



## **Effects of down-regulated steroidogenic factor-1 on ACTH and potassium chloride-induced steroid synthesis in H295R cells**

D. Hu<sup>✉</sup>, X. Wang, R. Cao and X. Ding

Department of urology, Zhongnan Hospital of Wuhan University, 169 Donghu road, Wuhan, China.

**Corresponding author:** Dongliang Hu, Department of Urology, Zhongnan Hospital of Wuhan University, 169 Donghu road, Wuhan, China.  
Email: [hdlhdl2008@163.com](mailto:hdlhdl2008@163.com)

### **Abstract**

The prevalence of adrenal diseases in the cortex is more common than that in the medulla in the form of hormone disorder or neoplasm. Steroidogenic factor-1 (SF-1) is important in regulating aldosterone synthase (CYP11B2) and cortisol synthase (CYP11B1). SF-1 is increased in aldosterone-producing adenoma (APA) and cortisol-producing adenoma (CPA). Overexpression of SF-1 has been extensively studied, but the available in-depth information regarding the effects of downregulated SF-1 on CYP11B2/CYP11B1 and their regulators is limited. In this paper, we attempted to investigate the effects of downregulated SF-1 on aldosterone to adrenocorticotrophic hormone (ACTH) and potassium chloride (KCl) stimulation and those on cortisol to ACTH stimulation through RNA interference in acute and chronic phases. Downregulated SF-1 decreased the sensitivity of aldosterone to ACTH/KCl and that of cortisol to ACTH stimulation. This study provides new insights into the influence of SF-1 on adrenocortical diseases by considering the effects of SF-1 on regulation.

**Key words:** Steroidogenic factor-1, Aldosterone, Cortisol, ACTH, H295R cells.

### **Introduction**

Steroidogenic factor-1 (SF-1) is an important transcription factor required for the synthesis of adrenal steroids (1). For adrenal gland, SF-1 is particularly expressed in the adrenal cortex rather than in the medulla (2). SF-1 possesses zone- and enzyme-specific characteristics in the adrenal cortex (3). Previous studies on adrenal steroids mainly focused on the regulation of aldosterone and cortisol. Thus, aldosterone synthase (CYP11B2) and cortisol synthase (CYP11B1) become a highlight for further research. The synthesis of aldosterone and cortisol can be divided into acute and chronic phases (4). The acute phase is controlled by the movement of cholesterol into the mitochondria by steroidogenic acute regulatory (StAR) protein. The chronic phase occurs hours later and mainly involves alteration in CYP11B2 and CYP11B1 expression.

Numerous studies on the correlation of SF-1 and CYP11B2/CYP11B1 have been conducted. However, a consensus among experts has not been completely reached. The detailed influence of SF-1 on adrenal steroid regulators, such as angiotensin II (AT-II), adrenocorticotrophic hormone (ACTH), and potassium chloride (KCl), was differentially reported during aldosterone and cortisol secretions. The majority of these studies focused on the effects of SF-1 overexpression in a doxycycline-induced way (5). Related studies on inhibition by human SF-1 are rarely reported. Thus far, Li made a relatively detailed study. The author determined the correlation between SF-1- and cyclic adenosine monophosphate (cAMP)-induced steroid synthesis by cotransfecting with SF-1 and Bpu-del mutant plasmid. Results were analyzed after 24 h without distinguishing acute and chronic stages (6). In our previous article, we identified that SF-1 played a differential role in basal and AT-II-

induced aldosterone secretion (7). In the present study, we attempted to investigate the effects of downregulated SF-1 on aldosterone to ACTH and KCl stimulation and those on cortisol to ACTH stimulation by detecting their production in acute and chronic phases.

### **Materials and methods**

#### **Cell culture**

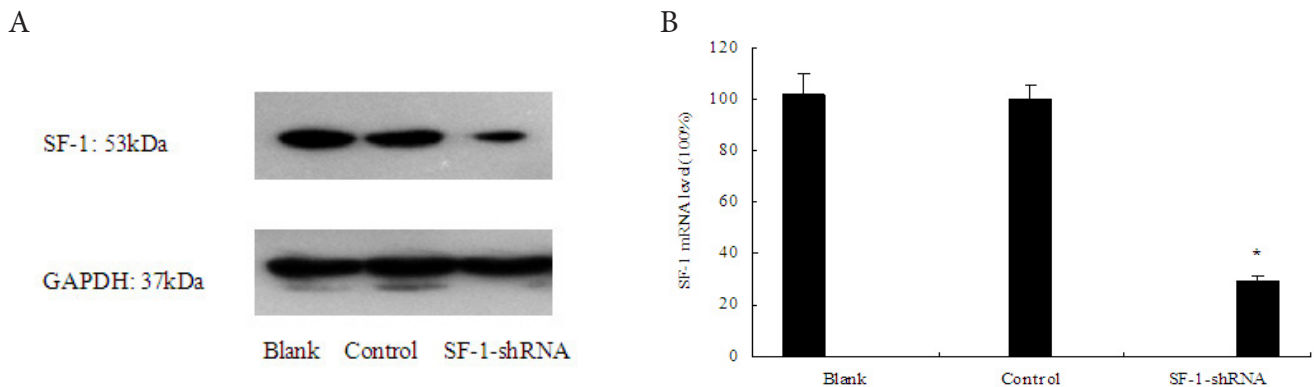
H295R human adrenocortical cells (ATCC, USA) were cultured in DMEM/F12 medium (Invitrogen), supplemented with 2.5% Nu Serum (BD Biosciences, USA), 1% ITS<sup>+</sup> (Sigma-Aldrich, USA) and antibiotics. Transfection or treatment was initiated 24 h after reseeding the cells.

#### **Lentiviral vector constructs and transfection**

To study RNA interference, we designed and cloned three SF-1-shRNA sequences into the downstream of a U6 promoter in pLVX-shRNA-SF-1 (Biowit, China). The most effective sequence was selected through verification in our previous article (8). The target gene sequence of SF-1 was GCAAGATCGACAAGACGCA. Lentiviral vector pLVX-shRNA-SF-1 was used to suppress SF-1 expression. It was transfected into the H295R cells. H295R cells transfected with the virus containing the expression vector with nonspecific shRNA were used as negative control. Normal H295R cells, without any transfections, were used as blank control. The cells were harvested to perform subsequent assays according to different experiments.

#### **Real-time PCR**

Total RNA was extracted from 6-well plates using the Total RNA Miniprep Kit (Axygen, USA) according to the manufacturer's instructions. For cDNA



**Figure 1.** RNA interference effectively inhibited SF-1 expression. **(A)** SF-1 is down-regulated in lentiviral vector pLVX- shRNA-SF-1 cells at the protein level. **(B)** The mRNA expression of SF-1 in different cells was quantified by real-time PCR.

synthesis, 1  $\mu$ g RNA was reverse transcribed using the First Strand cDNA Synthesis Kit (MBI Fermentas, Lithuania). The primers for related genes were as follows: (SF-1) forward, 5'-GTCTGCCTCAAGTTCATCATCC-3', reverse, 5'-TCCTGAGCGTCTTTCACCA-3'; (CYP11B1) forward, 5'-CTGGGACATTGGTGCGC-3', reverse, 5'-GTGTTTCAGCACATGGT-3'; (CYP11B2) forward, 5'-GGCAGAGGCAGAGATGCTG-3', reverse, 5'-CTTGAGTTAGTGTCTC-CACCAGGA-3'; (StAR) forward, 5'-CCAGATGTGGCAAGGTG-3'; reverse, 5'-CAGCGCACGCTCACAAAG-3' (GAPDH) forward, 5'-GTGAAGGTCGGAGTCAACG-3', reverse, 5'-TGAGGTCAATGAAGGGGTC-3'. A 25  $\mu$ l total volume consisted of 2  $\mu$ l cDNA, 12.5  $\mu$ l SYBR Green qPCR Master Mix (2 $\times$ ; MBI Fermentas, Lithuania), and 0.3  $\mu$ M forward and reverse primers. The cycling conditions were as follows: 95°C for 10 min, 40 cycles of 95 °C for 15 s and 60°C for 1 min. All samples were tested in triplicate under these conditions. The mRNA levels of each gene were standardized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

#### Western blot

Each sample was lysed in RIPA buffer on ice for 30 min. Protein concentration was measured with a standard BCA protein assay kit (Beyotime, China). Each sample was electrophoresed on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (Millipore, USA). The blots were probed with anti-SF-1 (1:200; Santa Cruz) and anti-GAPDH (1:500; Santa Cruz, USA). The expression was detected with an ultra-enhanced chemiluminescence kit (BioInd, Israel). Proteins were quantified using the software Quantity One (Bio-Rad, USA).

#### ACTH and KCl stimulation

The acute and chronic detection time were defined at 1 h and 48 h respectively after stimulation. Real-time PCR was used to determine the following gene expression levels. The StAR mRNA was detected at 1 h when we stimulated the experimental and negative control cells with 100 nM ACTH and 16 mM KCl respectively. Similarly, the CYP11B2 mRNA was detected at 48 h. To CYP11B1, it was investigated by incubating experimental and negative control H295R cells with 100 nM ACTH for 48 h.

#### Aldosterone and cortisol analysis

Similar to the preceding method, this analysis involves incubation of experimental and negative control cells for 1 and 48 h in the absence and presence of 100 nM ACTH and (or) 16 mM KCl to detect steroid levels. The supernatants were collected to determine aldosterone and cortisol production by using an ELISA kit (Bio-Swamp, China).

#### Statistical analysis

All experiments were repeated at least three times. Measurement data was analyzed for statistical significance using *t* tests and ANOVA (SPSS 15.0 software). Differences were considered significant at  $P < 0.05$ .

#### Results

##### Suppression of SF-1 expression in the transfected cells

When lentiviral vector pLVX- shRNA-SF-1 was used to transfect the H295R cells, Western blotting and real-time PCR showed that the SF-1 protein and mRNA levels in transfected cells were reduced by 69.7% and 71.2% ( $p < 0.05$ ), respectively (Figure 1), when compared with the negative control cells.

##### Effects of downregulated SF-1 on ACTH-induced aldosterone secretion

In acute phase, the StAR mRNA expression and aldosterone production after ACTH treatment of the experimental cells were higher than those of the negative control cells. The StAR mRNA in the SF-1-inhibited group was 1.4-fold higher than that in the control group ( $p < 0.05$ ). Similarly, the aldosterone productions in the SF-1-inhibited and control groups were  $91.59 \pm 4.28$  and  $79.90 \pm 2.41$  ng/L, respectively ( $p < 0.05$ ) (Figure 2). In chronic phase (48 h later), the CYP11B2 mRNA levels in the SF-1-inhibited group were 13.05-fold higher than those in the control group ( $p < 0.05$ ) (Figure 3). However, the amounts of aldosterone in the two groups were  $129.50 \pm 6.87$  and  $131.52 \pm 7.56$  ng/L ( $p > 0.05$ ) (Figure 4).

##### Effects of downregulated SF-1 on KCl-induced aldosterone secretion

In acute phase (1 h later), KCl slightly increased the StAR mRNA level (1.13-fold,  $p < 0.05$ ). The aldosterone productions in the SF-1-inhibited and control groups were  $80.40 \pm 3.11$  and  $79.90 \pm 2.41$  ng/L ( $p >$

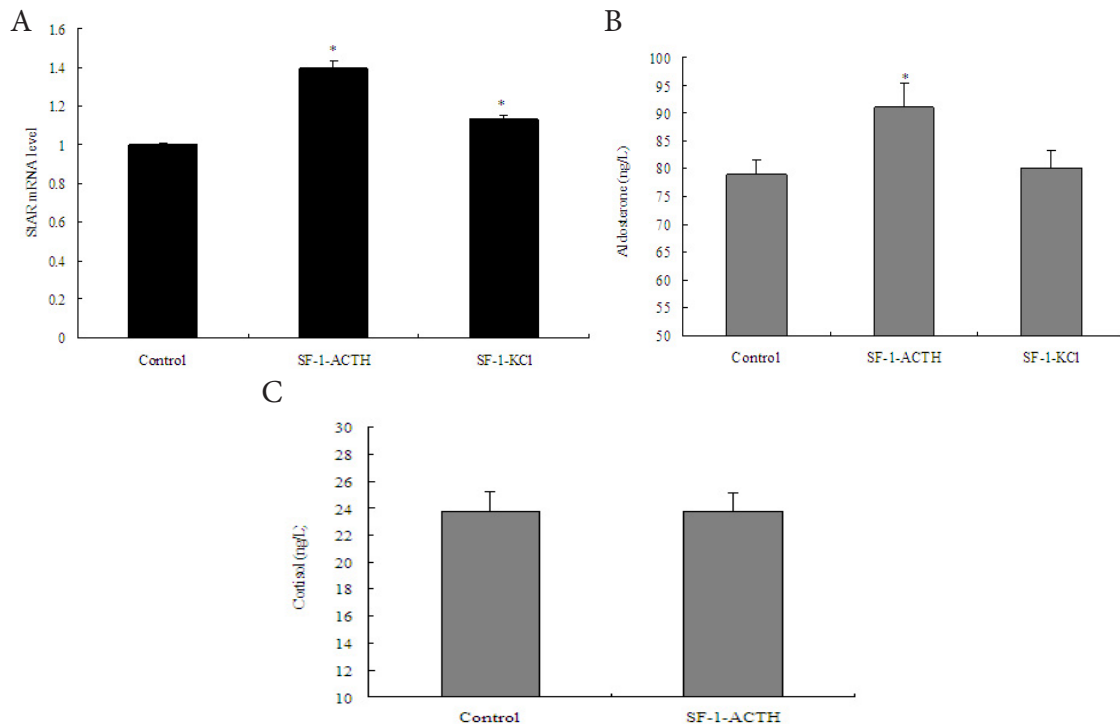
0.05) (Figure 2). In chronic phase, the CYP11B2 mRNA in the SF-1-inhibited group was 2.16-fold higher than that in the control group ( $p < 0.05$ ) (Figure 3). The aldosterone productions in the SF-1-inhibited and control groups were  $117.06 \pm 6.22$  and  $131.52 \pm 7.56$  ng/L, respectively ( $p < 0.05$ ) (Figure 4). By contrast, aldosterone increases in two groups were 37 ng/L and 52 ng/L.

#### Effects of downregulated SF-1 on ACTH-induced cortisol secretion

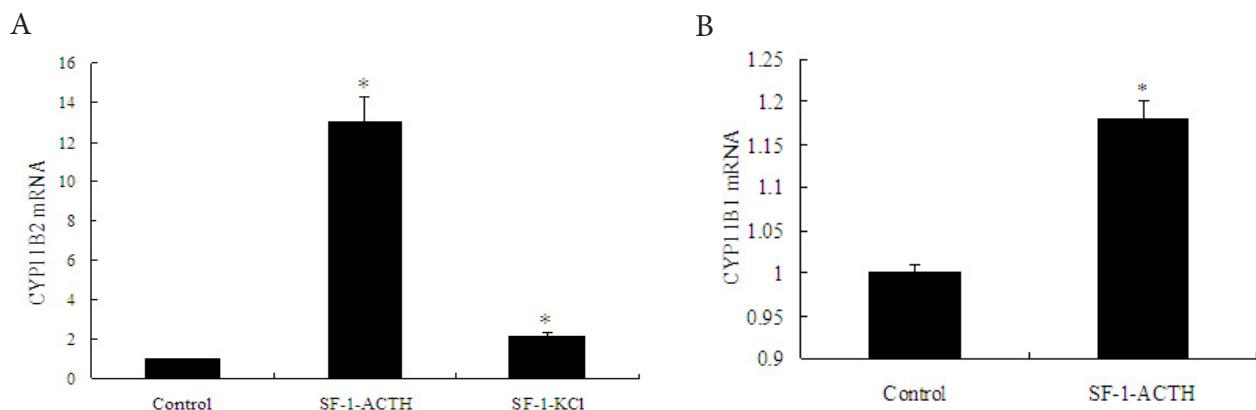
In acute phase, the StAR mRNA level was shown in the preceding section at 1 h after ACTH stimulation. The SF-1-inhibited and control groups exhibited no significant difference in cortisol production ( $23.72 \pm 1.5$  ng/L vs.  $23.74 \pm 1.4$  ng/L,  $p > 0.05$ ) (Figure 2). In chronic phase, the CYP11B1 mRNA in the SF-1-inhibited group was 1.18-fold higher than that in the control group ( $p < 0.05$ ) (Figure 3). The cortisol production was  $28.95 \pm 1.51$  ng/L and  $33.08 \pm 1.67$  ng/L ( $p < 0.05$ ) (Figure 4). By contrast, cortisol increases in two groups were 5.2 ng/L and 9.36 ng/L.

#### Decreased adrenal steroid sensitivity to ACTH/KCl stimulation through downregulated SF-1

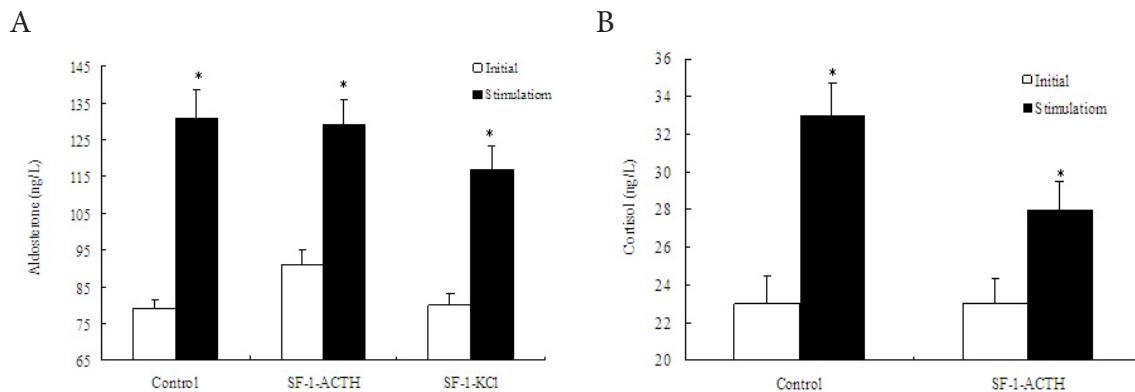
By comparing the preceding data, we determined that StAR, CYP11B1, and CYP11B2 mRNA were slightly increased except the obvious high expression of CYP11B2 mRNA in chronic phase. A decreased adrenal steroid sensitivity to ACTH/KCl stimulation was observed in SF-1-inhibited cells compared with control cells. Moreover, the control cells showed a 52 ng/L aldosterone increase from 1 h to 48 h after ACTH stimulation (initial: 79.90 ng/L; end: 131.52 ng/L), and SF-1-inhibited cells had a 38 ng/L increase (initial: 91.59 ng/L; end: 129.50 ng/L) (Figure 4). An approximately 1.37-fold increase occurred when an identical concentration of ACTH stimulated the cells with different SF-1 expression levels. By comparing the initial and end values in each group, a 1.40-fold aldosterone increase existed when the control and SF-1-inhibited cells were treated with the same concentration of KCl (52 ng/L vs. 37 ng/L). A 1.80-fold cortisol increase was observed when the control and SF-1-inhibited cells were treated



**Figure 2.** Effects of SF-1 downregulation on StAR mRNA and adrenal steroid response to external stimulation in acute phase. **(A)** StAR mRNA levels in the control and SF-1-inhibited cells at 1 h after ACTH and KCl stimulation ( $p < 0.05$ ). **(B)** Aldosterone production of each group at 1 h after ACTH ( $p < 0.05$ ) and KCl ( $p > 0.05$ ) stimulation. **(C)** Cortisol production of control and SF-1-inhibited cell at 1 h after ACTH stimulation ( $p > 0.05$ ).



**Figure 3.** Effects of down-regulated SF-1 on CYP11B2 and CYP11B1 mRNA levels in chronic phase. **(A)** CYP11B2 mRNA levels in the control and SF-1-inhibited cells at 48 h after ACTH and KCl stimulation ( $p < 0.05$ ). **(B)** CYP11B1 expression in the control and SF-1-inhibited cell at 48 h after ACTH stimulation ( $p < 0.05$ ).



**Figure 4.** Down-regulated SF-1 decreased adrenal steroids sensitivity to ACTH/KCl stimulation. **(A)** Effects of SF-1 downregulation on aldosterone response to ACTH/KCl stimulation. **(B).** Down-regulated SF-1 decreased cortisol sensitivity to ACTH stimulation.

with the same concentration of ACTH (9.36 ng/L vs. 5.2 ng/L). The differences of adrenal steroid production in the control and SF-1-inhibited cells were statistically significant ( $p < 0.05$ ) (Figure 4).

## Discussion

Adrenal diseases often cause damages to body in the form of hormone disorder or neoplasm. These two forms usually promote and influence mutually. Adrenalectomy and medical therapy are the two main methods for adrenal treatment. However, different disease needs distinct treatment. Therefore, the possible pathogenesis of adrenal diseases must be explored. Many authors have gradually focused on adrenocortical diseases because the diseases caused by adrenal medulla are relatively rare in clinical practice.

SF-1 (also called NR5A1/Ad4BP) is mainly expressed in steroid-related tissues. The target genes of SF-1 primarily involve several P450 steroid hydroxylases, StAR, and adrenocorticotrophic hormone receptor (ACTHR). SF-1 plays a pivotal role in regulating an array of genes that mediate steroidogenesis and sex differentiation either alone or in combination. Overexpression of SF-1 can lead to adrenal cell proliferation and tumor formation (9). On the contrary, several mutations of SF-1 often cause adrenal insufficiency and gonad agenesis (10). A recent research related to SF-1 has been based on doxycycline-inducible expression or SF-1 knockout mice (9). SF1 is clearly expressed in a time- and species-specific manner (3). Thus, many reports in other species are considered valuable only as references because of this specificity. The influence of SF-1 on human adrenocortical hormone regulation requires further investigation. In the present study, we focused on the effects of SF-1 gene silencing through shRNA experiments on aldosterone and cortisol after ACTH and/or KCl stimulation.

Many factors are involved in regulating adrenal steroid synthesis. AT-II, KCl, and ACTH are the most important regulators of aldosterone, and ACTH is the most important regulator of cortisol. In our previous research, we studied the differential role of SF-1 in basal and AT-II-induced aldosterone secretion (7). We continue to investigate the possible effects of SF-1 on ACTH- and KCl-induced adrenocortical hormones production by detecting their levels in acute and chronic phases.

In this work, adrenal steroids were detected at mRNA and hormone levels. In acute phase, StAR mRNA was

increased in each group. The increase in ACTH-induced cell was more evident than that in KCl-induced cell at the mRNA level. However, this increase was only statistically significant in ACTH-induced aldosterone group at the hormone level. SF-1 can regulate StAR expression positively (11). The downregulated SF-1 relatively decreased StAR expression level and largely counteracted the promoting effects of ACTH and KCl. Nerve growth factor-induced gene B (NGFI-B), which was expressed in the glomerulosa zone of the adrenal cortex, could be rapidly increased after ACTH stimulation within 30 min (12). The elevated NGFI-B combined with StAR promoter region and played a role as an important transcription factor or rapidly inducible gene. Numerous data show that CYP11B2 promoter possesses an NGFI-B response element (NBRE), but CYP11B1 does not possess an NBRE (13). Thus, no significant differences existed in ACTH-induced cortisol and KCl-induced aldosterone groups. In chronic phase, ACTH-induced CYP11B2 mRNA showed the highest expression. By contrast, KCl-induced CYP11B2 and ACTH-induced CYP11B1 mRNA slightly increased. During the subsequent hormone assay, we determined that the aldosterone and cortisol productions in SF-1-inhibited cells were lower than those in control cells. Compared with the levels at the initial stage, downregulated SF-1 decreased the sensitivity of aldosterone and cortisol to their regulator stimulation, especially ACTH-induced cortisol. High SF-1 expression may increase the reactivity and sensitivity of aldosterone and cortisol to regulators. This finding is consistent with that of the published articles, which showed that the SF-1 expression in cortisol adenoma and aldosterone adenoma is higher than that in normal adrenal (14).

The decreased sensitivity of aldosterone and cortisol to ACTH/KCl in SF-1-inhibited cells may be attributed to the following. (1) ACTHR promoter region, as a target gene, has abundant SF-1 binding sites. SF-1 can positively promote and regulate ACTHR expression (15). Moreover, ACTH performs its functions by activating ACTHR. Thus, the downregulated SF-1 decreased ACTHR expression and caused the downregulation of aldosterone and cortisol secretion. However, the difference between the two hormones is that SF-1 regulates CYP11B1 positively but CYP11B2 negatively (16). In addition, the role of ACTH in cortisol is more crucial than that in aldosterone. Therefore, the decline in ACTH-induced cortisol group was evident. (2) SF-1 is essential for adrenal growth and cell proliferation

(7,17). Downregulated SF-1 often leads to less cell number and atrophic cell states, thereby decreasing adrenal steroid production and stimulus response. (3) cAMP, as an important signaling molecule, has an important role in ACTH and KCl regulatory processes, particularly to ACTH. It resembles a consensus cAMP response element that can combine with SF-1 (18). Thus, downregulated SF-1 affects hormone secretion and regulatory ability via ACTH/cAMP signaling pathway.

Regulations of adrenal steroids occur in the body's internal environment. Thus, regulatory mechanisms and influencing factors were more complex than those in vitro. The complexity and multi-sources of adrenocortical diseases require us to consider various factors when diagnosing and forming a comprehensive view of these diseases.

### Acknowledgements

This study was supported by the grants from the National Natural Science Foundation of China (No. 81200579).

### References

- Parker, K.L., Schimmer, B.P., Steroidogenic factor 1: a key determinant of endocrine development and function. *Endocr Rev.* 1997; **18**:361-377. doi: 10.1210/edrv.18.3.0301
- Shibata, H., Ikeda, Y., Mukai, T., et al. Expression profiles of COUP-TF, DAX-1, and SF-1 in the human adrenal gland and adrenocortical tumors: possible implications in steroidogenesis. *Mol Genet Metab.* 2001; **74**:206-216. doi: 10.1006/mgme.2001.3231
- Hoivik, E.A., Aumo, L., Aesoy, R., et al. Deoxyribonucleic acid methylation controls cell type-specific expression of steroidogenic factor 1. *Endocrinology.* 2008; **149**:5599-5609. doi: 10.1210/en.2008-0104
- Bassett, M.H., White, P.C., and Rainey, W.E., The regulation of aldosterone synthase expression. *Molecular and Cellular Endocrinology.* 2004; **217**: 67-74. doi: 10.1016/j.mce.2003.10.011
- Ye, P., Nakamura, Y., Lalli, E., and Rainey, W.E., Differential effects of high and low steroidogenic factor-1 expression on CYP11B2 expression and aldosterone production in adrenocortical cells. *Endocrinology.* 2009; **150**: 1303-1309. doi: 10.1210/en.2008-0667
- Li, L.A., Chang, Y.C., Wang, C.J., et al. Steroidogenic factor 1 differentially regulates basal and inducible steroidogenic gene expression and steroid synthesis in human adrenocortical H295R cells. *J Steroid Biochem Mol Biol.* 2004, **91**:11-20. doi: 10.1016/j.jsbmb.2004.02.002
- Ouyang, J., Hu, D., Wang, B., et al. Differential effects of down-regulated steroidogenic factor-1 on basal and angiotensin II-induced aldosterone secretion. *J Endocrinol Invest.* 2011; **34**:671-675. doi: 10.3275/7413
- Dongliang, H., Jinzhi, O., Zhun, W., et al. Elementary studies on the elevated expression of steroidogenic factor-1 in aldosterone-producing adenoma. *Urologic Oncology.* 2012, **30**:457-462. doi: 10.1016/j.urolonc.2010.03.001
- Doghman, M., Karpova, T., Rodrigues, G.A., et al. Increased Steroidogenic factor-1 dosage triggers adrenocortical cell proliferation and cancer. *Molecular Endocrinology.* 2007; **21**:2968-2987. doi: 10.1210/me.2007-0120
- Correa, R.V., Domenice, S., Bingham, N.R., et al. A microdeletion in the ligand binding domain of human steroidogenic factor 1 causes XY sex reversal without adrenal insufficiency. *J Clin Endocrinol Metab.* 2004; **89**:1767-1772. doi: 10.1210/jc.2003-031240
- Ansurudeen, I., Kopprasch, S., Ehrhart-Bornstein, M., et al. Endothelial cell-mediated regulation of aldosterone release from human adrenocortical cells. *Mol Cell Endocrinol.* 2007; **266**:150-156. doi: 10.1016/j.mce.2006.12.011
- Wilson, T.E., Mouw, A.R., Weaver, C.A., et al. The orphan nuclear receptor NGFI-B regulates expression of the gene encoding steroid 21-hydroxylase. *Mol Cell Biol.* 1993; **13** :861-868. doi: 10.1128/MCB.13.2.861
- Bassett, M.H., Suzuki, T., Sasano, H., et al. The orphan nuclear receptors NURR1 and NGFI-B regulate adrenal aldosterone production. *Mol Endocrinol.* 2004; **18**: 279-290. doi: 10.1210/me.2003-0005
- Bassett MH, Mayhew B, Rehman K, et al. Expression profiles for steroidogenic enzymes in adrenocortical disease. *J Clin Endocrinol Metab.* 2005; **90**: 5446-5455. doi: 10.1210/jc.2005-0836
- Mountjoy, K.G., Bird, I.M., Rainey, W.E., et al. ACTH induces up-regulation of ACTH receptor mRNA in mouse and human adrenocortical cell lines. *Molecular and cellular endocrinology.* 1994, **99**: 17-20. doi:10.1016/0303-7207(94)90160-0
- Bassett, M.H., Zhang, Y., Clyne, C., White, P.C., and Rainey, W.E., Differential regulation of aldosterone synthase and 11beta-hydroxylase transcription by steroidogenic factor-1. *Journal of Molecular Endocrinology.* 2002; **28**: 125-135. doi: 10.1677/jme.0.0280125
- Hammer, G.D., Parker, K.L., Schimmer, B.P., et al. Minireview: transcriptional regulation of adrenocortical development. *Endocrinology.* 2005; **146**: 1018-1024. doi: 10.1210/en.2004-1385
- Bassett, M.H., Suzuki, T., Sasano, H., et al. The orphan nuclear receptors NURR1 and NGFI-B regulate adrenal aldosterone production. *Mol Endocrinol.* 2004; **18**: 279-290. doi: 10.1210/me.2003-0005