threshold cycles were required for quantification by real time PCR Thus we overexpressed YPEL4 in the cells, and YPEL4 mRNA levels were increased to 120-fold by lentiviral transduction carrying YPEL4 in HAC15 (data not shown). To assess the effect of YPEL4 on aldosterone production, we measured aldosterone concentration in the medium of HAC15 cells with YPEL4 or control, with and without stimulation by several concentrations of A-II or K^+. Fig. 2 shows that HAC15 cells infected with YPEL4 had higher basal aldosterone levels than the controls (38.7 ± 8.7 pg/ml *vs.* 55.4 ± 4.3 pg/ml, $P < 0.05$), whereas no significant differences between YPEL4 and control were observed after A-II or K^+ stimulation.

3.3. The mRNA expression of steroid synthesis enzymes and YPEL4

Since the YPEL4 infected cells had higher aldosterone levels, we investigated the effect of YPEL4 on mRNA levels of steroid synthesis enzymes. There were no differences in expression levels of mRNA for STAR, CYP11A1, HSD3B2, CYP21A2, CYP11B1 or CYP11B2 normalized to GAPDH between YPEL4 and control cells (Fig. 3). These results indicated that YPEL4 had no effects on aldosterone production via the steroid synthesis enzymes.

3.4. The influence of YPEL4 on cell proliferation

We determined the effect of YPEL4 in HAC15 on cell proliferation because the increase of aldosterone production was observed without a change in message for steroidogenic enzymes. The effect of YPEL4 overexpression compared to control using the XTT assay after 0 h, 24 h, 48 h and 72 h is shown in Fig. 4A. Although the number of YPEL4 and control cells were identical at the beginning of the experiment, the rate of proliferation of the YPEL4 cells exceeded that of controls after 24 h (24 h, 1.31 ± 0.09-fold increase; 48 h, 1.33 ± 0.10-fold increase; 72 h, 1.72 ± 0.26-fold increase).

Crystal violet stains proteins and DNA of viable cells, and can reflect cell proliferation, density, and/or expansion. HAC15 cells with YPEL4 and control were assessed by crystal violet staining 12 h, 24 h, 48 h and 72 h after plating (Fig. 4B). There were no differences in staining between YPEL4 and control 24 h, after plating, but by 48 h the rate of increase in

staining of YPEL4 cells was significant greater than that of controls (YPEL4, 4.07 ± 0.12; control, 3.23 ± 0.08; $P < 0.01$) and 72 h (YPEL4, 5.30 ± 0.15; control, 4.23 ± 0.13; $P < 0.01$).

Effect of YPEL4 on aldosterone production and aldosterone concentration adjusted by protein concentration.

Aldosterone concentrations in the culture media were also assessed 12 h, 24 h, 48 h and 72 h after plating. Aldosterone secretion by YPEL4 infected cells after 48 h and 72 h, were 1.11 ± 0.04-fold ($P < 0.05$) and 1.27 ± 0.04-fold ($P < 0.01$) greater than that by control cells, respectively (Fig. 5A). The increase of aldosterone concentration paralleled the increase in cell proliferation; there was no significant difference between YPEL4 and control cell when aldosterone concentrations in the media was normalized to cell protein concentrations after the 48 h and 72 h incubations (48 h, 1.02 ± 0.04-fold; 72 h, 1.03 ± 0.05-fold) (Fig. 5B).

3.5. The association between pathological characteristics and YPEL4 expression in human APA and NF samples

The clinical characteristics of the patients are presented in Table 2. YPEL4 levels in APA were 2.4-fold higher than in NF (Fig. 6A). While there was a positive association between tumor diameter and YPEL4 levels in APA samples (Fig. 6B), YPEL4 expression levels did not correlate with CYP11B2 levels in APA ($r = -0.235$, $P = 0.103$, data not shown).

4. Discussion

We showed that over-expression of YPEL4 caused a significant increase in aldosterone in the cell culture media associated with increased proliferation of HAC15 human adrenocortical cells. The dependence of aldosterone production upon adrenal cell proliferation was not evident initially. Normalization of aldosterone production with protein as the surrogate for cell mass abrogated the apparent increase in aldosterone synthesis. We also found that APA had higher YPEL4 levels compared with NF adenomas and that there was a positive relationship between YPEL4 levels and tumor diameter in APA.

Our results describe a novel potential factor by which A-II or K^+ modulates adrenal cell growth, since mechanisms of adrenal cell proliferation have not been clearly elucidated as yet. Previous reports suggest that YPEL4 has roles in cell cycle and proliferation because of its subcellular localization on or close to the mitotic apparatus during the mitotic phase and in the nuclei and nucleoli in the interphase (Hosono et al., 2010; Hosono et al., 2004). Additionally, some single nucleotide polymorphisms of YPEL family were reported to be a risk factor for the development of breast cancer (Olson et al., 2011). These findings are consistent with our results demonstrating that YPEL4 increased adrenal cell proliferation. Reduction in YPEL5 expression in COS-7 cells with siRNA resulted in a significant reduction in cell proliferation and in doubling time due to prolongation of the G_1 phase and the G_2+M (Hosono et al., 2010). The G_2+M prolongation, albeit small, implies a possible function of YPEL4 during mitosis and cytokinesis (Hosono et al., 2010).

The normal adrenal zona glomerulosa increases both in number and size of cells upon chronic stimulation, most often by activation of the renin-angiotensin-aldosterone system. The effect of K^+ on cell proliferation has been uncertain in human adrenal cells. In this study we demonstrated that K^+ stimulated YPEL4 mRNA expression by approximately 3.8-fold in native HAC15 (Fig. 1), whereas HAC15 cells with YPEL4 had 120-fold increase in YPEL4 mRNA levels (data not shown). The relatively small increase in YPEL4 by K^+ stimulation may not have a large effect on adrenal cell proliferation, but it may be one of myriad mitogenic signals induced by K^+ that contribute to a significant effect.

The increased proliferative effects byYPEL4 determined through XTT assay was higher than those determined through crystal violet staining and the increased ratio of aldosterone production. Although the XTT method can be effectively used for the detection of cell proliferation, the result is dependent on the number or function of mitochondria (Berridge et al., 2005). The XTT cell proliferation assay is based on the conversion of the colorless XTT to an orange colored formazan product of XTT detected by an absorbance reader. It is considered that YPEL4 regulates the number or function of mitochondria as well as cell cycle or mitosis.

Other factors or proteins demonstrated or postulated to be involved in adrenal cell proliferation using adrenocortical cell lines or cells isolated from the zona glomerulosa involve MAPK. Rho-GTP proteins stimulate cell proliferation via activation of ERK1 (Otis and Gallo-Payet, 2006), and Neuropeptides B and W, both ligands for G protein-coupled receptors, activate MAPK to increase cell proliferation (Andreis et al., 2005). Orexin is a hypothalamic hormone that also modulates the growth of rat adrenocortical cells through MAPK (Spinazzi et al., 2005). However, this is the first study demonstrating that YPEL4 expression in a human adrenocortical cell line is increased by A-II or K^+ stimulation and that YPEL4 has a potentially relevant role in stimulating the proliferation and perhaps growth of adrenal cells.

There is not a good correlation between the size of an APA and degree of abnormality in aldosterone production. Expression of the aldosterone synthase (CYP11B2), the last and rate limiting enzyme for aldosterone biosynthesis, is expressed in tumors in a highly variable manner. Many large tumors express the enzyme in a minority of cells interspersed with cells that do not express this crucial enzyme for aldosterone biosynthesis (Nakamura et al., 2016). Importantly, there was a positive correlation between the size of the adenoma and expression of YPEL4 (Fig. 6B), and YPEL4 did not have any effects on CYP11B2 expression the in vitro study (Fig. 3) or any relationship with CYP11B2 expression in APA (data not shown). Taken together, these suggest that YPEL4 might have a potential role of cell growth and the cells expresses relatively normal levels of CYP11B2 in APA. CYP11B2 expression would be regulated by other intracellular cascades such as calcium signaling in each cells of APA.

In conclusion, we clarified that YPEL4, which is a highly up-regulated gene in HAC15 cells after A-II or K^+ stimulation, stimulates adrenal cell proliferation and consequently aldosterone production. This is a newly described intracellular signaling pathway for the modulation of adrenal cell physiology by A-II and K^+. The cumulative effects of many genetic and molecular alterations are required to regulate cell growth; our results suggest

that YPEL4 is one of the important molecular mechanisms regulating of adrenal zona glomerulosa cell proliferation.

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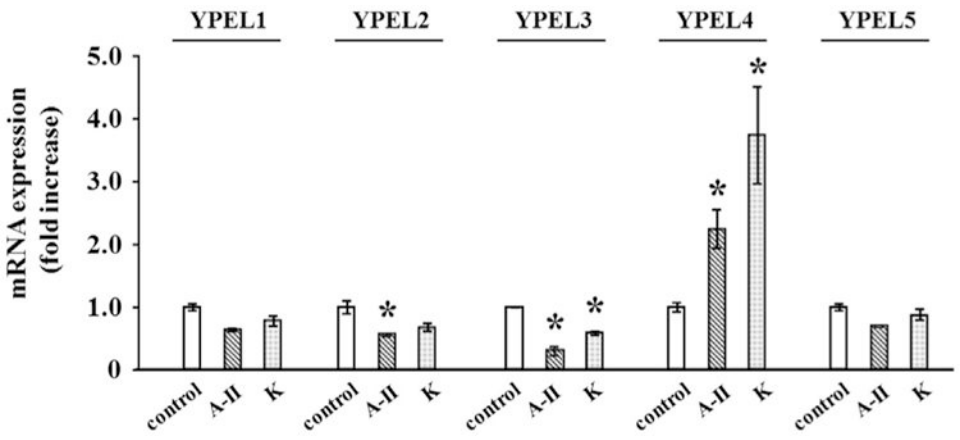

Fig. 1.

Effect of A-II or K on YPEL1-5 mRNA expression in HAC15. After reaching confluence, cells were serum starved with DMEM:F12 containing 0.1% Cosmic Calf serum for 24 h, and then incubated with the fresh media containing 0.1% serum with A-II, K^+, or no secretagogue for 3 h. After the aspiration of media, the cells were collected for RNA extraction and real time RT-PCR using specific primers. All of results were normalized by GAPDH mRNA expression and expressed as fold increase versus control. n = 3.

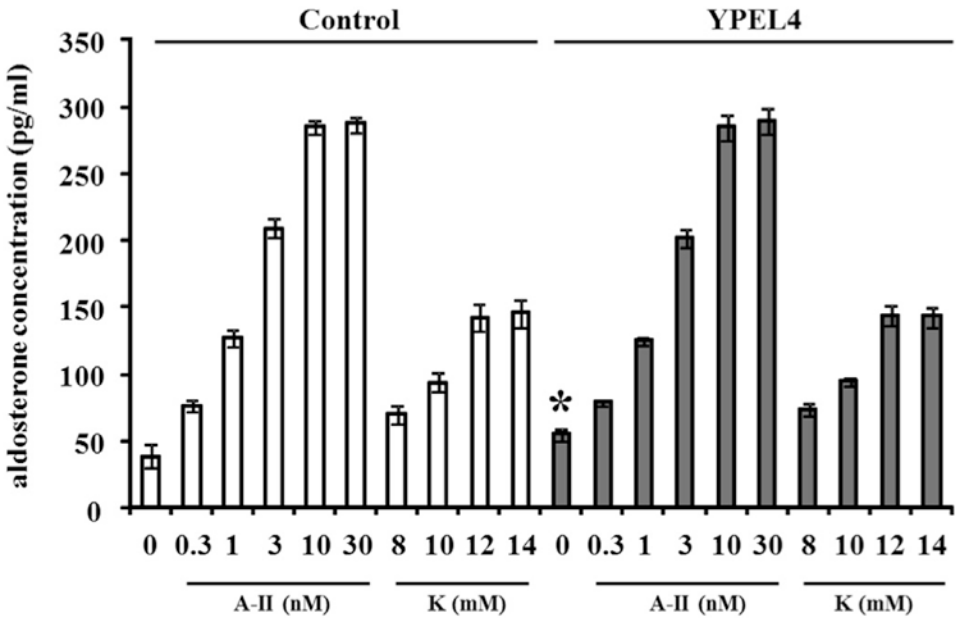

Fig. 2.

Effect of YPEL4 on aldosterone production after stimulation by several concentrations of A-II and K^+ in HAC15 cells infected with YPEL4 or control lentivirus. The culture media was replaced with DMEM:F12 supplemented with 0.1% Cosmic Calf serum 72 h after infection and the cells cultured with the indicated concentrations of A-II or K^+ for 24 h. Aldosterone concentrations were measured in the media. *, $P < 0.05$ vs. control, n = 4.

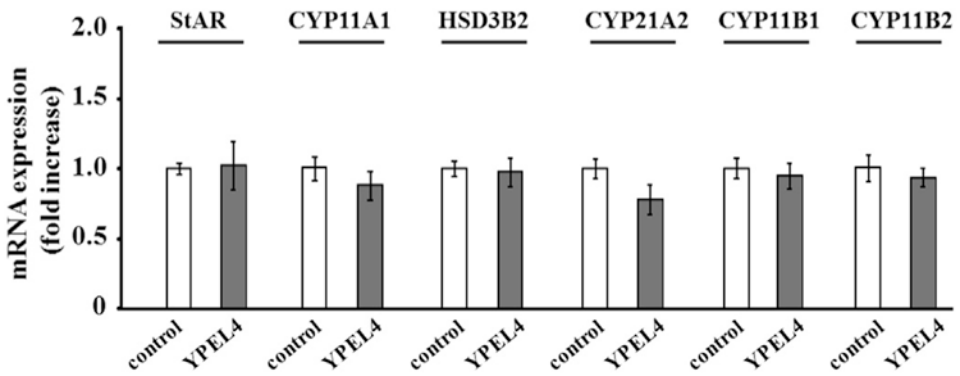

Fig. 3.

The mRNA expression of the steroid synthesis enzymes STAR, CYP11A1, HSD3B2, CYP21A2, CYP11B1 and CYP11B2 in HAC15 cells transduced with YPEL4 or control vectors. HAC15 cells were incubated for 72 h after transduction, then serum starved with DMEM:F12 containing 0.1% Cosmic Calf serum for 24 h, and then incubated with the fresh media with 0.1% serum for 3 h. After the aspiration of media, the cells were collected for RNA extraction and real time RT-PCR using specific primers. All of results were normalized by GAPDH mRNA expression and expressed as fold increase versus control. n = 3.

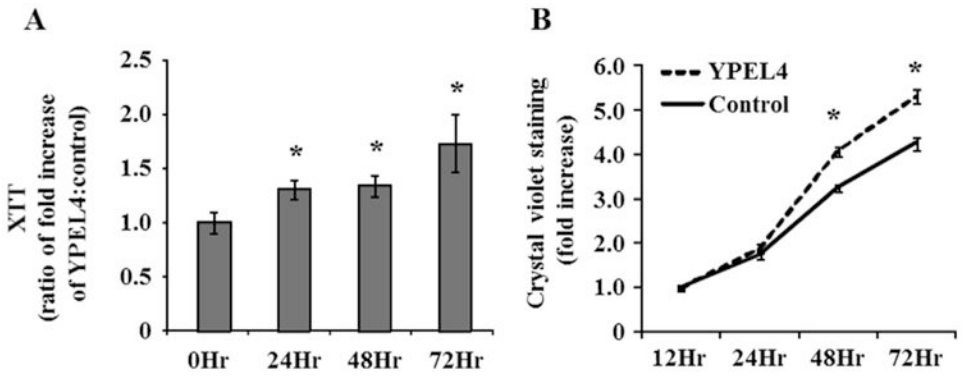

Fig. 4.

Effect of YPEL4 on cell proliferation and aldosterone concentration. (A) HAC15 cells stably transduced with YPEL4 or control lentivirus were plated in 96 well plates with the identical number of cells. After the indicated times, the XTT assay was performed. The results are shown as fold increase in cell proliferation of YPEL4 compared with each control. (B) The stably infected cells were seeded in 48 well plates. Media were collected for measurement of aldosterone concentration after indicated time. Cells were fixed and stained with crystal violet. Data are shown as fold increases in cell proliferation of YPEL4 compared with control after indicated times. *, $P < 0.01$ vs. control, n = 4.

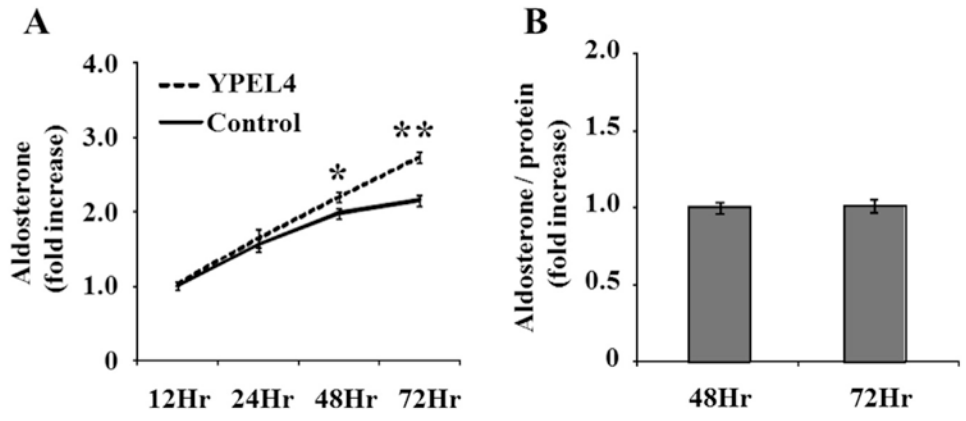

Fig. 5.

Effect of YPEL4 on aldosterone production and aldosterone concentration adjusted by protein concentration. (A) Stably infected cells were seeded in 48 well plates. Media were collected for measurement of aldosterone concentration after 12 h, 24 h, 48 h and 72 h. *, $P < 0.05$ vs. control. **, $P < 0.01$ vs. control, n = 4. (B) After the aspiration of media, cells were harvested for measurement of protein concentration. The aldosterone levels adjusted by protein concentrations are shown after 48 h and 72 h. n = 4.

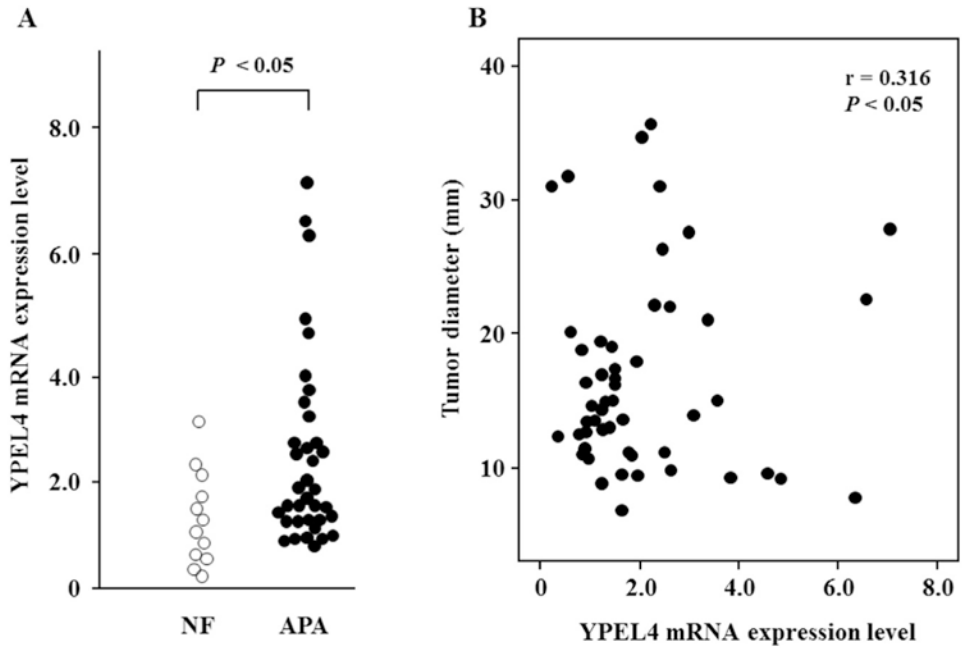

Fig. 6.

(A) YPEL4 mRNA expression levels in NF (n = 12) and APA (n = 39) samples. (B) A relationship between maximum diameter and YPEL4 mRNA levels in 39 APA.

Table 1

Real-time PCR primers.

Gene symbol	Forward primer	Reverse primer
YPEL1	AAGTCCTTTCAGGGGAGCC	TGAGAAGGACCCTCTCCTCTG
YPEL2	ACTAATTTCCAAGTCATTCCAAGG	CAGTCCTGTTAGCAACACTCG
YPEL3	TCAGGCCTACTTGGATGATTG	ACGTTCACCACTGAGTTGAAGAG
YPEL4	TTAACTCCGTGGTCAACGTG	CCTTCACCATGTGTGACATTTC
YPEL5	TTTCCTTGATCATATCGGTGG	ACTGTACTGCAGGTTAACTACCTTG
GAPDH	CCCCTTCATTGACCTCAACTAC	GATGACAAGCTTCCCGTTCTC

Table 2

Clinical characteristics

	NF (n = 12)	APA (n = 39)	*P* value
Male/female (%)	4/8	20/19	0.335
Age (years)	54.3 ± 12.2	50.1 ± 12.2	0.297
BMI	27.2 ± 7.0	23.7 ± 4.2	0.038
Systolic blood pressure (mmHg)	127.0 ± 25.2	144.6 ± 22.5	0.026
Diastolic blood pressure (mmHg)	75.2 ± 16.2	86.2 ± 12.7	0.017
Plasma aldosterone revel (ng/dl)	11.3 ± 9.8	54.3 ± 45.3	0.002
Aldosterone renin ratio	13.9 ± 13.8	208.5 ± 199.6	<0.001
Tumor diameter (mm)	22.5 ± 9.4	15.1 ± 5.6	0.002

Data are expressed as means ± SD. *P* values were determined by χ^2 test or *t*-test. BMI, body mass index.