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

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24

27

Abstract

Ovulation is the pinnacle of folliculogenesis, a process that requires an interplay between the oocyte, the
30 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 in humans and
animals. Therefore, uncovering novel mechanisms regulating androgen synthesis in TCs is of great
33 significance. Previously, our group showed that salt-inducible kinases (SIKs) regulate granulosa cell
steroidogenesis. Here, we investigated whether SIKs play any role in the regulation of androgen
production in TCs. SIK2 and SIK3 were detected in the TCs of mouse ovaries and isolated TCs. Next, TCs
36 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
39 of steroidogenic genes and increased androgen production. Activation of adenylyl cyclase with forskolin or
emulation of increased intracellular cyclic AMP levels stimulated steroidogenic gene expression, an effect
that was also enhanced by the inhibition of SIK activity. The stimulatory effect of downstream targets of
42 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
45 of TC function and androgen production. This information could contribute to uncovering therapeutic
targets to treat hyperandrogenic diseases.

48 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, Walters, Middleton, Joseph et al., 2012, Sen and Hammes, 2010). TC-produced androgens enhance follicle development in several species by activating the androgen receptor in primates (Vendola, Zhou, Wang et al., 1999, Vendola, Zhou, Adesanya et al., 1998) and rodents (Wang, Andoh, Hagiwara et al., 2001, Xue, Liu, Murphy et al., 2012, Murray, Gosden, Allison et al., 1998). 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 (Gleicher, Kim, Weghofer et al., 2013, Wisner, Gonen, Ghetler et al., 2010, Noventa, Vitagliano, Andrisani et al., 2019, Bercaire, Nogueira, Lima et al., 2018, Katsika, Bosdou, Goulis et al., 2022). 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 17 α -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 (Wein, Foretz, Fisher et al., 2018, Sakamoto, Bultot and Goransson, 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 (Wein et al., 2018, Sakamoto et al., 2018, Darling and Cohen, 2021). 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 et al., 2020, Armouti, Rodriguez-Esquivel and Stocco, 2022). 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 (Wein et al., 2018, Sakamoto 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.

99 **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

105 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
108 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.

111 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^{ff} mice with ZP3Cre mice. ZP3Cre mice express Cre expression
under the control of zona pellucida 3 (Zp3) promoter in growing oocytes before the completion of the first
114 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
117 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
120 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).

123 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
126 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.
129 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
132 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, 135 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 138 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 141 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) 144 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 147 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 150 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 (1:500), SIK1 (1:1000), SIK2 (1:1000), and SIK3 (1:1000), all from Cell Signaling (Danvers, MA). 153 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 156 (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).

159 **Testosterone measurement** – Undiluted cell culture medium was used for testosterone level
determinations by ELISA (Sensitivity = 0.07 ng/ml, Range 0.2
ng/ml - 16 ng/ml; intra and inter assay % variable 5.1% and
162 6%, respectively; DGR International, Springfield, NJ).

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

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

177

Results

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

First, SIK isoforms in isolated mouse TCs were
examined using western blotting. For this purpose,
183 mouse TCs were isolated, and the purity of the cell
preparation was verified using immunofluorescence
with antibodies against CYP17A1, a TC-specific

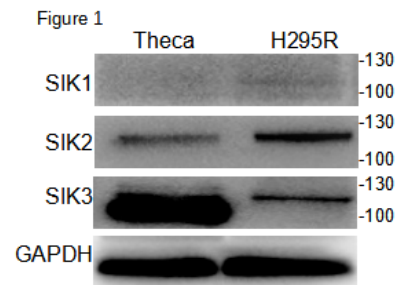


Figure 1: SIKs expression in mouse TCs and human H295R cells. SIKs protein expression using Western blotting. A representative blot is shown n=4.

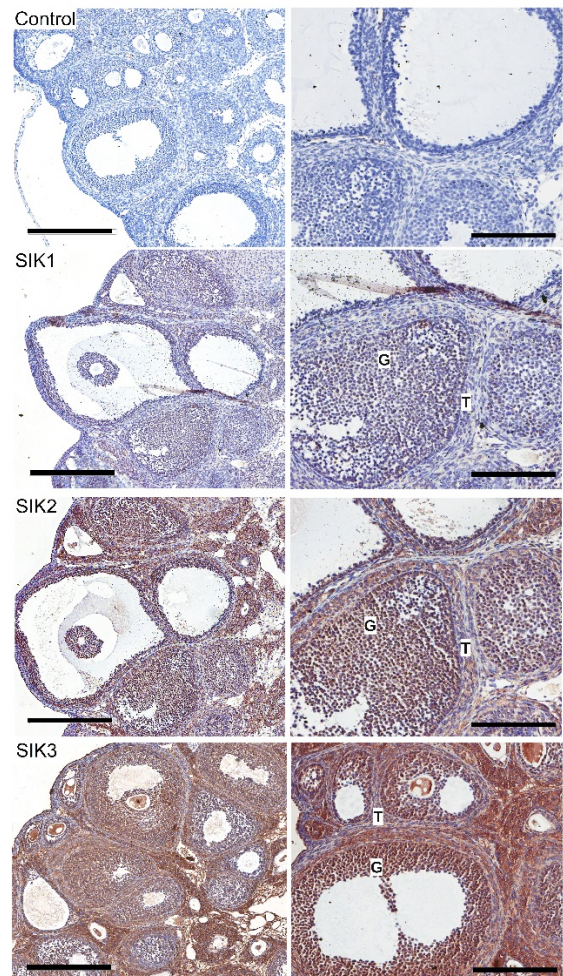


Figure 2: SIK3 is highly expressed in theca and interstitial cells. IHC was performed on serial sections from the ovaries of adult female mice at proestrus using specific primary antibodies and a secondary HRP-labeled antibody (brown). Sections were counterstained with hematoxylin (blue). n=5, representative pictures are shown. T: Theca; G: Granulosa.

186 marker, and counterstained with DAPI to detect
 nuclear DNA. **Supplemental Figure 1** shows
 that 100% of the cells were stained for CYP17A1,
 189 confirming the identity of the isolated TCs. We
 also examined the expression of SIKs in H295R
 cells, a human adrenocortical cell line that
 192 expresses high CYP17A1 activity and produces
 androgens (Samandari et al., 2007). Western
 blotting demonstrated the presence of SIK2 and
 195 SIK3 isoforms in mouse TCs, whereas SIK1 was
 undetectable. SIK3 protein was more strongly
 expressed than SIK2. In contrast, in H295R cells,
 198 all isoforms were detected although SIK1 levels
 were lower in comparison to SIK2 and SIK3, of
 which SIK2 was the most highly expressed (**Figure**
 201 **1**).

To determine if similar expression levels for each
 SIK isoform are found *in vivo*, we used the ovaries of
 204 adult mice to perform immunohistochemical (IHC)
 studies. The results showed that SIK2 and SIK3, but
 not SIK1, are highly expressed in the ovaries (**Figure**
 207 **2**). Higher magnification analysis also showed that
 SIK3 is especially highly expressed in the theca and
 interstitial cells (**Figure 2**, right). Taken together these
 210 studies show that the interstitial tissue and the TCs
 express mostly SIK3.

**SIK inhibition enhances androgen synthesis and
 213 LH responses in TCs.**

Figure 3

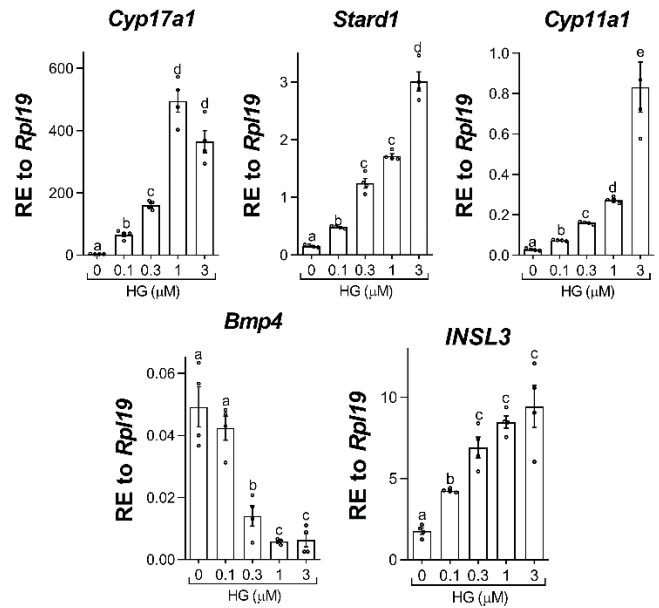


Figure 3: SIK inhibition regulates theca cell genes in a concentration-dependent manner. TCs were treated with the SIK inhibitor HG for 24 h. The mRNA levels of the depicted genes were quantified by qPCR. Different letters differ significantly ($n = 3$).

Figure 4

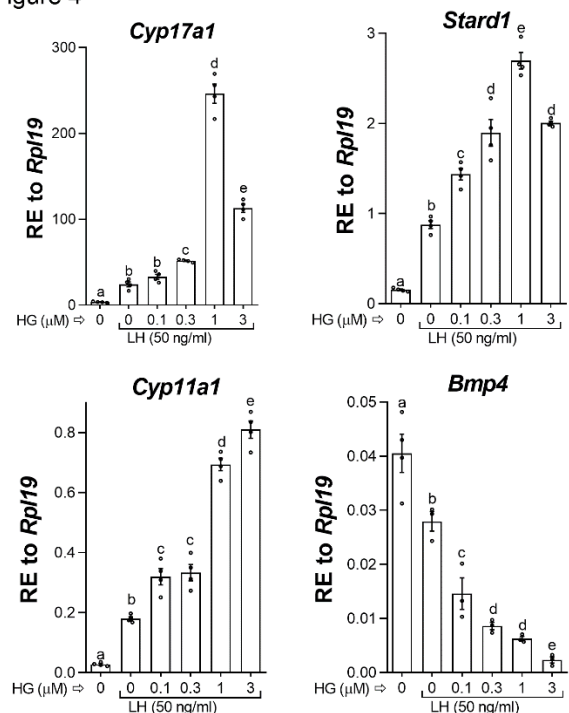


Figure 4: SIK inhibition enhances LH stimulation of enzymes involved in androgen synthesis. Cells were treated with the SIK inhibitor HG (1 μM) for 1 h before the addition of LH (50 ng/mL). The mRNA levels of the depicted genes were quantified 24h later by qPCR. Different letters differ significantly ($n = 5$).

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 (*InsI3*) (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 *InsI3* in a concentration-dependent manner (Figure 3). The lowest concentration used (0.1 μ M) significantly stimulated the expression of *Stard1*, *Cyp17a1*, *Cyp11a1*, and *InsI3*. For *Cyp17a1*, a stronger effect was observed at a 1 μ M concentration compared to 3 μ M. SIK inhibition decreased the expression of *Bmp4* mRNA levels also in a concentration-dependent manner showing a maximal inhibitory effect at 1 μ M.

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 μ M) for *Stard1* and *Cyp11a1* and at 0.3 μ M for *Cyp17a1*. However, the 3 μ M concentration was less effective in enhancing LH actions on the expression of *Cyp17a1* and *Stard1* when compared to 1 μ M (Figure 4). SIK inhibition also enhanced the inhibitory effect of LH on *Bmp4* expression in a concentration-dependent manner. SIK inhibition with 1 μ M

Figure 5

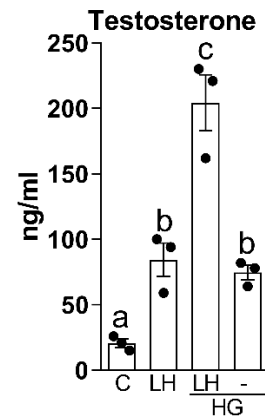


Figure 5: SIK inhibition increases testosterone production in mouse TCs. Cells were treated with the SIK inhibitor HG (1 μ M) for 1 h before the addition of LH (50 ng/mL). Testosterone levels in the media were quantified 24 h later by ELISA. Different letters differ significantly ($n = 3$).

HG also enhanced the stimulatory effects of LH on *Cyp17a1* and *Stard1* in rat TCs (**Supplemental Figure 2**).

243 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 μ M in these experiments. As expected, LH
246 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**).

249

SIK inhibition enhances the effects of LH

252 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

264 *Bmp4* (Figures 6 and 7). As with LH, SIK inhibition enhanced forskolin and dbcAMP induction of *Cyp17a1*, *Stard1*, and *Cyp11a1*

267 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

273 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).

Figure 6

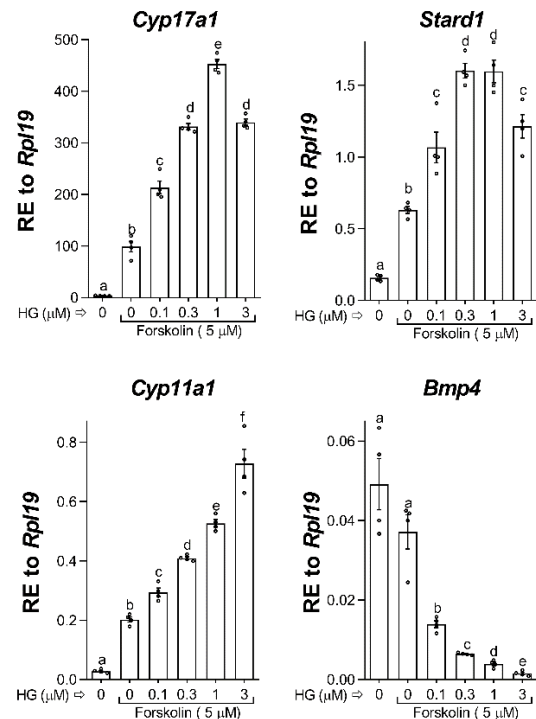


Figure 6: SIK inhibition enhances the response of TCs to forskolin signaling. Mouse TCs were treated with forskolin (5 μM) in the presence or absence of increasing concentration of HG. Different letters differ significantly, $n = 3$.

Figure 7

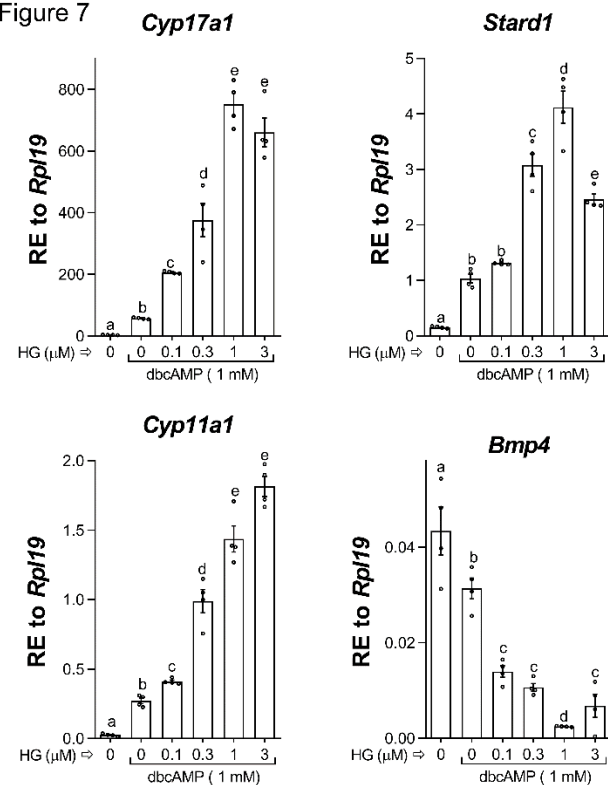


Figure 7: SIK inhibition enhances the response of TCs to cAMP signaling. Mouse TCs were treated with dbcAMP (1 mM) in the presence or absence of increasing concentration of HG. Different letters differ significantly, $n = 3$

279 Importantly, SIK inhibition also enhanced dbcAMP stimulation
 282 of testosterone production (**Figure 8**).

Figure 8

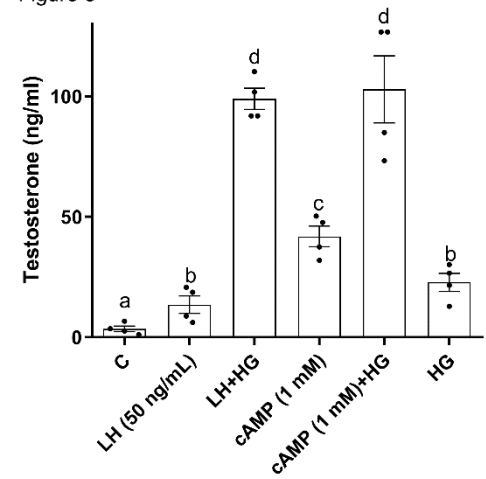


Figure 8: SIK inhibition enhances the response of TCs to cAMP signaling. Mouse TCs were treated with an analog of cAMP (dbcAMP) or with LH (50 ng/ml) in the presence or absence of the SIK inhibitor HG (1 μ M). Testosterone levels in the media were quantified 48 h later. Different letters differ significantly, $n = 4$

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

294 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

297 increased mRNA

expression of

STARD1, *CYP11A1*,

300 and *CYP17A1* when
 compared to controls

(**Figure 9**). The

303 induction of these
 genes by forskolin or
 dbcAMP was

306 enhanced by SIK

inhibition. SIK

Figure 9

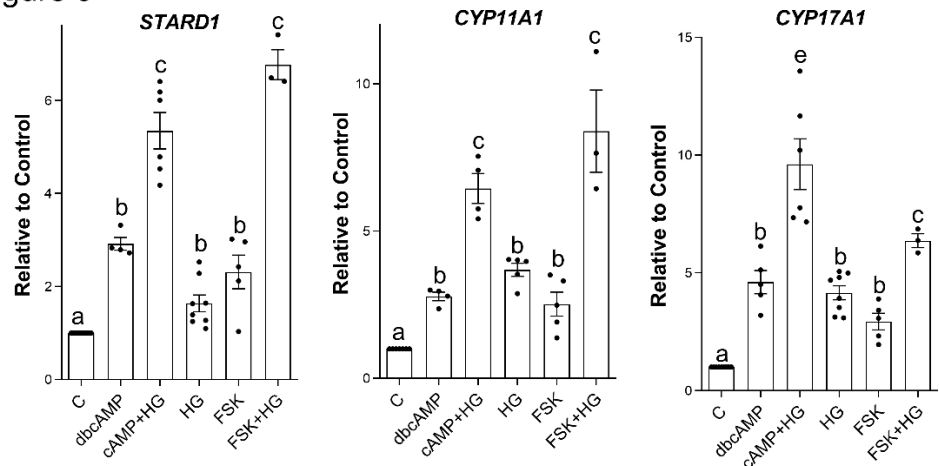


Figure 9: SIK inhibition enhances the response of human H295R cells to cAMP signaling. H295R cells were treated with dbcAMP (1 mM) or forskolin (5 μ M) in the presence or absence of HG (1 μ M). Different letters differ significantly, $n = 4-8$

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).

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

315 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

336 phosphorylation and consequent activation of CREB-regulated genes. Therefore, the impact of SIK

Figure 10

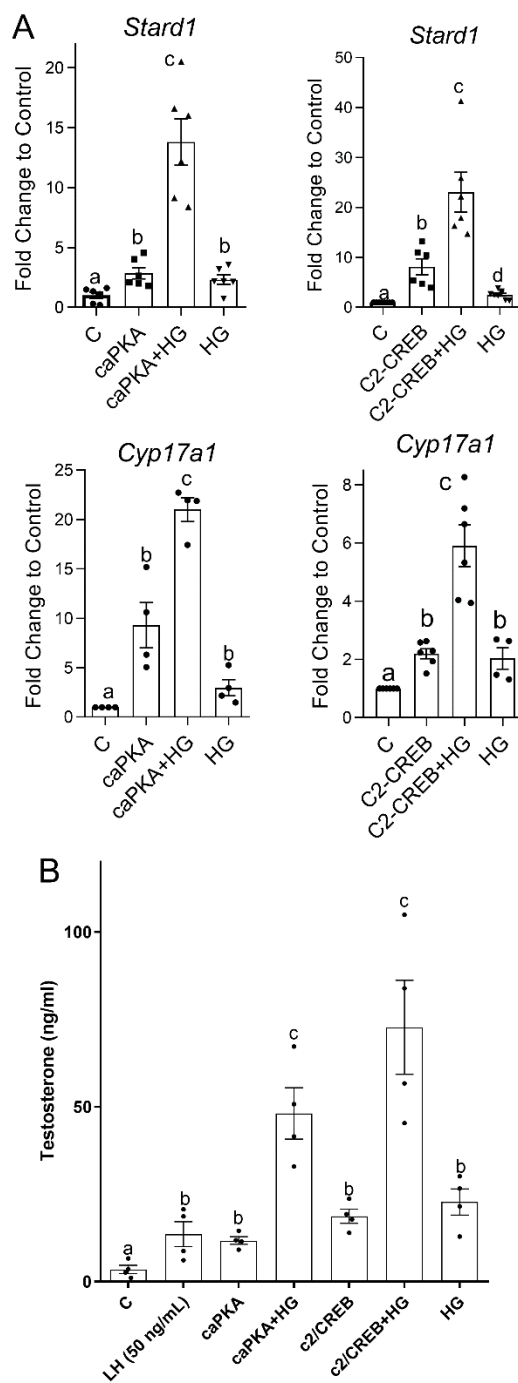


Figure 10: SIK inhibition enhances the response of TCs to PKA and CREB. TCs were infected with lentivirus carrying the pGPs empty plasmid (C), caPKA, or C2/CREB. 24 h after infection, cells were treated with the vehicle of SIK inhibitor HG (1 μ M). 48 h later, gene expression (A) and testosterone (B) were quantified using qPCR or ELISA, respectively. Different letters differ significantly, n = 3-4.

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 production. As shown in **Figure 11**, SIK2 knockout mice have significantly higher levels of testosterone in circulation when compared to control mice.

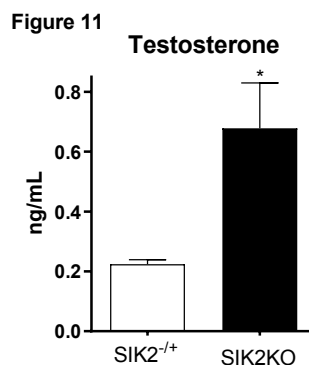
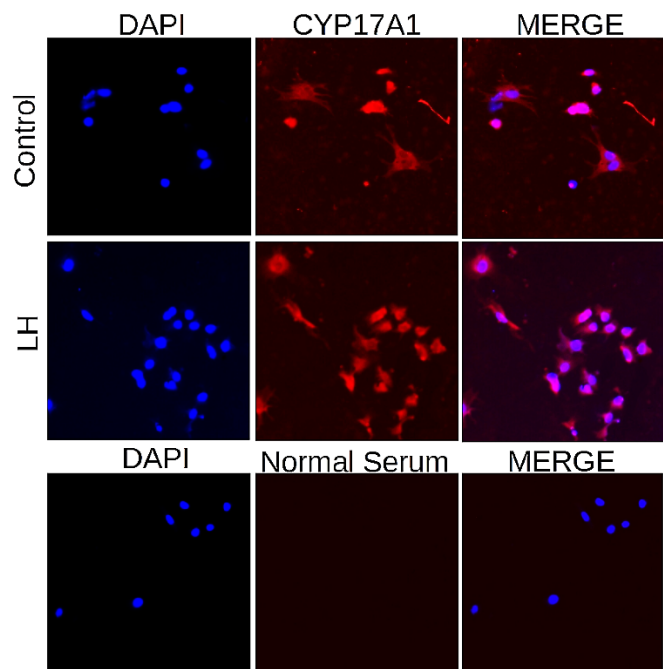


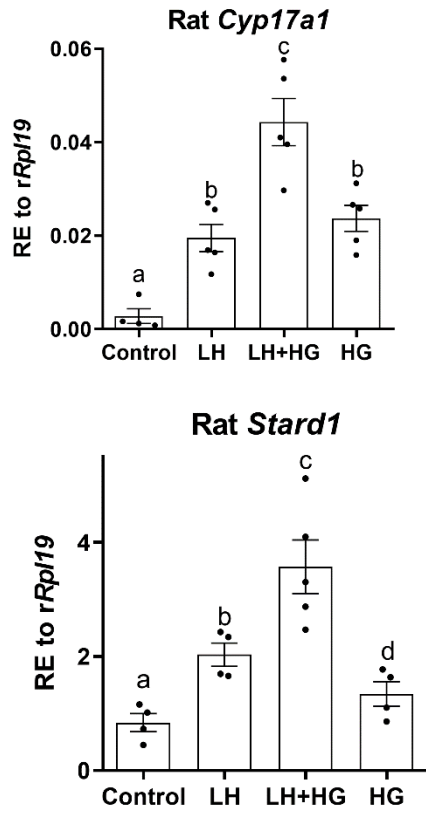
Figure 11: Serum testosterone levels in SIK2 knockout mice. Testosterone levels were quantified ELISA, respectively. * P < 0.05 vs control. n = 5.

Supplemental Figure 1: Confirmation of mouse TC identity after cell purification using discontinuous Percoll gradients as described in the Materials and Methods Section.



Supplemental Figure 2: SIK inhibition enhances the response of rat TCs to LH. TCs were treated

357 with LH (50 ng/ml) in the presence or absence of HG (1 μ M). Different letters differ significantly, n = 4.



360 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, analysis of SIK knockout mice demonstrated that SIKs are critical regulators of female fertility (Armouti et al., 2020, Armouti et al., 2022). This report aimed to determine the role that SIKs play in the regulation of other cell types in the ovary, more specifically the TCs. Our findings demonstrate that TCs express mainly SIK3 and at lower levels SIK2, suggesting that SIK3 may play a major role in the regulation of theca cell function. The results also 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. Based on the levels of expression of SIK2 and SIK3, it may be possible that SIK3 plays a more prominent role in TCs, however, further research is needed to elucidate the specific roles of SIK2 and SIK3.

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 specific factors such as *InsI3* 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 μ M 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 μ M 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 μ M HG was less effective than 1 μ M 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 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 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
417 *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.

420 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,
423 Barnhart, Schlaff et al., 2008), a disease associated with increased androgen biosynthesis in TCs due to
the augmented expression of steroidogenic enzymes (Wood, Nelson, Ho et al., 2003, Nelson, Legro,
Strauss et al., 1999, Franks, White, Gilling-Smith et al., 1996). Overexpression of LKB1 was also shown
426 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.
429 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.

SIKs are drug targets (Sundberg, Liang, Wu et al., 2016), therefore, targeting SIK activity could be used
432 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,
435 including humans (Gleicher et al., 2013, Wiser et al., 2010, Noventa et al., 2019, Bercaire et al.,
2018, Katsika et al., 2022). Therapeutically, androgen priming has been used to improve the ovarian
response to gonadotropin stimulations in patients with diminished ovarian reserve or poor responders
438 (Noventa et al., 2019, Bercaire et al., 2018). 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
441 (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

444 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,
447 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.

450 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
453 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"
456 (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
459 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.

Finally, we provide evidence that SIK2 may be the main isoform involved in the upregulation of
462 **testosterone production. 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. Whereas SIK1 knockout mice have no fertility
465 **problems therefore we do not expect changes in testosterone in these animals. Our findings open**
up a new and exciting research area, which must include the determination of the specific effects
that each SIK isoform has in the ovary.

468 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
471 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. Further experiments are needed to determine the involvement of SIKs in regulating CREB activity and whether SIK activity targets additional PKA substrates in TCs.

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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.

Author contributions

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

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