HHS Public Access

Author manuscript

*Mol Cell Endocrinol*. Author manuscript; available in PMC 2024 November 01.

Published in final edited form as:

*Mol Cell Endocrinol*. 2023 November 01; 577: 112030. doi:10.1016/j.mce.2023.112030.

# Salt-Inducible Kinases Regulate Androgen Synthesis in Theca Cells by Enhancing CREB Signaling

**Miriam Rodriguez Esquivel**[1], **Emily Hayes**[1], **Oliwia Lakomy**[1], **Mariam Hassan**[1], **Marc Foretz**[2], **Carlos Stocco**[1]

[1]Department of Physiology and Biophysics, University of Illinois at Chicago. Chicago, IL, 60612

[2]Université Paris Cité, Institut Cochin, CNRS, INSERM, F-75014 Paris, FRANCE.

## Abstract

Ovulation is the pinnacle of folliculogenesis, a process that requires an interplay between the oocyte, the granulosa cells, and the theca cells (TCs). TCs are the only source of ovarian androgens, which play a vital role in female fertility. However, abnormally elevated androgen levels reduce fertility. Therefore, uncovering novel mechanisms regulating androgen synthesis in TCs is of great significance. We have shown that salt-inducible kinases (SIKs) regulate granulosa cell steroidogenesis. Here, we investigated whether SIKs regulate androgen production in TCs. SIK2 and SIK3 were detected in the TCs of mouse ovaries and isolated TCs. Next, TCs in culture were treated with luteinizing hormone (LH) in the presence or absence of a highly specific SIK inhibitor. SIK inhibition enhanced the stimulatory effect of LH on steroidogenic gene expression and androgen production in a concentration-dependent manner. SIK inhibition alone stimulated the expression of steroidogenic genes and increased androgen production. Activation of adenylyl cyclase with forskolin or emulation of increased intracellular cyclic AMP levels stimulated steroidogenesis, an effect that was enhanced by the inhibition of SIK activity. The stimulatory effect of downstream targets of cyclic AMP was also significantly augmented by SIK inhibition, suggesting that SIKs control targets downstream cyclic AMP. Finally, it is shown that SIK2 knockout mice have higher circulating testosterone than controls. This evidence shows that TCs express SIKs and reveal novel roles for SIKs in the regulation of TC function and

Correspondence and reprint requests to Carlos Stocco (costocco@uic.edu).

Author contributions

Conceptualization: MR-E, EH, and CS; Methodology: MR-E, EH, OL, and MH; Formal analysis and investigation: EH, MRE, and CS; Resources: MF; Draft preparation: CS, MRE, and MRE; Review and editing: MR-E, EH, OL, MH, MF, and CS; Project administration and funding acquisition: CS.



Disclosure Summary: The authors have nothing to disclose.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The authors have no financial/personal interest or belief that could affect the objectivity of the results presented.

Credit Author Statement

Conceptualization: MR-E, EH, and CS; Methodology: MR-E, EH, OL, and MH; Formal analysis and investigation: EH, MRE, and CS; Resources: MF; Draft preparation: CS, MRE, and MRE; Review and editing: MR-E, EH, OL, MH, MF, and CS; Project administration and funding acquisition: CS.

androgen production. This information could contribute to uncovering therapeutic targets to treat hyperandrogenic diseases.

### Keywords

Ovary; Theca Cells; SIKs; Androgens; Steroidogenesis

## Introduction

The follicle is the functional unit of the ovary. At the core of the ovarian follicle are the oocyte and the granulosa cells starting at primordial follicle formation, while theca cells (TCs) differentiate as the outer layer of the follicle at the preantral stage and remain an essential part of the follicle until ovulation. The theca layer provides structural support and a place for vasculature development that secures appropriate blood supply for follicle growth. In addition, TCs are the only source of ovarian androgens (Young and McNeilly, 2010). Androgens play vital roles in reproduction as essential precursors for estrogen synthesis or acting directly through the androgen receptors located throughout the ovary (Hu, Wang, Yeh et al., 2004,Sen and Hammes, 2010,Walters, Middleton, Joseph et al., 2012). TC-produced androgens enhance follicle development by activating the androgen receptor in primates (Vendola, Zhou, Wang et al., 1999,Vendola, Zhou, Adesanya et al., 1998) and rodents (Murray, Gosden, Allison et al., 1998,Wang, Andoh, Hagiwara et al., 2001,Xue, Liu, Murphy et al., 2012). In humans, androgen depletion reduces fertility (Astapova, Minor and Hammes, 2019). Indeed, androgen priming improves the ovarian response to gonadotropins in patients with diminished ovarian reserve or poor responders (Bercaire, Nogueira, Lima et al., 2018,Gleicher, Kim, Weghofer et al., 2013,Katsika, Bosdou, Goulis et al., 2022,Noventa, Vitagliano, Andrisani et al., 2019,Wiser, Gonen, Ghetler et al., 2010). Therefore, a better understanding of the mechanisms controlling androgen synthesis in TCs may have important clinical consequences.

TCs are highly differentiated with structural features characteristic of steroid-secreting cells, including numerous mitochondria and the expression of steroidogenic factors such as cholesterol side-chain cleavage (CYP11A1) and steroidogenic acute regulator (STARD1) (Young and McNeilly, 2010). Chiefly, TCs express high levels of 17α-hydroxylase-17,20-lyase (CYP17A1), which is the only enzyme able to transform progestogens into androgens (Miller and Tee, 2015,Zhang, Compagnone, Fiore et al., 2001). The capacity of TCs to synthesize androgens also relies on the expression of the luteinizing hormone (LH) receptor. LH activates Gs alpha stimulating adenylyl cyclase activity and cyclic AMP (cAMP) production, which activates protein kinase A (PKA) leading to the phosphorylation of cAMP response element-binding protein (CREB). However, the molecular mechanisms by which PKA and CREB regulate TC function and androgen production remain to be fully understood.

Salt-inducible kinases (SIKs) are serine/threonine kinases of which three isoforms are known: SIK1, SIK2, and SIK3 (Sakamoto, Bultot and Goransson, 2018,Wein, Foretz, Fisher et al., 2018). SIK1 was identified in the adrenal gland of rats fed a high-salt diet (Wang,

Takemori, Halder et al., 1999). SIK2 and SIK3 are expressed ubiquitously but especially at high levels in adipose tissue and the brain (Darling and Cohen, 2021,Sakamoto et al., 2018,Wein et al., 2018). We recently reported that SIK1 knockout (KO) female mice are fertile, producing average litter sizes and releasing a similar number of oocytes as control females (Armouti, Winston, Hatano et al., 2020). SIK2KO mice are also fertile and produce average litter sizes. However, in response to a superovulation protocol, they release three times more oocytes than controls. In contrast, SIK3KO females are infertile and do not ovulate when stimulated with a superovulation protocol. We have also recently shown that inhibition of SIK activity in granulosa cells enhances the response of these cells to follicle-stimulating hormone (FSH) (Armouti, Rodriguez-Esquivel and Stocco, 2022,Armouti et al., 2020). However, whether SIKs regulate any aspect of theca cell physiology remains to be seen.

As mentioned above, cAMP is the main second messenger in the LH signaling pathway. Interestingly, cAMP stimulation of gene expression is negatively regulated by SIKs (Sakamoto et al., 2018,Wein et al., 2018). The reported capacity of SIKs to regulate cAMP signaling led to the hypothesis that SIKs influence the response of TCs to LH. Here, we examined the role of SIKs in the regulation of TCs and the effects of these kinases on the production of androgens and the response of TCs to LH. The results demonstrate that SIK inhibition led to an increase in basal androgen synthesis and an enhancement of the stimulatory effect of LH on androgen synthesis. The findings could help identify new players regulating LH actions and reveal novel roles for SIKs in TCs and female fertility. Thus, targeting SIK activity could increase ovarian androgen synthesis and improve fertility in some patients.

## Materials and Methods

### Animals and Cell Cultures –

Mice and rats were obtained from Charles Rivers. The Institutional Animal Care and Use Committee at the University of Illinois at Chicago approved all animal experiments (Protocol number 20–173). To isolate TCs, mouse or rat ovaries were digested using a mixture of Collagenase type I (4 mg/ml), DNAse (10 ug/mL), BSA (10 mg/ml), Dispase Grade II (0.5 mg/ml) (all from Sigma, St. Louis, MO) for 1h at 37C with gentle rotation. The resulting cell suspension was filtered through a 40 μM filter to eliminate oocytes. A discontinuous Percoll (GE) gradient was prepared using Hank's Balanced Salt Solution. Cells were washed, loaded on top of a 35% Percoll layer, and spun for 15 minutes at 200 *g*. TCs were recovered from the 35% layer, washed, and cultured in serum-free DMEM/F12 media with the addition of insulin (10 μg/ml), transferrin (5.5 μg/ml), sodium selenite (5 ng/ml), and antibiotic (all from Sigma). TC preparations were routinely verified using immunofluorescence staining against CYP17A1 (Proteintech, Cat#14447–1-AP, 1:50) and counterstained with DAPI to detect nuclear DNA.

SIK2 knockout mice: Mice carrying one floxed allele and one null allele for the SIK2 gene (denoted as $SIK2^{F/-}$) were produced by crossing $SIK2^{F/F}$ mice with ZP3Cre mice. ZP3Cre mice express Cre recombinase under the control of zona pellucida 3 (Zp3) promoter in growing oocytes before the completion of the first meiotic division, causing the deletion

of floxed sequences in the female germ line (de Vries, Binns, Fancher et al., 2000). $SIK2^{F/-}$ were crossed to produce $SIK2^{-/-}$ mice. ZP3Cre mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice carrying floxed alleles of the SIK2 genes were previously described (Patel, Foretz, Marion et al., 2014).

Human H295R cells: NCI-H295R (ATCC CRL-2128) cells, an adrenal cell line isolated from an adrenocortical carcinoma (Samandari, Kempna, Nuoffer et al., 2007), were cultured following vendor recommendations in DMEM:F12 with the addition of the following components 0.00625 mg/ml insulin, 0.00625 mg/ml transferrin, 6.25 ng/ml selenium, 1.25 mg/ml bovine serum albumin, 0.00535 mg/ml linoleic acid, and 2.5% Nu-Serum I (Corning).

Theca and H295R cells were treated with the SIK inhibitor HG-9–91-01 (HG, Tocris, Bristol, United Kingdom) for 30 minutes before the addition of ovine LH (National Hormone and Pituitary Program, NIH, Rockville, MD), forskolin (Sigma), or dibutyryl cyclic AMP (dbcAMP, Sigma), as indicated in the figure legends.

### Lentivirus Infections -

Overexpression experiments – Expression plasmids encoding constitutively active PKA or C2/CREB were kindly provided by Dr. Anthony J. Zeleznik (University of Pittsburgh) and Dr. Thiel (University of Saarland, Germany), respectively. C2/CREB cDNA was subcloned into the pGPcs vector, which was derived from the pCDH vector (System Biosciences, Mountain View, CA). The capacity of these plasmids to activate the CRE-Luc reporter was tested by transfecting a CRE-Luc reporter along with pGPcs, caPKA, or C2/CREB in HEK293 cells as previously reported (Armouti et al., 2022). Lentivirus stocks were generated in HEK293 cells (Invitrogen) transfected with pGPcs (empty), caPKA, or C2/CREB lentiviral vector along with the packaging and envelope plasmids psPAX2 and pMD2G (Addgene, Watertown, MA). Cell supernatants were concentrated by ultracentrifugation. Viral stocks were titrated in HEK293 cells aided by a fluorescence (GFP) marker. Viral stocks carrying pGPcs (control), caPKA, or C2/CREB were added directly to the cells 2 h after plating at a multiplicity of infection of 20 and cultured for 24 h before the initiation of the treatments described in each figure.

### RNA Isolation and Quantification –

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) and reverse-transcribed using anchored oligo-dT primers (IDT, Coralville, IA) and Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen). Intron-spanning primers were used to amplify the gene of interest (GOI) along with a standard curve containing serial dilutions of the cDNA of the GOI. Real-time PCR amplifications were performed with Brilliant II qPCR SYBR master mix (Agilent, Santa Clara, CA) using an AriaMx instrument (Agilent). For each sample, the number of copies per microliter of cDNA was computed for each GOI and the ribosomal protein L19 mRNA (*Rpl19*). The expression of each GOI is reported as the ratio between the number of copies of the GOI and *Rpl19* for rat and mouse TCs and for H295R cells.

### Immunoblotting –

Protein extracts prepared using RIPA lysis buffer supplemented with protease inhibitors. Protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, Illinois). Proteins were subjected to gel electrophoresis, transferred to nitrocellulose membranes, and processed by routine procedures. The primary antibodies and the dilutions used were GAPDH (ProteinTech, #60004, 1:500), SIK1 (LS Bio, LS-B9981, 1:1000), SIK2 (Cell Signaling, #6919, 1:1000), and SIK3 (LS Bio, #LS-B9603, 1:1000). The secondary antibodies used were anti-rabbit IgG-HRP (goat, 1:10,000) from Abcam (Cambridge, United Kingdom) or anti-mouse IgG-HRP (goat, 1:10,000) from Jackson ImmunoResearch Laboratory Inc. (West Grove, PA). Detection was performed with Supersignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL) and detected using ChemiDoc MP Imaging System (BioRad, Hercules, CA). Protein expression quantification was performed with ImageJ software (National Institutes of Health, Bethesda, Maryland).

### Testosterone Measurement –

Undiluted cell culture medium was used for testosterone level determinations by ELISA. The intra- and inter-assay variabilities are 3.6% and 7.1%, respectively; DGR International, Springfield, NJ).

### Immunohistochemistry –

Mouse ovaries were embedded in paraffin to prepare 5 μm sections, which were stained using primary antibodies diluted in PBS: SIK1, SIK2, and SIK3 previously described (Armouti et al., 2020). Antibodies were detected using Vectastain Elite (Vector Laboratories, Burlingame, CA) and counterstained with Gill's hematoxylin.

### Statistics –

Data were analyzed using Prism 6 (San Diego, CA). Differences between two groups were determined by Student's *t*-test. For multiple groups, one-way ANOVA was used, and differences between individual means were determined by the Tukey test. Data are represented as mean ± SEM. Significant differences were recognized at $p < 0.05$.

## Results

### SIKs are expressed in mouse TCs and human theca-like cells.

First, SIK isoforms in isolated mouse TCs were examined using western blotting. For this purpose, mouse TCs were isolated, and the purity of the cell preparation was verified using immunofluorescence with antibodies against CYP17A1, a TC-specific marker, and counterstained with DAPI to detect nuclear DNA. Supplemental Figure 1 shows that 100% of the cells were stained for CYP17A1, confirming the identity of the isolated TCs. We also examined the expression of SIKs in H295R cells, a human adrenocortical cell line that expresses high CYP17A1 activity and produces androgens (Samandari et al., 2007). Western blotting demonstrated the presence of SIK2 and SIK3 isoforms in mouse TCs, whereas SIK1 was undetectable. SIK3 protein was more strongly expressed than SIK2. In contrast, in

H295R cells, all isoforms were detected although SIK1 levels were lower in comparison to SIK2 and SIK3, of which SIK2 was the most highly expressed (Figure 1).

To determine if similar expression levels for each SIK isoform are found *in vivo*, we used the ovaries of adult mice to perform immunohistochemical (IHC) studies. The results showed that SIK2 and SIK3, but not SIK1, are highly expressed in the ovaries (Figure 2). Higher magnification analysis also showed that SIK3 is especially highly expressed in the theca and interstitial cells (Figure 2, right). Taken together these studies show that the interstitial tissue and the TCs express mostly SIK2 and SIK3.

### SIK inhibition enhances androgen synthesis and LH responses in TCs.

Next, we examined the effects of SIK inhibition on TC function. We previously demonstrated that SIK inhibition by itself can regulate gene expression in granulosa cells (Armouti et al., 2022). Therefore, we first examined the effect of HG-9–91-01 (HG), a highly specific SIK inhibitor (Patel et al., 2014), on the expression of steroidogenic and theca-specific genes such as *Cyp17a1*, bone morphogenetic protein 4 (*Bmp4*) (Chang, Cheng and Leung, 2013), and insulin-like peptide 3 (*Insl3*) (Nef and Parada, 1999). We also examined the effect of SIK inhibition on *Stard1* and *Cyp11a1*, which are required for the production of androgens. As shown in Figure 3, inhibition of SIK activity stimulated the expression of *Stard1*, *Cyp17a1*, *Cyp11a1*, and *Insl3* in a concentration-dependent manner (Figure 3). The lowest concentration used (0.1 μM) significantly stimulated the expression of *Stard1*, *Cyp17a1*, *Cyp11a1*, and *Insl3*. For *Cyp17a1*, a stronger effect was observed at a 1 μM concentration compared to 3 μM. SIK inhibition decreased the expression of *Bmp4* mRNA levels also in a concentration-dependent manner showing a maximal inhibitory effect at 1 μM.

To investigate the role played by SIKs on the hormonal regulation of TC function, we next treated mouse TCs with LH (50 ng/ml) in the presence of vehicle (DMSO) or the SIK activity inhibitor HG. The dose of LH used is based on previously published reports (Magoffin and Erickson, 1982).

As expected, LH treatment of TCs stimulated the expression of *Stard1*, *Cyp11a1*, and *Cyp17a1*, whereas LH inhibited the expression of *Bmp4* (Figure 4). SIK inhibition enhanced the stimulatory effect of LH on *Stard1*, *Cyp11a1*, and *Cyp17a1* mRNA expression in a concentration-dependent manner. Enhancing effects were observed at the lowest concentration used (0.1 μM) for *Stard1* and *Cyp11a1* and at 0.3 μM for *Cyp17a1*. However, the 3 μM concentration was less effective in enhancing LH actions on the expression of *Cyp17a1* and *Stard1* when compared to 1 μM (Figure 4). SIK inhibition also enhanced the inhibitory effect of LH on *Bmp4* expression in a concentration-dependent manner. SIK inhibition with 1 μM HG also enhanced the stimulatory effects of LH on *Cyp17a1* and *Stard1* in rat TCs (Supplemental Figure 2).

The effects of SIK inhibition on the basal production of testosterone or the stimulatory effect of LH on the production of this androgen were examined next. Based on the concentration-dependent effect observed in Figures 3 and 4, HG was used at a concentration of 1 μM in these experiments. As expected, LH treatment of TCs stimulated the production of

testosterone (Figure 5). SIK inhibition alone also increased testosterone production. Finally, we also observed that SIK inhibition enhanced the stimulatory effect of LH on testosterone production, suggesting that SIK inhibition enhances TC function (Figure 5).

### SIK inhibition enhances the effects of LH downstream targets.

In light of the enhancing effects observed between LH and SIK inhibition, we postulated that SIK inhibition amplifies LH-stimulated signaling pathways. Thus, we examined if SIK inhibition enhances the effect of LH receptor downstream targets on the expression of steroidogenic genes and the production of testosterone. We observed that activation of adenylyl cyclase with forskolin or emulation of increased intracellular cAMP with dbcAMP stimulated steroidogenic genes and inhibited *Bmp4* (Figures 6 and 7). As with LH, SIK inhibition enhanced forskolin and dbcAMP induction of *Cyp17a1, Stard1*, and *Cyp11a1* and the inhibition of *Bmp4* expression in a concentration-dependent manner (Figures 6 and 7). Significant effects of HG were observed at the lowest concentration tested (0.1 μM). As observed above, 3 μM of HG was less effective than 1 μM in enhancing forskolin or dbcAMP effects on *Cyp17a1* and *Stard1* but not for *Cyp11a1* and *Bmp4*.

The stimulatory effect of cAMP on the expression of steroidogenic genes was also translated into significantly higher levels of testosterone production (Figure 8). Importantly, SIK inhibition also enhanced dbcAMP stimulation of testosterone production (Figure 8).

### SIK inhibition enhances the response of human H295R cells to cAMP signaling.

As shown above, H295R cells express all three SIK isoforms (Figure 1). To study the role of SIKs on androgen production in human cells, we examined if inhibition of SIK activity affects the response of H295R cells to cAMP. Like TCs, H295R cells responded to forskolin and dbcAMP with significantly increased mRNA expression of *STARD1*, *CYP11A1*, and *CYP17A1* when compared to controls (Figure 9). The induction of these genes by forskolin or dbcAMP was enhanced by SIK inhibition. SIK inhibition alone was sufficient to stimulate the expression of *STARD1*, *CYP17A1*, and *CYP11A1* in H295R cells, which agrees with our observations in mouse and rat TCs (Figure 9).

### SIK inhibition enhances PKA and CREB stimulation of testosterone synthesis and production.

Next, we examined if SIK inhibition enhances the effect of cAMP downstream targets on the expression of steroidogenic genes and the production of testosterone in mouse TCs. Since the main target of cAMP is PKA, we first used a lentivirus to express a constitutively active PKA (caPKA) protein, which carries His87Gln and Trp196Arg mutations rendering it insensitive to the regulatory units (Orellana and McKnight, 1992). The overexpression of caPKA significantly stimulated the expression of *Stard1* and *Cyp17a1* mRNA levels over controls (Figure 10A). Cotreatment with HG significantly enhanced the effects of caPKA on the expression of these genes. Consistent with this finding, overexpression of caPKA was also sufficient to stimulate testosterone production (Figure 10B). This stimulatory effect of caPKA on testosterone production was significantly enhanced by inhibiting SIK activity.

PKA targets mainly CREB leading to its phosphorylation and consequent activation of CREB-regulated genes. Therefore, the impact of SIK inhibition on the effect of C2/CREB, a fusion protein that activates CREB-responsive genes in the absence of cAMP (Thiel, Al Sarraj, Vinson et al., 2005), on steroidogenic gene expression and testosterone production was tested next. Overexpression of C2/CREB significantly increased the expression of *Stard1* and *Cyp17a1* over the basal levels found in cells infected with a control expression vector (Figure 10A). This stimulatory effect of C2/CREB was enhanced by the inhibition of SIK activity. As expected, C2/CREB stimulated testosterone production, an effect that was also enhanced by SIK inhibition (Figure 10B).

### SIK2 knockout mice produce more testosterone.

We have previously demonstrated that of the three SIK isoforms, only SIK2 is involved in the regulation of steroidogenesis (Armouti et al., 2020). Therefore, we tested if SIK2 deficiency *in vivo* leads to changes in circulating testosterone. As shown in Figure 11, SIK2 knockout mice have significantly higher levels of testosterone in circulation when compared to control mice.

## Discussion

We recently showed that inhibition of SIK activity enhances FSH induction of steroidogenic gene expression and estradiol production in human and rodent granulosa cells (Armouti et al., 2020). In addition, an analysis of SIK knockout mice demonstrated that SIKs are critical regulators of female fertility (Armouti et al., 2022,Armouti et al., 2020). This report aimed to determine the role that SIKs play in the regulation of other cell types in the ovary, more specifically the TCs. The results show that overall SIK activity has an inhibitory effect on the capacity of TCs to synthesize and secrete androgens, which is evident in the stimulatory effect that SIK inhibition has on these parameters.

Inhibition of SIKs alone is sufficient to stimulate the expression of genes that are key for normal TC function. However, full activation of the TC capacity to produce androgens or to express TC-specific factors such as *Insl3* and *Bmp4* is only reached in the presence of LH. Considering this finding and the fact that SIK inhibition mimics and enhances LH action, it is possible to propose that SIK activity may be inhibited by LH. However, the mechanisms involved remain to be explored. In contrast to the stimulatory effect of SIK inhibition on androgen synthesis, inhibition of SIK activity or treatment with LH strongly suppressed the expression of BMP4. Of interest, BMP4 was shown to suppress androgen synthesis in TCs (Liu, Du, Ding et al., 2017). Therefore, we cannot rule out that the stimulatory effect of SIK inhibition or LH treatment on androgen production could be mediated at least in part by a decrease in BMP4.

Recently, it was shown that HG-9–91-01 can also inhibit receptor-interacting protein kinase 3 (RIPK3), a central regulator of necroptosis (Huang, Chen, Huang et al., 2022). However, this report mostly used HG-9–91-01 at 5 μM to inhibit RIPK3, which is a higher concentration than the one we used to inhibit SIK activity in TCs. Further, significant effects were seen in our study using only 0.1 μM of HG. Moreover, we did not observe cell death or apoptosis after the treatment of TCs with HG. In addition, RIPK3 has been

found expressed in the granulosa and luteal cells, but not in the TCs (Li, Chen, Guo et al., 2021). Therefore, we are confident that the effects observed in TCs are mostly likely not due to the inhibition of RIPK3. However, we cannot rule out that higher concentrations of HG may have off-target effects in TCs. For instance, we observed that treatment with 3 μM HG was less effective than 1 μM in enhancing the effects of LH, forskolin, and dbcAMP. Taken together, the evidence points to a specific effect of SIKs on the regulation of androgen production in TCs.

We have elucidated in part the mechanism by which SIK activity inhibition increases androgen synthesis and secretion by TCs. SIK inhibition enhances *Cyp17a1*, *Stard1*, and *Cyp11a1* expression induced by forskolin and dbcAMP. SIK inhibition also maximized the inhibitory effects of forskolin and dbcAMP on *Bmp4* expression. Therefore, the robust enhancement of forskolin and dbcAMP effects by SIK inhibition indicates that SIK activity controls targets downstream of cAMP. This conclusion is supported experimentally in cells overexpressing a constitutively active form of PKA (caPKA), the main target of LH and cAMP. Our findings show for the first time that overexpression of caPKA in TCs is enough to stimulate androgen synthesis and testosterone secretion. Further, our findings show that SIK inhibition also augments the stimulatory effects of caPKA on *Cyp17a1*, *Stard1*, and *Cyp11a1* expression, suggesting that SIKs act downstream of PKA in TCs.

One of the main roles of PKA is the phosphorylation of CREB. To study the role of CREB on androgen synthesis and its interaction with SIK independently of PKA activation, we overexpressed C2/CREB, a CREB fusion protein that stimulates CRE-responsive genes in the absence of PKA. As with PKA, we also show for the first time that overexpression of C2/CREB is enough to stimulate the expression of steroidogenic genes and the secretion of testosterone in TCs. Of great interest, we also found that SIK inhibition enhances the stimulatory effect of C2/CREB in TCs. This evidence demonstrates that SIK activity does not directly regulate CREB activity but contributes to the activation of factors that might enhance the activity of CREB. Consistent with this observation, SIK1 inhibition of adrenal steroidogenesis is mediated by the inactivation of a CREB cofactor known as CREB-regulated transcription coactivator 2 (CRTC2) (Hu, Hu, Shen et al., 2015). Previous reports have demonstrated that SIKs phosphorylate and inhibit CRTCs resulting in their nuclear exclusion (Katoh, Takemori, Lin et al., 2006,Lee, Tong, Takemori et al., 2015). Because the binding of CRTCs to CREB also leads to increased CREB occupancy over cognate binding sites in the chromatin (Wang, Inoue, Ravnskjaer et al., 2010), the nuclear exclusion of CRTCs by SIK leads to a decrease in the expression of CREB-targeted genes. The translocation of CRTCs to the nucleus of TCs in the absence of SIK activity may also explain the stimulatory effect of SIK inhibition on *Cyp17a1*, *Stard1*, and *Cyp11a1* in the absence of LH. We propose that SIK inhibition is enough to increase CREB transcriptional activity possibly by increasing CRTCs binding to CREB. However, the mechanisms involved and the role of CRTCs in TCs remain to be investigated.

SIK activity depends on the phosphorylation of Thr residues in the N-terminal kinase domain by liver kinase 1 (LKB1) (Hashimoto, Satoh, Okamoto et al., 2008). Of interest, LKB1 polymorphisms have been associated with inadequate ovulation in women affected by polycystic ovarian syndrome (PCOS) (Legro, Barnhart, Schlaff et al., 2008), a disease

associated with increased androgen biosynthesis in TCs due to the augmented expression of steroidogenic enzymes (Franks, White, Gilling-Smith et al., 1996,Nelson, Legro, Strauss et al., 1999,Wood, Nelson, Ho et al., 2003). Overexpression of LKB1 was also shown recently to suppress androgen synthesis in a mouse model of hyperandrogenism (Xu, Gao, Huang et al., 2019). Thus, a deficit in LKB1 signaling in TCs could be a contributory factor in the development of hyperandrogenisms in PCOS. Conversely, increased LKB1 signaling could lead to androgen insufficiency. Both situations compromise normal follicle development, leading to subfertility or infertility in animals and humans. However, LKB1 expression and function in the ovary require further exploration.

We also provide *in vivo* evidence that SIK2 may be the main isoform involved in the upregulation of testosterone production. Thus, we show that animals lacking SIK2 have higher levels of testosterone in circulation than the controls. Although we have not determined testosterone levels in SIK1 and SIK3 knockout mice, we predict that testosterone may be significantly decreased in SIK3 knockout mice, since SIK3 knockout mice are infertile (Armouti et al., 2020). Whereas since SIK1 knockout mice have no fertility problems, we do not expect changes in testosterone in these animals.

SIKs are drug targets (Sundberg, Liang, Wu et al., 2016), therefore, targeting SIK activity, particularly SIK2, could be used to improve or control fertility. For instance, androgens play vital roles in reproduction as essential precursors for estrogen synthesis or via acting directly through the androgen receptor expressed throughout the ovary (Walters et al., 2012). Androgens enhance follicle development in several species, including humans (Bercaire et al., 2018,Gleicher et al., 2013,Katsika et al., 2022,Noventa et al., 2019,Wiser et al., 2010). Therapeutically, androgen priming has been used to improve the ovarian response to gonadotropin stimulations in patients with diminished ovarian reserve or poor responders (Bercaire et al., 2018,Noventa et al., 2019). However, support for androgen priming in assisted reproduction is not robust and optimal protocols have not been designed. A reason for this is that androgen concentrations in human follicular fluid are much higher (>100 fold) than in the circulation (Braunstein, Reitz, Buch et al., 2011), suggesting that exogenous administration of androgens may not increase the intrafollicular concentration of androgens to levels that could enhance follicle growth. Since SIK inhibition stimulates androgen production in TCs, we propose that SIK inhibitors could be used to achieve a temporary (days) enhancement of local androgen exposure or “intra-ovarian androgen priming” before ovarian stimulation with gonadotropins. This approach also has the potential advantage of supplying androgens at their place of physiological production and action, avoiding systemic exposure, and minimizing side effects. Thus, understanding SIK-regulated mechanisms involved in androgen synthesis could contribute to the identification of therapeutic targets to restore intraovarian levels of this steroid in patients with diminished ovarian reserve or poor responders.

This report also provides evidence that SIKs regulate androgen synthesis in human cells. We observed that human H295R cells express SIKs. H295R cells are an established model for studying androgen synthesis. Like TCs, H295R cells express high CYP17A1 activity and produce androgens (Samandari et al., 2007). Recently, H295R cells were used to study the molecular mechanisms involved in the regulation of CYP17A1 and CYP11A1

and the consequent excessive production of androgens in women with PCOS (McAllister, Han, Modi et al., 2019). Some of these reports refer to H295R as a “theca-like cell line” (Abdeljabar El Andaloussi, 2019). We demonstrated that H295R cells respond to forskolin and dbcAMP by increasing *Cyp17a1* expression. Interestingly, SIK inhibition enhances the effects of both stimuli. Moreover, SIK inhibition alone was sufficient to stimulate gene expression in H295R cells, which agrees with our observations in primary mouse and rat TCs. Thus, H295R cells could be used to further elucidate the mechanisms by which SIKs regulate the synthesis of androgens in humans.

In summary, these findings show, for the first time, the involvement of SIKs in the regulation of TC function in rodents and a human cell line. We described the expression of all SIK isoforms in TCs and show that SIK2 and SIK3 are the most relevant isoforms. In addition, it was demonstrated that SIK activity functions as a negative regulator not only of basal steroid synthesis but also of the stimulatory effect of LH. We also demonstrate that SIK negatively regulates androgen synthesis in a human cell line known to produce androgens. Moreover, our findings outline the intracellular signaling pathway downstream of the LH receptor showing that SIK inhibition enhances the actions of all the components of the LH signaling transduction pathway, including adenylyl cyclase, cAMP, PKA, and CREB. *In vivo*, findings demonstrated that deletion of SIK2 is enough to significantly increase circulating testosterone levels in mice. Further experiments are needed to determine the involvement of SIKs in regulating CREB activity and whether SIK activity targets additional PKA substrates in TCs. Finally, our findings open up a new and exciting research area, which must include the determination of the specific effects that each SIK isoform has on the different ovarian cell types.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

The authors thank the National Institute of Health (NIH) for the financial support grant R01HD097202 to CS.

**Funding:**

NIH grant R01HD097202 to CS.

## Bibliography

Abdeljabar El Andaloussi NI, Garcia Natalia, MacAllister Jan, Al-Hendy Ayman, 2019. Human Mesenchymal Stem Cells Secretome Inhibits Androgen Production by Human Theca-Like Cells: Implications for Stem Cell Therapy of Polycystic Ovarian Syndrome.. Reproductive Sciences Supplement 1.

Armouti M, Rodriguez-Esquivel M and Stocco C, 2022. Mechanism of negative modulation of FSH signaling by salt-inducible kinases in rat granulosa cells, Front Endocrinol (Lausanne). 13, 1026358. [PubMed: 36246922]

Armouti M, Winston N, Hatano O, Hobeika E, Hirshfeld-Cytron J, Liebermann J, Takemori H and Stocco C, 2020. Salt Inducible Kinases Are Critical Determinants of Female Fertility, Endocrinology. 161, 1–13.

Astapova O, Minor BMN and Hammes SR, 2019. Physiological and Pathological Androgen Actions in the Ovary, Endocrinology. 160, 1166–1174. [PubMed: 30912811]

Bercaire L, Nogueira SM, Lima PC, Alves VR, Donadio N, Dzik A and Cavagna M, 2018. ANDRO-IVF: a novel protocol for poor responders to IVF controlled ovarian stimulation, JBRA Assist Reprod. 22, 52–55. [PubMed: 29303236]

Braunstein GD, Reitz RE, Buch A, Schnell D and Caulfield MP, 2011. Testosterone reference ranges in normally cycling healthy premenopausal women, J Sex Med. 8, 2924–34. [PubMed: 21771278]

Chang HM, Cheng JC and Leung PC, 2013. Theca-derived BMP4 and BMP7 down-regulate connexin43 expression and decrease gap junction intercellular communication activity in immortalized human granulosa cells, J Clin Endocrinol Metab. 98, E437–45. [PubMed: 23386650]

Darling NJ and Cohen P, 2021. Nuts and bolts of the salt-inducible kinases (SIKs), Biochem J. 478, 1377–1397. [PubMed: 33861845]

de Vries WN, Binns LT, Fancher KS, Dean J, Moore R, Kemler R and Knowles BB, 2000. Expression of Cre recombinase in mouse oocytes: a means to study maternal effect genes, Genesis. 26, 110–2. [PubMed: 10686600]

Franks S, White D, Gilling-Smith C, Carey A, Waterworth D and Williamson R, 1996. Hypersecretion of androgens by polycystic ovaries: the role of genetic factors in the regulation of cytochrome P450c17 alpha, Baillieres Clin Endocrinol Metab. 10, 193–203.

Gleicher N, Kim A, Weghofer A, Shohat-Tal A, Lazzaroni E, Lee HJ and Barad DH, 2013. Starting and resulting testosterone levels after androgen supplementation determine at all ages in vitro fertilization (IVF) pregnancy rates in women with diminished ovarian reserve (DOR), J Assist Reprod Genet. 30, 49–62. [PubMed: 23212832]

Hashimoto YK, Satoh T, Okamoto M and Takemori H, 2008. Importance of autophosphorylation at Ser186 in the A-loop of salt inducible kinase 1 for its sustained kinase activity, J Cell Biochem. 104, 1724–39. [PubMed: 18348280]

Hu YC, Wang PH, Yeh S, Wang RS, Xie C, Xu Q, Zhou X, Chao HT, Tsai MY and Chang C, 2004. Subfertility and defective folliculogenesis in female mice lacking androgen receptor, Proc Natl Acad Sci U S A. 101, 11209–14. [PubMed: 15277682]

Hu Z, Hu J, Shen WJ, Kraemer FB and Azhar S, 2015. A Novel Role of Salt-Inducible Kinase 1 (SIK1) in the Post-Translational Regulation of Scavenger Receptor Class B Type 1 Activity, Biochemistry. 54, 6917–30. [PubMed: 26567857]

Huang D, Chen P, Huang G, Sun H, Luo X, He C, Chen F, Wang Y, Zeng C, Su L, Zeng X, Lu J, Li S, Huang D, Gao H and Cao M, 2022. Salt-inducible kinases inhibitor HG-9–91-01 targets RIPK3 kinase activity to alleviate necroptosis-mediated inflammatory injury, Cell Death Dis. 13, 188. [PubMed: 35217652]

Katoh Y, Takemori H, Lin XZ, Tamura M, Muraoka M, Satoh T, Tsuchiya Y, Min L, Doi J, Miyauchi A, Witters LA, Nakamura H and Okamoto M, 2006. Silencing the constitutive active transcription factor CREB by the LKB1-SIK signaling cascade, FEBS J. 273, 2730–48. [PubMed: 16817901]

Katsika ET, Bosdou JK, Goulis DG, Grimbizis GF and Kolibianakis EM, 2022. Higher live birth rate following transdermal testosterone pretreatment in poor responders: a systematic review and meta-analysis, Reprod Biomed Online.

Lee J, Tong T, Takemori H and Jefcoate C, 2015. Stimulation of StAR expression by cAMP is controlled by inhibition of highly inducible SIK1 via CRTC2, a co-activator of CREB, Mol Cell Endocrinol. 408, 80–9. [PubMed: 25662274]

Legro RS, Barnhart HX, Schlaff WD, Carr BR, Diamond MP, Carson SA, Steinkampf MP, Coutifaris C, McGovern PG, Cataldo NA, Gosman GG, Nestler JE, Giudice LC, Ewens KG, Spielman RS, Leppert PC, Myers ER and Reproductive Medicine N, 2008. Ovulatory response to treatment of polycystic ovary syndrome is associated with a polymorphism in the STK11 gene, J Clin Endocrinol Metab. 93, 792–800. [PubMed: 18000088]

Li D, Chen J, Guo J, Li L, Cai G, Chen S, Huang J, Yang H, Zhuang Y, Wang F and Wang X, 2021. A phosphorylation of RIPK3 kinase initiates an intracellular apoptotic pathway that promotes prostaglandin(2alpha)-induced corpus luteum regression, Elife. 10.

Liu Y, Du SY, Ding M, Dou X, Zhang FF, Wu ZY, Qian SW, Zhang W, Tang QQ and Xu CJ, 2017. The BMP4-Smad signaling pathway regulates hyperandrogenism development in a female mouse model, J Biol Chem. 292, 11740–11750. [PubMed: 28572510]

Magoffin DA and Erickson GF, 1982. Primary culture of differentiating ovarian androgen-producing cells in defined medium, J Biol Chem. 257, 4507–13. [PubMed: 7068648]

McAllister JM, Han AX, Modi BP, Teves ME, Mavodza GR, Anderson ZL, Shen T, Christenson LK, Archer KJ and Strauss JF, 2019. miRNA Profiling Reveals miRNA-130b-3p Mediates DENND1A Variant 2 Expression and Androgen Biosynthesis, Endocrinology. 160, 1964–1981. [PubMed: 31184707]

Miller WL and Tee MK, 2015. The post-translational regulation of 17,20 lyase activity, Mol Cell Endocrinol. 408, 99–106. [PubMed: 25224484]

Murray AA, Gosden RG, Allison V and Spears N, 1998. Effect of androgens on the development of mouse follicles growing in vitro, J Reprod Fertil. 113, 27–33. [PubMed: 9713373]

Nef S and Parada LF, 1999. Cryptorchidism in mice mutant for Insl3, Nat Genet. 22, 295–9. [PubMed: 10391220]

Nelson VL, Legro RS, Strauss JF 3rd and McAllister JM, 1999. Augmented androgen production is a stable steroidogenic phenotype of propagated theca cells from polycystic ovaries, Mol Endocrinol. 13, 946–57. [PubMed: 10379893]

Noventa M, Vitagliano A, Andrisani A, Blaganje M, Vigano P, Papaelo E, Scioscia M, Cavallin F, Ambrosini G and Cozzolino M, 2019. Testosterone therapy for women with poor ovarian response undergoing IVF: a meta-analysis of randomized controlled trials, J Assist Reprod Genet. 36, 673–683. [PubMed: 30610664]

Orellana SA and McKnight GS, 1992. Mutations in the catalytic subunit of cAMP-dependent protein kinase result in unregulated biological activity, Proc Natl Acad Sci U S A. 89, 4726–30. [PubMed: 1584809]

Patel K, Foretz M, Marion A, Campbell DG, Gourlay R, Boudaba N, Tournier E, Titchenell P, Peggie M, Deak M, Wan M, Kaestner KH, Goransson O, Viollet B, Gray NS, Birnbaum MJ, Sutherland C and Sakamoto K, 2014. The LKB1-salt-inducible kinase pathway functions as a key gluconeogenic suppressor in the liver, Nat Commun. 5, 4535. [PubMed: 25088745]

Sakamoto K, Bultot L and Goransson O, 2018. The Salt-Inducible Kinases: Emerging Metabolic Regulators, Trends Endocrinol Metab. 29, 827–840. [PubMed: 30385008]

Samandari E, Kempna P, Nuoffer JM, Hofer G, Mullis PE and Fluck CE, 2007. Human adrenal corticocarcinoma NCI-H295R cells produce more androgens than NCI-H295A cells and differ in 3beta-hydroxysteroid dehydrogenase type 2 and 17,20 lyase activities, J Endocrinol. 195, 459–72. [PubMed: 18000308]

Sen A and Hammes SR, 2010. Granulosa cell-specific androgen receptors are critical regulators of ovarian development and function, Mol Endocrinol. 24, 1393–403. [PubMed: 20501640]

Sundberg TB, Liang Y, Wu H, Choi HG, Kim ND, Sim T, Johannessen L, Petrone A, Khor B, Graham DB, Latorre IJ, Phillips AJ, Schreiber SL, Perez J, Shamji AF, Gray NS and Xavier RJ, 2016. Development of Chemical Probes for Investigation of Salt-Inducible Kinase Function in Vivo, ACS Chem Biol. 11, 2105–11. [PubMed: 27224444]

Thiel G, Al Sarraj J, Vinson C, Stefano L and Bach K, 2005. Role of basic region leucine zipper transcription factors cyclic AMP response element binding protein (CREB), CREB2, activating transcription factor 2 and CAAT/enhancer binding protein alpha in cyclic AMP response element-mediated transcription, J Neurochem. 92, 321–36. [PubMed: 15663480]

Vendola K, Zhou J, Wang J, Famuyiwa OA, Bievre M and Bondy CA, 1999. Androgens promote oocyte insulin-like growth factor I expression and initiation of follicle development in the primate ovary, Biol Reprod. 61, 353–7. [PubMed: 10411511]

Vendola KA, Zhou J, Adesanya OO, Weil SJ and Bondy CA, 1998. Androgens stimulate early stages of follicular growth in the primate ovary, J Clin Invest. 101, 2622–9. [PubMed: 9637695]

Walters KA, Middleton LJ, Joseph SR, Hazra R, Jimenez M, Simanainen U, Allan CM and Handelsman DJ, 2012. Targeted loss of androgen receptor signaling in murine granulosa cells of preantral and antral follicles causes female subfertility, Biol Reprod. 87, 151. [PubMed: 23115271]

Wang H, Andoh K, Hagiwara H, Xiaowei L, Kikuchi N, Abe Y, Yamada K, Fatima R and Mizunuma H, 2001. Effect of adrenal and ovarian androgens on type 4 follicles unresponsive to FSH in immature mice, Endocrinology. 142, 4930–6. [PubMed: 11606461]

Wang Y, Inoue H, Ravnskjaer K, Viste K, Miller N, Liu Y, Hedrick S, Vera L and Montminy M, 2010. Targeted disruption of the CREB coactivator Crtc2 increases insulin sensitivity, Proc Natl Acad Sci U S A. 107, 3087–92. [PubMed: 20133702]

Wang Z, Takemori H, Halder SK, Nonaka Y and Okamoto M, 1999. Cloning of a novel kinase (SIK) of the SNF1/AMPK family from high salt diet-treated rat adrenal, FEBS Lett. 453, 135–9. [PubMed: 10403390]

Wein MN, Foretz M, Fisher DE, Xavier RJ and Kronenberg HM, 2018. Salt-Inducible Kinases: Physiology, Regulation by cAMP, and Therapeutic Potential, Trends Endocrinol Metab. 29, 723–735. [PubMed: 30150136]

Wiser A, Gonen O, Ghetler Y, Shavit T, Berkovitz A and Shulman A, 2010. Addition of dehydroepiandrosterone (DHEA) for poor-responder patients before and during IVF treatment improves the pregnancy rate: a randomized prospective study, Hum Reprod. 25, 2496–500. [PubMed: 20729538]

Wood JR, Nelson VL, Ho C, Jansen E, Wang CY, Urbanek M, McAllister JM, Mosselman S and Strauss JF 3rd, 2003. The molecular phenotype of polycystic ovary syndrome (PCOS) theca cells and new candidate PCOS genes defined by microarray analysis, J Biol Chem. 278, 26380–90. [PubMed: 12734205]

Xu Y, Gao Y, Huang Z, Zheng Y, Teng W, Zheng D and Zheng X, 2019. LKB1 suppresses androgen synthesis in a mouse model of hyperandrogenism via IGF-1 signaling, FEBS Open Bio. 9, 1817–1825.

Xue K, Liu JY, Murphy BD and Tsang BK, 2012. Orphan Nuclear Receptor NR4A1 Is a Negative Regulator of DHT-Induced Rat Preantral Follicular Growth, Mol Endocrinol. 26, 2004–15. [PubMed: 23028064]

Young JM and McNeilly AS, 2010. Theca: the forgotten cell of the ovarian follicle, Reproduction. 140, 489–504. [PubMed: 20628033]

Zhang P, Compagnone NA, Fiore C, Vigne JL, Culp P, Musci TJ and Mellon SH, 2001. Developmental gonadal expression of the transcription factor SET and its target gene, P450c17 (17alpha-hydroxylase/c17,20 lyase), DNA Cell Biol. 20, 613–24. [PubMed: 11749720]

## Highlights

- SIK2 and SIK3 are expressed in theca cells of mouse ovaries and isolated theca cells.
- SIK inhibition enhances steroidogenic gene expression and androgen production.
- SIKs control targets downstream cyclic AMP involved in androgen production.
- SIK2 knockout mice produce more testosterone than controls.

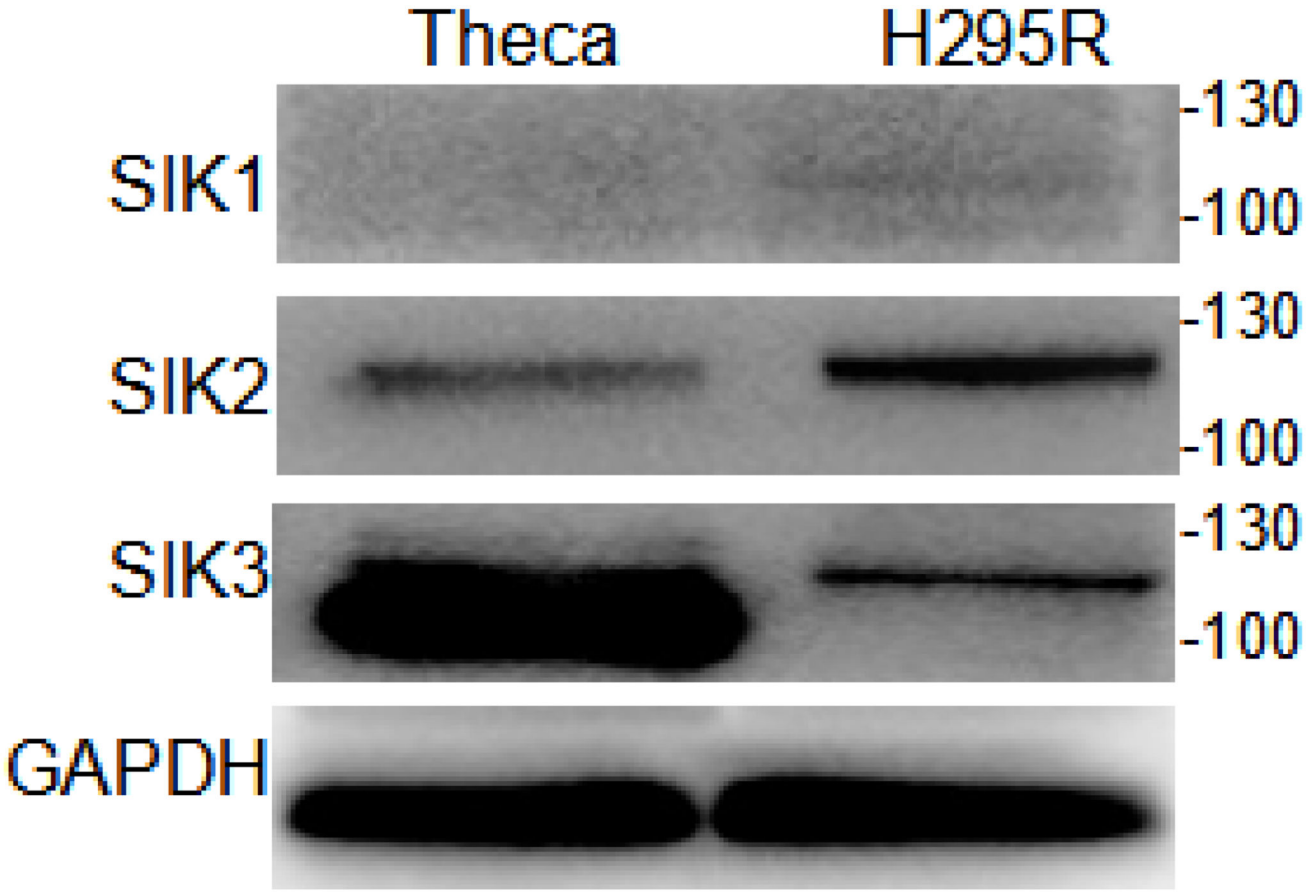


**Figure 1: SIKs expression in mouse TCs and human H295R cells.**

SIKs protein expression using Western blotting. A representative blot is shown n=4.

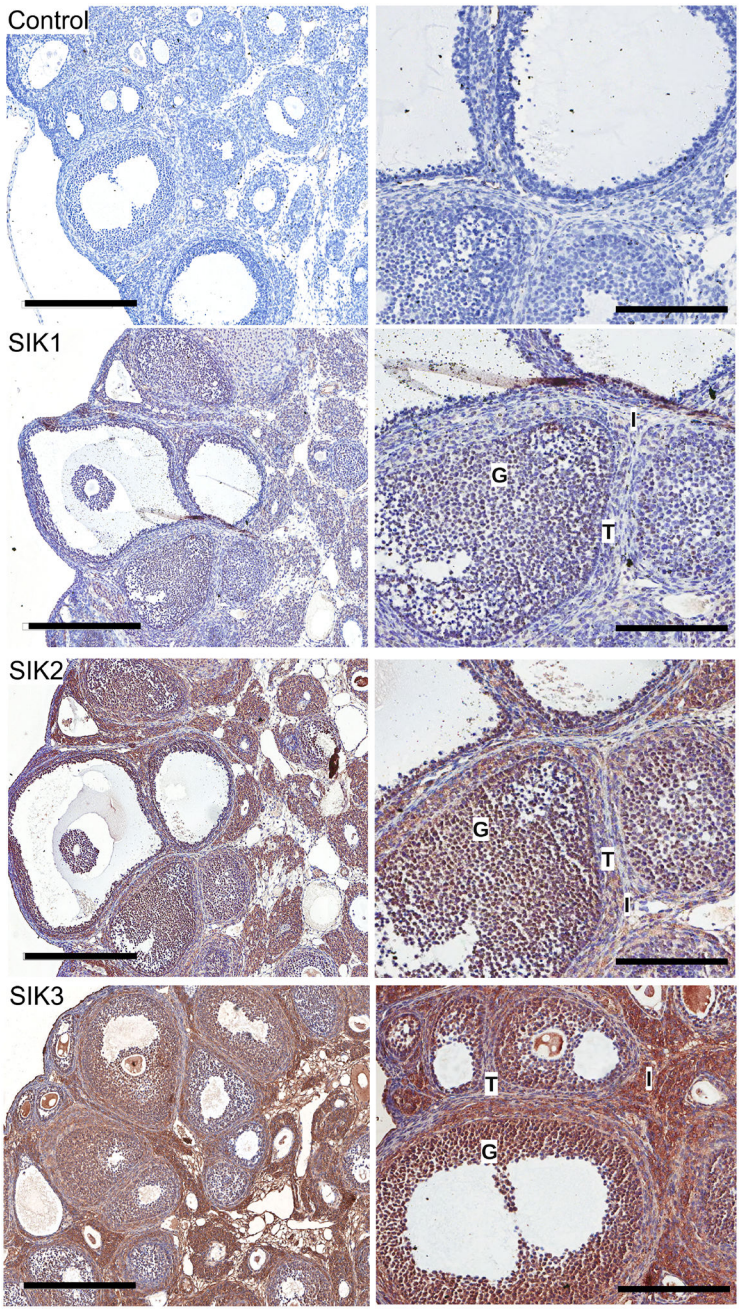


**Figure 2: SIK3 is highly expressed in theca and interstitial cells.**

IHC was performed on serial sections from the ovaries of adult female mice at proestrus using specific primary antibodies and a secondary HRP-labeled antibody (brown). Sections were counterstained with hematoxylin (blue). n=5, representative pictures are shown. I: interstitial; T: Theca; G: Granulosa. Left column bars = 300 μM on the right column bars = 150 μM.

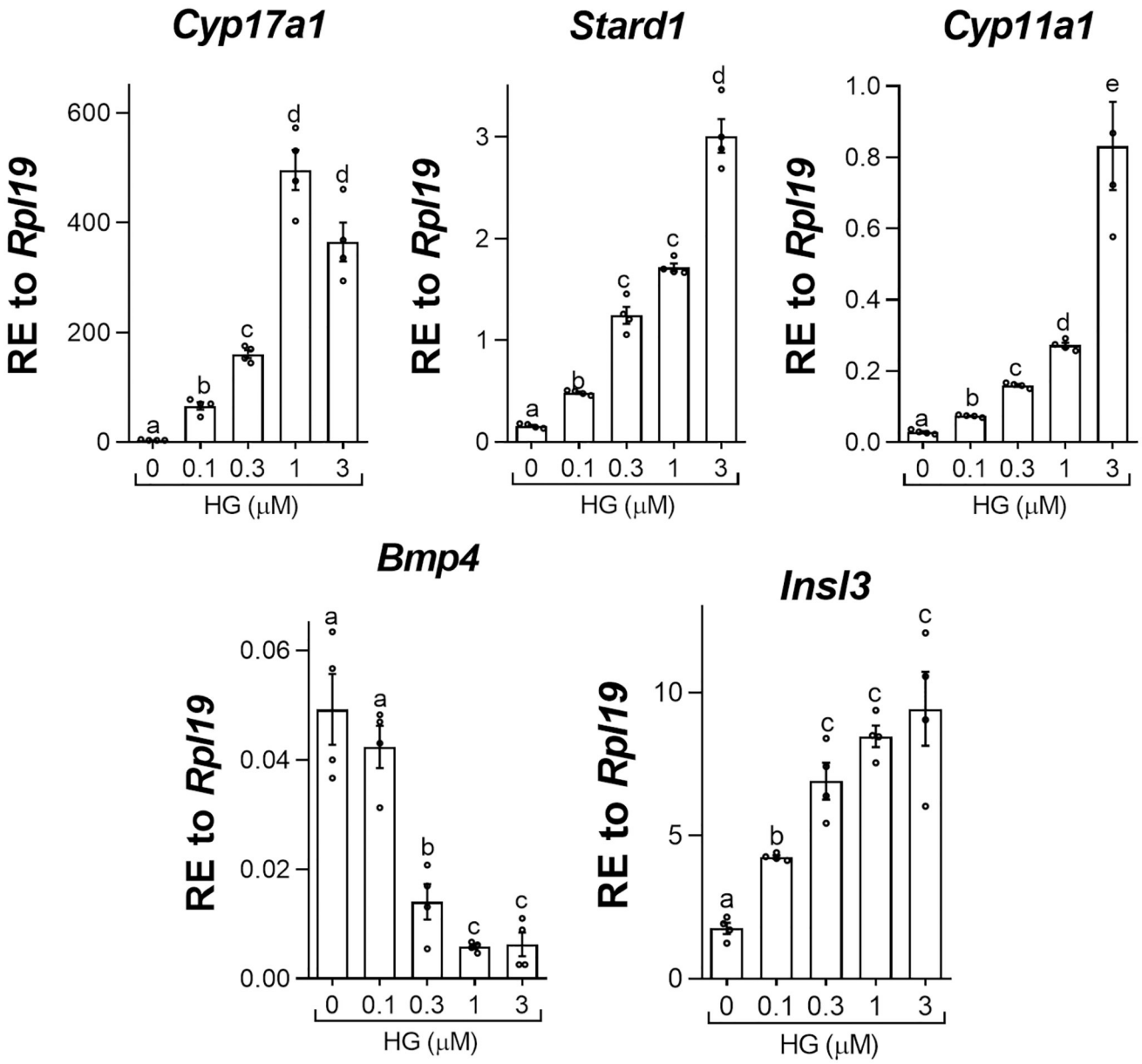


**Figure 3: SIK inhibition regulates theca cell genes in a concentration-dependent manner.**
TCs were treated with the SIK inhibitor HG for 24 h. The mRNA levels of the depicted genes were quantified by qPCR. Different letters differ significantly ($n = 3$).

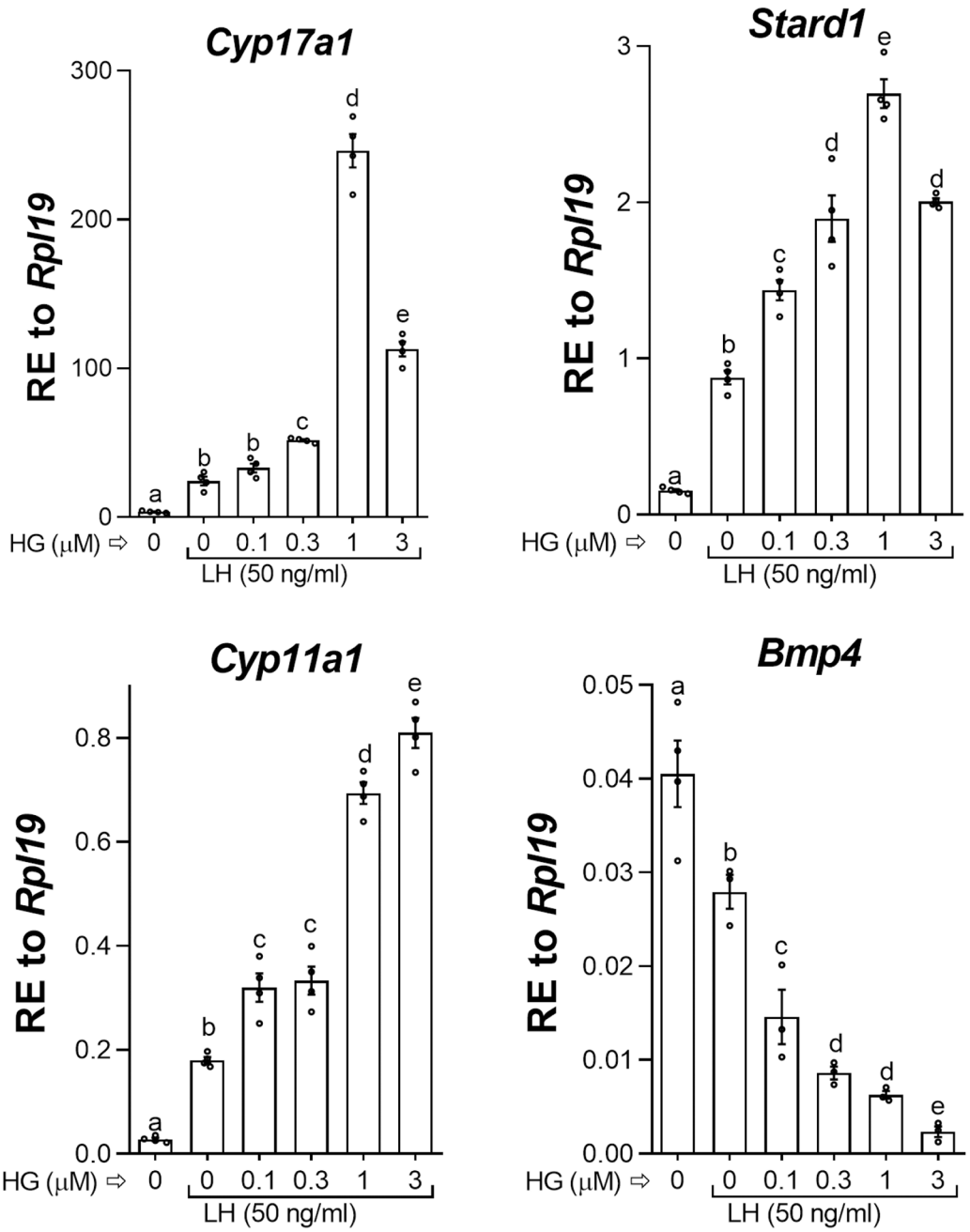


**Figure 4: SIK inhibition enhances LH stimulation of enzymes involved in androgen synthesis.**

Cells were treated with the SIK inhibitor HG (1 μM) for 1 h before the addition of LH (50 ng/mL). The mRNA levels of the depicted genes were quantified 24h later by qPCR. Different letters differ significantly ($n = 5$).

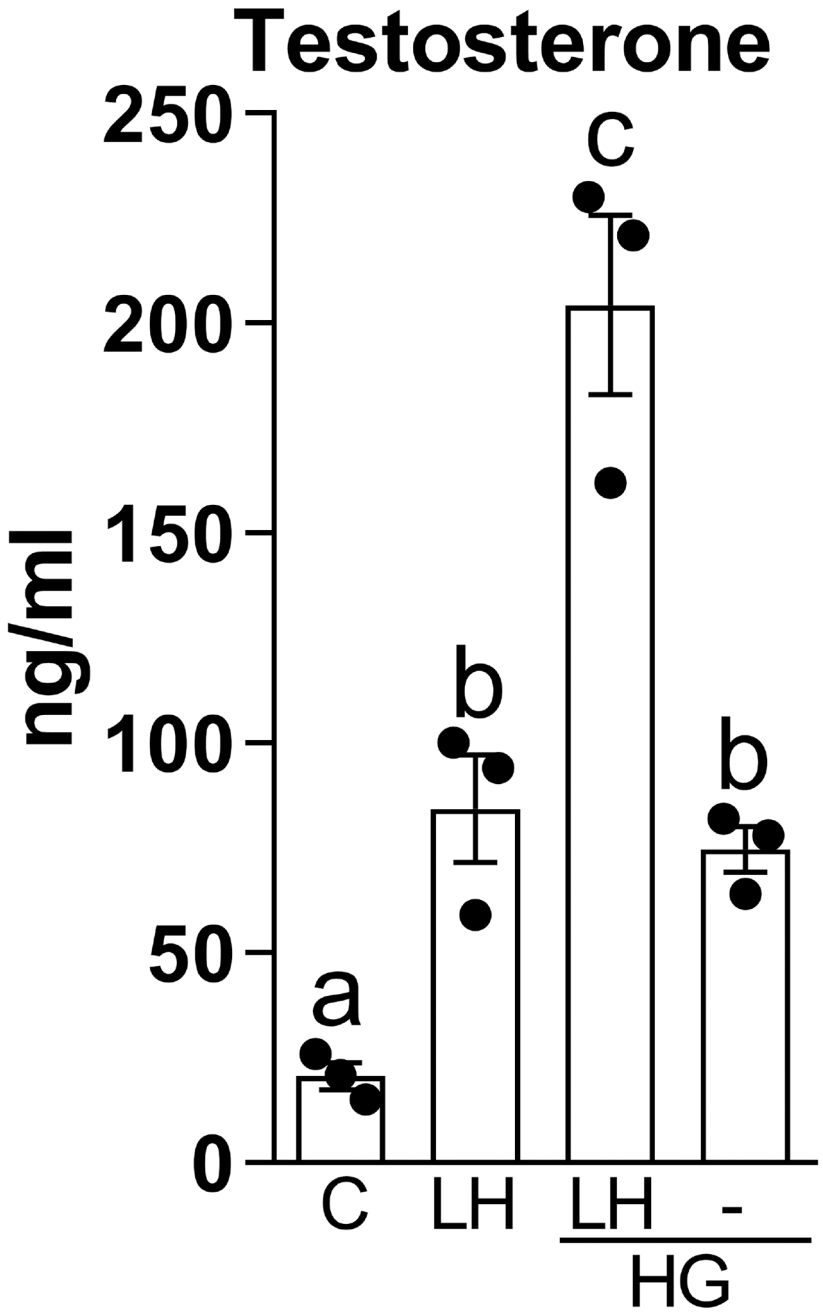


**Figure 5: SIK inhibition increases testosterone production in mouse TCs.**

Cells were treated with the SIK inhibitor HG (1 μM) for 1 h before the addition of LH (50 ng/mL). Testosterone levels in the media were quantified 24 h later by ELISA. Different letters differ significantly ($n = 3$).

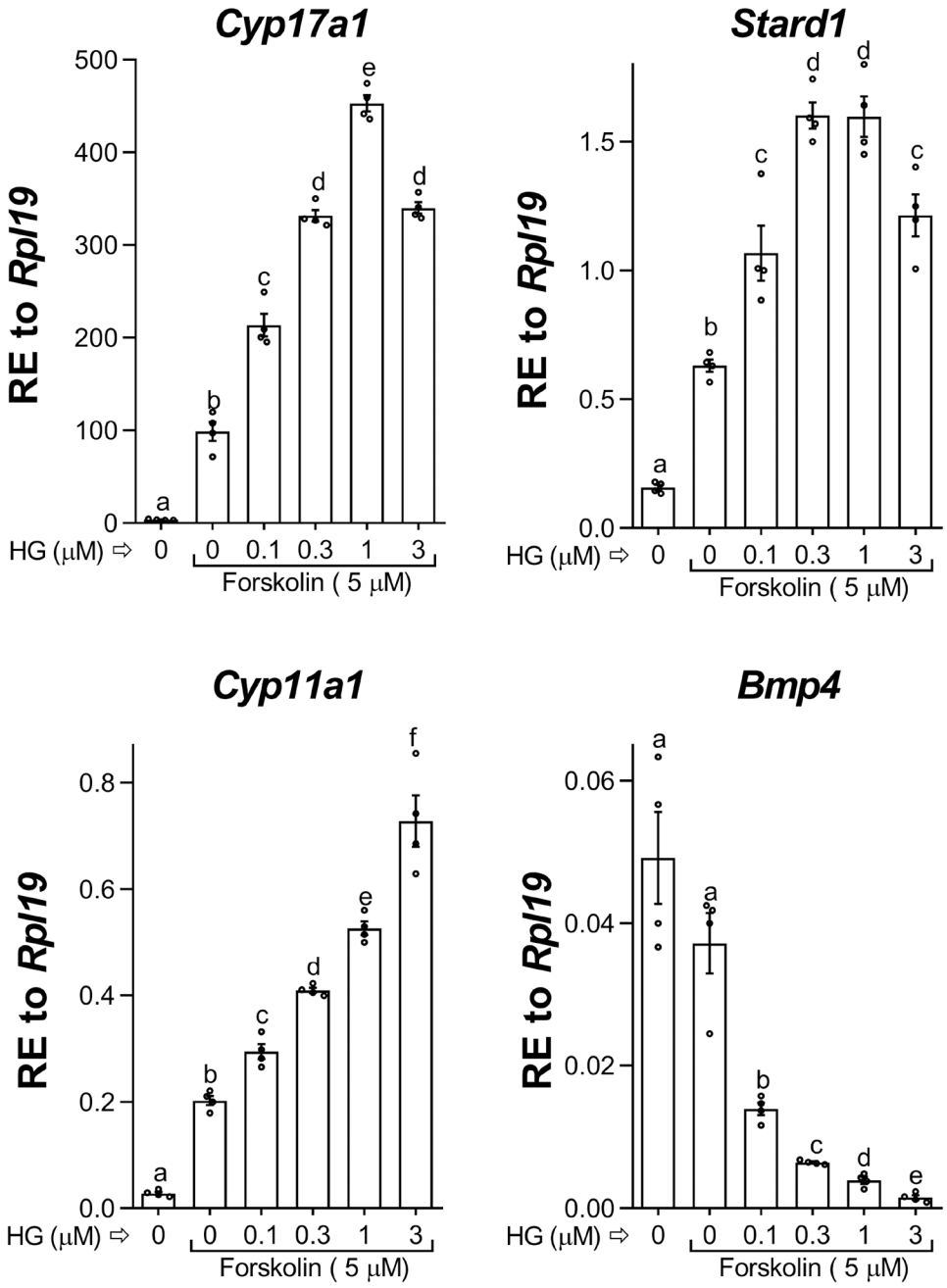


**Figure 6: SIK inhibition enhances the response of TCs to forskolin signaling.**

Mouse TCs were treated with forskolin (5 μM) in the presence or absence of increasing concentration of HG. Different letters differ significantly, $n = 3$.

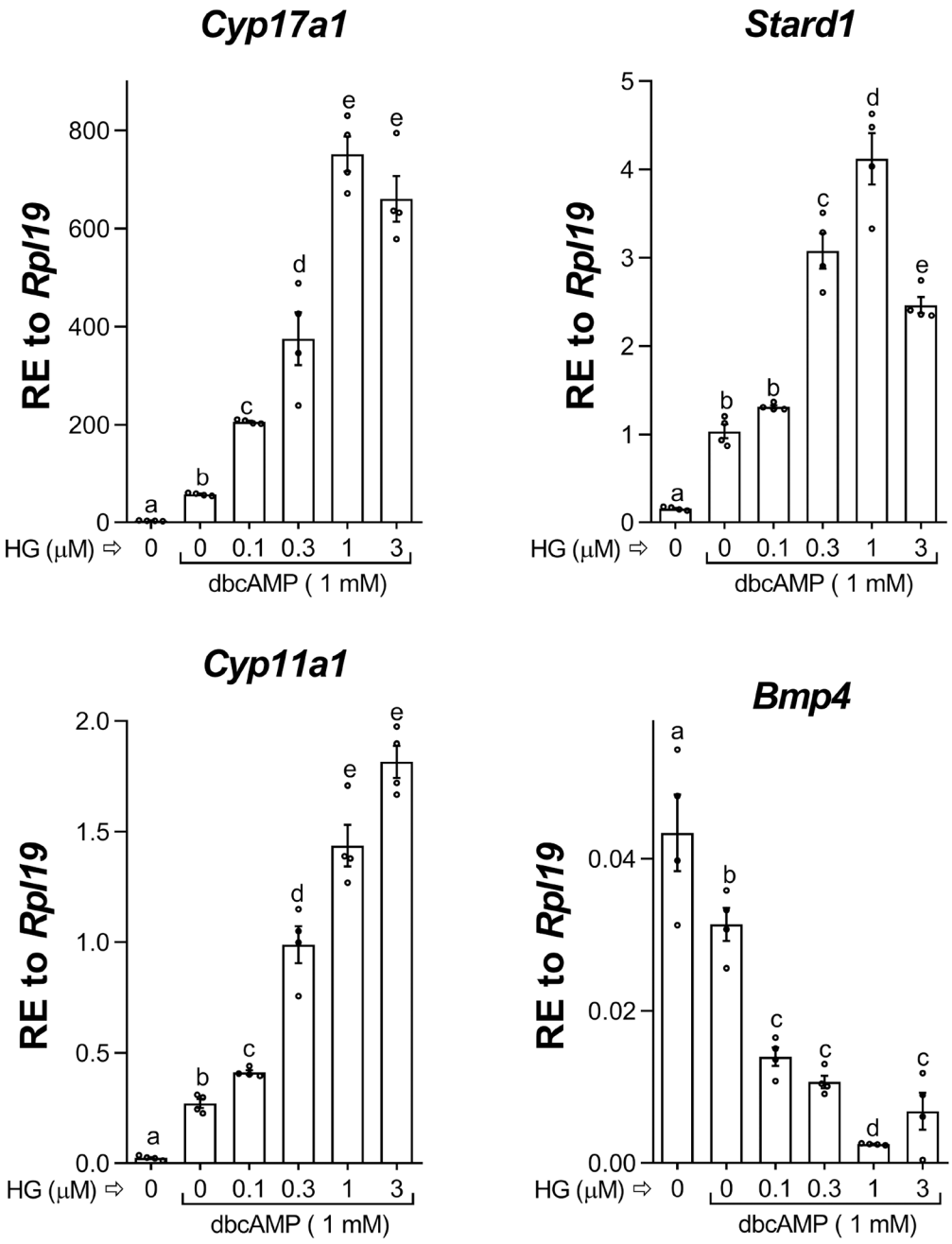


**Figure 7: SIK inhibition enhances the response of TCs to cAMP signaling.**

Mouse TCs were treated with dbcAMP (1 mM) in the presence or absence of increasing concentration of HG. Different letters differ significantly, $n = 3$

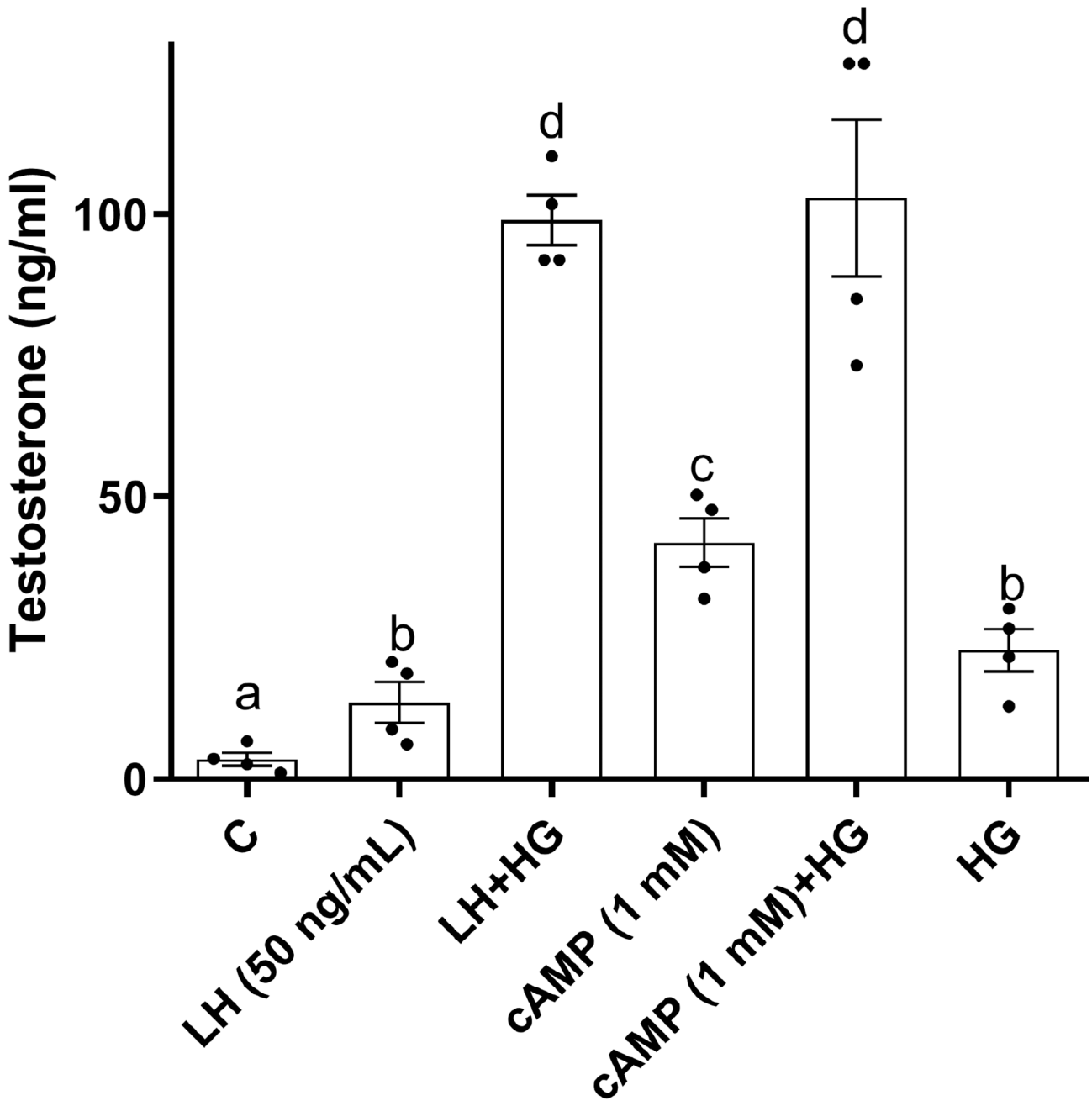


**Figure 8: SIK inhibition enhances the response of TCs to cAMP signaling.**

Mouse TCs were treated with an analog of cAMP (dbcAMP) or with LH (50 ng/ml) in the presence or absence of the SIK inhibitor HG (1 μM). Testosterone levels in the media were quantified 48 h later. Different letters differ significantly, $n = 4$.

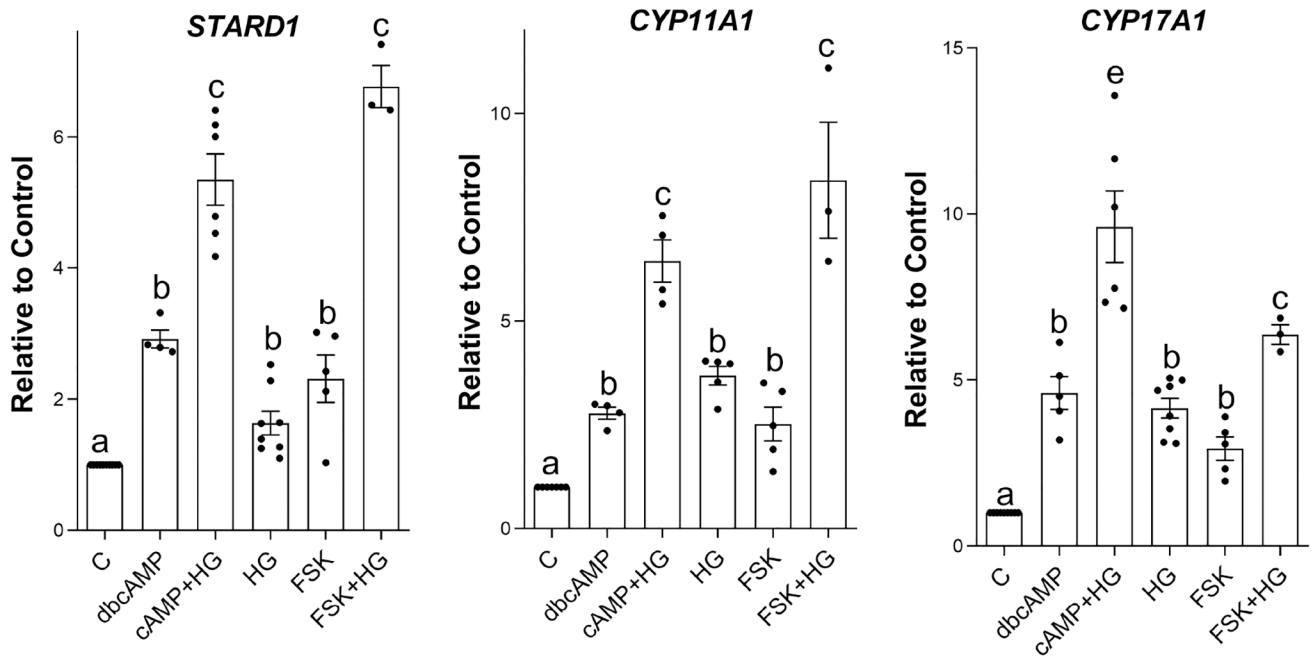


**Figure 9: SIK inhibition enhances the response of human H295R cells to cAMP signaling.**

H295R cells were treated with dbcAMP (1 mM) or forskolin (5 μM) in the presence or absence of HG (1 μM). Different letters differ significantly, n = 4–8

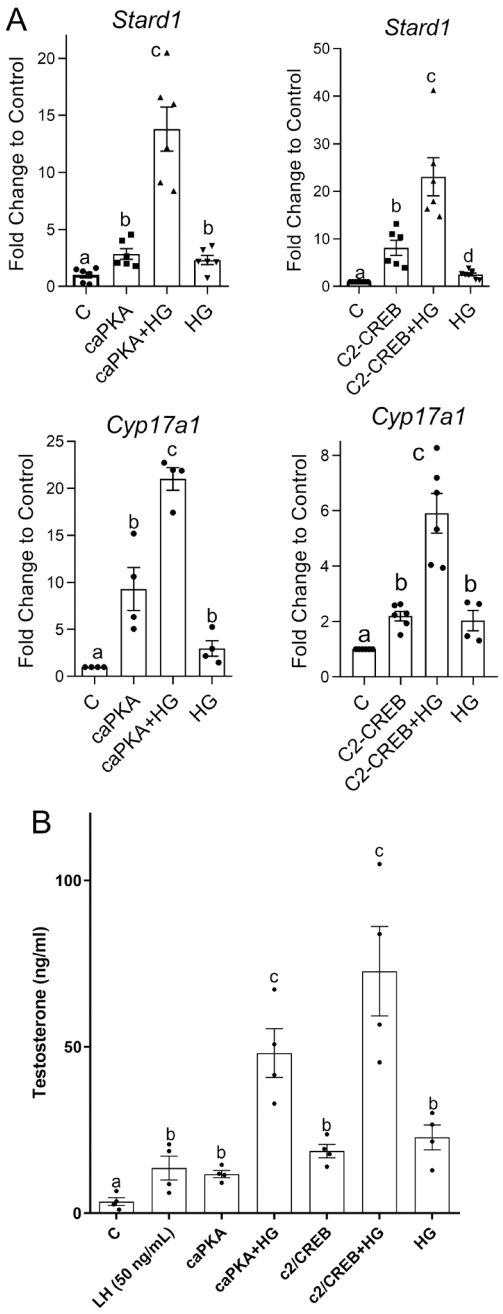


**Figure 10: SIK inhibition enhances the response of TCs to PKA and CREB.**

TCs were infected with lentivirus carrying the pGPcs empty plasmid (C), caPKA, or C2/CREB. 24 h after infection, cells were treated with the vehicle of SIK inhibitor HG (1 μM). 48 h later, gene expression (A) and testosterone (B) were quantified using qPCR or ELISA, respectively. Different letters differ significantly, n = 3–4.

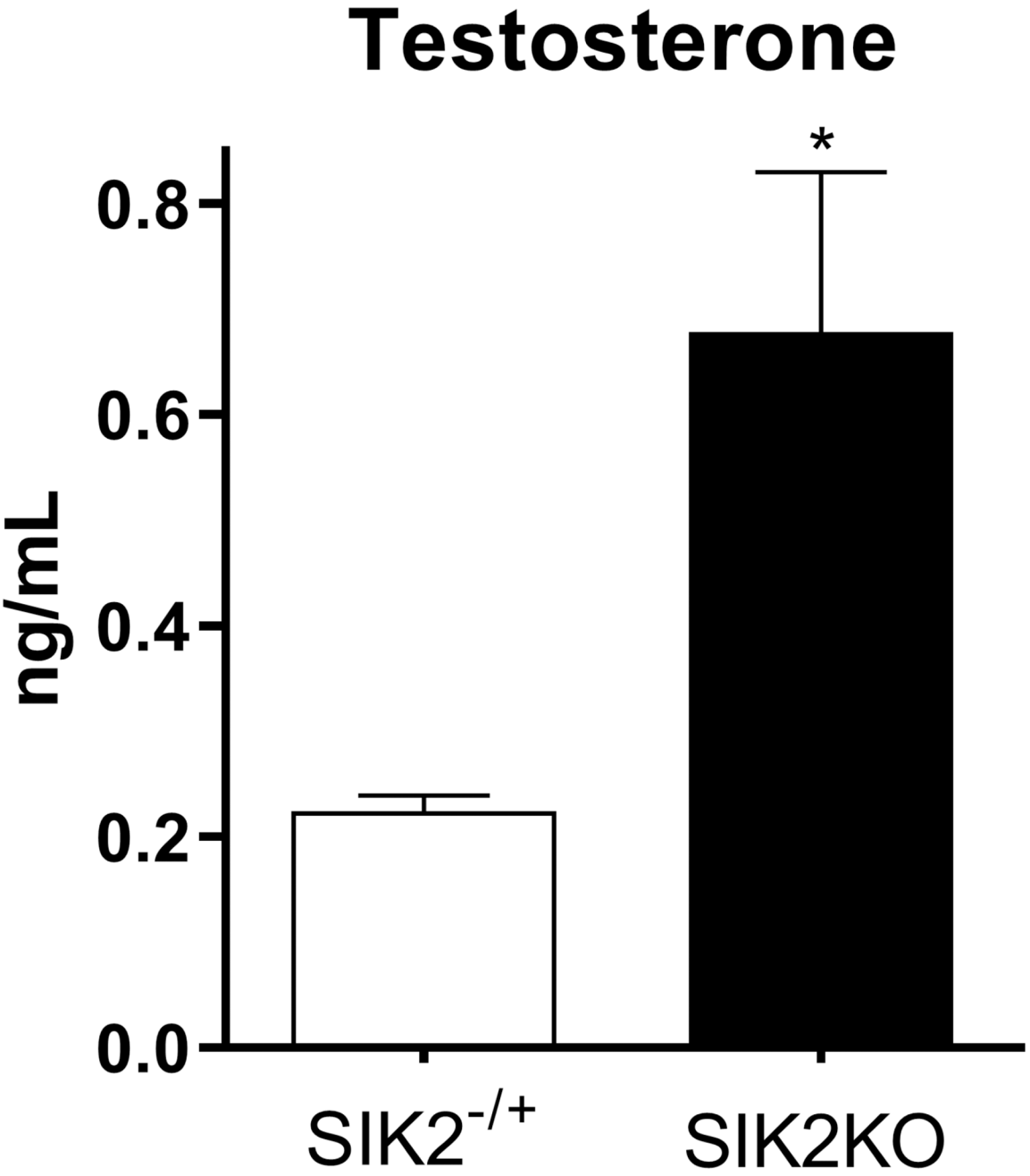


**Figure 11: Serum testosterone levels in SIK2 knockout mice.**

Testosterone levels were quantified ELISA, respectively. * $P < 0.05$ vs control. n = 5.