Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

KISS1R Signaling Modulates Gonadotropin Sensitivity in Mouse Leydig Cell

Meng-Chieh Hsu[1], Leang-Shin Wu[1], De-Shien Jong[1], Chih-Hsien Chiu[1*]

[1]Department of Animal Science and Technology, College of Bio-Resources and Agriculture, National Taiwan University, No. 50, Ln. 155, Sec. 3, Keelung Rd., Taipei 106, Taiwan, ROC

[*]**Corresponding author**

Chih-Hsien Chiu, Ph.D., Department of Animal Science and Technology, College of Bio-Resources and Agriculture, National Taiwan University

No. 50, Ln. 155, Sec. 3, Keelung Rd., Taipei 106, Taiwan, ROC

E-mail: chiuchihhsien@ntu.edu.tw; Tel: +886-2-3366-4171

Short title: KISS1R signaling regulates steroidogenesis

Key words: Kisspeptin, KISS1R, LHCGR, Steroidogenesis, Gonadotropin sensitivity

Word count: approximate 4682

Abstract

Kisspeptin and its receptor KISS1R have been proven as pivotal regulators on controlling the hypothalamus-pituitary-gonad axis. Inactivating mutations in one of them cause idiopathic hypogonadotropic hypogonadism in human as well as rodent models. Notably, gonadotropin insensitivity, failure in hCG response, was presented in the male patients with loss-function-mutations in *KISS1R* gene; this reveals the essential role of KISS1R signaling in regulating testosterone production beyond the hypothalamic functions of kisspeptin. In this study, we hypothesized that the autocrine action of kisspeptin on Leydig cells may modulate steroidogenesis. Based on the mouse cell model, we firstly demonstrated that the cAMP/ protein kinase A (PKA)/ cAMP response element-binding protein (CREB) signaling pathway mediated gonadotropin-induced kisspeptin expression. By using siRNA interfering technique, knockdown of *Kiss1r* in MA-10 cells, a mouse Leydig tumor cell line, significantly reduced progesterone productions in both basal and hCG-treated conditions. Integrating the results from both quantitative real-time PCR and steroidogenic enzyme-activity assay, we found that this steroidogenic defect was associated with decreased luteinizing hormone/choriogonadotropin receptor (*Lhcgr*) and steroidogenic acute regulatory protein (*Star*) expressions. Furthermore, exogenous expression of human LHCGR completely rescued hCG-stimulated progesterone production in the KISS1R-deficient cells. In conclusion, we proposed that the reproductive functions of KISS1R signaling in Leydig cell include modulating *Lhcgr* and steroidogenic gene expressions, which may shed the light on the pathophysiology of gonadotropin insensitivity.

 Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

Introduction

In mammals, kisspeptin is thought to be an indispensable regulator for the activation of hypothalamus-pituitary-gonad axis by stimulating the gonadotropin-releasing hormone (GnRH) neuron, which expresses the kisspeptin receptor, KISS1R, also known as GPR54. Inactivating mutations in *KISS1* or *KISS1R* cause idiopathic hypogonadotropic hypogonadism, a syndrome characterized by deficient production of GnRH, gonadotropins and sex steroid hormones, leading to incomplete sexual maturation (Ohtaki et al., 2001, de Roux et al., 2003, Funes et al., 2003, Seminara et al., 2003, Messager et al., 2005). The human kisspeptin precursor contains 145 amino acids and can be proteolytically cleaved into peptides of different lengths, including kisspeptin-54, kisspeptin-14, kisspeptin-13 and kisspeptin-10. These peptides contain a common C-terminal decapeptide sequence and have the similar biological activities triggering intracellular calcium mobilization (Kotani et al., 2001). Aside from the hypothalamus, several non-neuronal tissues express *Kiss1*, *Kiss1r* or both, and their local functions are continuously investigated nowadays (Ohtaki et al., 2001, Funes et al., 2003, Hussain et al., 2015, Chianese et al., 2018).

The previous study had demonstrated that *Kiss1* expression in mouse testis was correlated to postnatal testicular development (Wang et al., 2015). Further, luteinizing hormone (LH) is regarded as a stimulator to prompt kisspeptin production in the Leydig cell (Salehi et al., 2015). However, the intracellular signaling pathway in LH-induced *Kiss1* expression has not yet been well described. In the Leydig cell, the primarily downstream signal transduction pathway of luteinizing hormone/choriogonadotropin receptor (LHCGR) is the cyclic adenosine monophosphate (cAMP)/ protein kinase A (PKA) pathway (Hansson et al., 2000). Following the signaling cascade, a transcription factor cAMP responsive element binding protein (CREB), a target protein of PKA with the serine 133 phosphorylation,

is activated and regulates several steroidogenic enzyme expressions, such as steroidogenic acute regulatory protein (StAR) (Gonzalez and Montminy, 1989, Manna et al., 2002). In the view of LHCGR signaling cascade, we proposed that, in the mouse Leydig cells, CREB might mediate LH-induced *Kiss1* expression.

As both kisspeptin and KISS1R exist in the mammalian testes, and *Kiss1* expression is correlated to testicular development (Salehi et al., 2015, Wang et al., 2015, Han et al., 2020), it raised a hypothesis that autocrine and paracrine actions of kisspeptin might be involved in regulating two major testicular functions, testosterone biosynthesis and spermatogenesis (Chianese et al., 2016, Wahab et al., 2016, Sharma et al., 2020). Indeed, several studies had examined the effect of kisspeptin on steroidogenesis. However, the conclusions contained some discrepancies. In the *in vitro* experiments, kisspeptin-10 did not directly increase steroid hormone production or synergize with the hCG stimulations in the mouse primary and tumor Leydig cells (Mei et al., 2013, Wang et al., 2015). Nevertheless, the acutely inhibitory effects of kisspeptin-10 treatment on the testosterone and estradiol productions in the frog testis explants has been described (Chianese et al., 2017). By contrast, kisspeptin-10 directly increases testosterone production in the primary horse Leydig cells (Petrucci et al., 2020). Due to the divergent results of the kisspeptin influence on steroid hormone productions, this concept needed further studies to clarify its role in steroidogenesis. More importantly, the human male patients with inactivating mutations in *KISS1R* gene were completely irresponsive to gonadotropin treatment for stimulating testosterone secretion (Semple et al., 2005, Nimri et al., 2011), suggesting that testicular KISS1R signaling locally interacts with LHCGR signaling, and is crucial for steroidogenesis.

In the present study, we hypothesized that the autocrine action of kisspeptin is involved in regulating steroidogenesis in mouse Leydig cells. At first, we examined

Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

100 whether the LH-induced kisspeptin expression depended on cAMP/PKA/CREB
101 signaling pathway. Next, we investigated the possible regulations of kisspeptin and
102 KISS1R on steroidogenesis by manipulating cellular *Kiss1* or *Kiss1r* expressions
103 endogenously and exogenously.
104

Materials and methods

Reagents, compounds and antibodies

Recombinant human chorionic gonadotropin (hCG, C1063) and gelatin (G1890) were purchased from Sigma-Aldrich (St. Louis, MA, USA). 8-Bromo-cAMP sodium salt (8-Br-cAMP), Forskolin, 3-Isobutyl-1-methylxanthine (IBMX) and H89 were purchased from Tocris Bioscience (Bristol, UK). Synthesized mouse kisspepitn-10 was purchased from Kelowna International Scientific (Taipei, Taiwan). All nucleotide primers were obtained from Genomics (Taipei, Taiwan). Predesigned siGENOME SMARTpool siRNAs, against mouse *Creb1* (#M-040959-01), *Kiss1* (#M-057640-01) and *Kiss1r* (#M-059823-01), were purchased from GE Healthcare (Little Chalfont, UK). Lipofectamine RNAiMAX, Lipofectamine 3000, pcDNA3.1(+) vector, Restore Stripping Buffer and goat anti-rabbit IgG HRP antibody (#31460) were purchased from Thermo Fisher Scientific (Waltham, USA). Anti-kisspeptin antibody (PAC559Mu01) was purchased from Cloud-Clone Corp. (Houston, USA) and the antibody validation was shown in Supplemental Figure 1. Anti-CREB (#9197), anti-phospho-CREB(Ser133) (#9198), anti-StAR (#8449), and anti-GAPDH (#2118) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, USA). Anti-β-actin antibody (sc-47778) and goat anti-mouse IgG HRP antibody (sc-2005) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The chemicals used in medium and buffer preparations without specific mention were purchased from Sigma-Aldrich or Merck Millipore (Billerica, MA, USA)

Animals

Male imprinting control region (ICR) mice, purchased from the Laboratory Animal Center, College of Medicine, National Taiwan University, were maintained in a 12 hour light: 12 hour darkness cycle at 25 ± 2°C, 50-70% relative humidity

 Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

environment, and provided a chow diet and water *ad libitum* for the duration of the study. Five adult mice, 12 to 16-week old, were used in the experiment of primary Leydig cell culture. All of animal experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of National Taiwan University (IACUC No.: NTU-100-EL-102).

Primary mouse Leydig cell isolation and culture

The mouse testis tunica albuginea were removed and the testicular contents were washed once with Hanks' balanced salt solution (HBSS). Next, the testicular contents were transferred to fresh 10 mL HBSS in centrifuge tube and dissociated by shaking the tubes with hand for 5-10 times. The dissociated tissues were passed through a cell strainer, and interstitial cells were suspended in the filtrate. The crude Leydig cells were collected by centrifugation at 300×*g* for 5 min, and resuspended in serum-free Medium 199 (Sigma-Aldrich). In the experiment of hCG dose response, the crude Leydig cells were counted and seeded on 12-well culture plate with 2 x 10^5 cells/well, and then treated with hCG for 8 hours. The cell lysates and media were collected for further analyses.

Cell line culture

MA-10 cell was purchased from American Type Culture Collection (ATCC, Manassas, USA; #CRL-3050) and cultured in DMEM/F-12 medium (Sigma-Aldrich) supplemented with 15% horse serum (Thermo Fisher Scientific), penicillin-streptomycin, and additional 20 mM HEPES as ATCC instruction described. The culture plates or flasks were coated with 0.1% sterile gelatin solution for at least 1 hour before using. The cells were maintained at 37°C with 5% CO_2. In

the general experiments, the cells were counted and seeded on 24-well culture plate with 1.25 or 1.6 x 10^5 cells/ well for western blotting and progesterone releasing assay, or on 12-well culture plate with 2.5 x 10^5 cells/ well for real-time PCR analysis. After 24 to 48 hour post-seeding, the cells were treated with the different indicated materials for 4 or 8 hours in the fresh complete growth media. At the end of treatment, the media were collected for progesterone measurement, and the cell lysates were collected for Western blotting or real-time PCR analyses.

In the gene knockdown experiments, 10μM siRNA for the indicated genes were reverse-transfected into the MA-10 cell using Lipofectamine RNAiMAX Reagent. For the experiments in exogenous gene expression, the indicated plasmids were transfected into the MA-10 cell using Lipofectamine 3000 Reagent. Transfection procedures were carried out following manufacture's protocol.

Plasmid construction

The insert DNA fragments were amplified by using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) and further cut the 5' and 3' ends by using FastDigest Restriction Enzymes (Thermo Fisher Scientific). The DNA fragment ligation was under a molar ratio of 1:3 vector to insert with T4 DNA Ligase (Takara Bio Inc.) at 16°C for 1 hour. The ligated plasmid was transferred to DH5α competent cell ECOS™ 101 (Yeastern Biotech Co., Ltd, Taipei, Taiwan) and further selected the bacterial clones under antibiotic-containing agar plates. In this study, the indicated genes were inserted into pcDNA3.1(+) mammalian expression vector between EcoRI and XbaI sites. pcDNA3.1(+)-EGFP contains the reporter gene, enhanced green fluorescent protein (EGFP); pcDNA3.1(+)-Kiss1-T2A-GFP contains the coding sequences of ICR mouse *Kiss1*, *Thoseaasigna* virus 2A (T2A) element, and EGFP reporter. pcDNA3.1(+)-hLHCGR contains the coding sequences of human *Lhcgr*,

 Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

183 subcloned from LHCGR-Tango plasmid (#66417, Addgene, Watertown, USA).

184

185 *Western blotting*

186 The cultured cells were rinsed with cold PBS once and lysed with 1× Laemmli
187 buffer containing 50 mM dithiothreitol on the ice, and further heated at 98ºC for 10
188 min to achieve protein denaturation. The cell lysates were then subjected to 10% or
189 13% Tris-Glycine SDS-PAGE electrophoresis, and then transferred to PVDF
190 membranes. The membrane was blocked with 5% non-fat milk for 1 hour and then
191 incubated with primary antibody in TBS containing 0.1% Tween-20 (TBST)
192 containing 1% BSA at 4ºC overnight. After washing in TBST, the membrane was
193 incubated for 1 hour at room temperature with horseradish peroxidase
194 (HRP)-conjugated secondary antibody in TBST with 5% non-fat milk. After washing
195 in TBST 3 times, signals were detected by ECL (GE Healthcare) using a ChemiDoc™
196 Imaging System (Bio-Rad Laboratories, Inc., Hercules, USA). For detection of
197 internal control protein, the same membrane was incubated with stripping buffer for
198 20 min at room temperature, and re-probed with internal control primary antibody.
199 The protein levels were determined by obtaining the signal density in a defined area
200 using Image Lab version 6.0 (Bio-Rad Laboratories, Inc.) or ImageJ version 1.49b
201 (NIH, Bethesda, MD, USA).

202

203 *Enzyme-linked immunosorbent assay (ELISA)*

204 Medium progesterone and testosterone concentrations were determined using the
205 direct competitive enzyme immunoassay (Wu et al., 2000). Briefly, 50 μL diluted
206 sample aliquot and 150 μL diluted HRP-conjugated progesterone solution (Fitzgerald
207 Industries International, Acton, MA, USA) were added to microtiter plates, which had
208 coated with anti-progesterone antibody (G-7 clone). For testosterone measurement,

anti-testosterone antibody and HRP-conjugated testosterone (Cosmo Bio Co., Tokyo, Japan) were used in the assay. After incubation for 20 to 40 min at room temperature with gentle shaking, the plates were washed three times with washing buffer (0.01 M phosphate buffer containing 0.1% Tween-20, pH 7.0). Color was developed for 20 to 40 min by using substrate solution, 0.1 M pH 6.0 phosphate buffer containing 3.7 mM o-phenylenediamine and 0.03% H_2O_2. Reaction was stopped by the addition of 50 μL 8N sulfuric acid solution. Absorbance was determined using a dual wavelength reader at 490/630 nm. All standards and samples were assayed in duplicate. The detection limits of progesterone and testosterone assays were 104 pg/mL and 52 pg/mL, respectively. The intra- and inter-assay coefficients of variation were 8.5% and 8.6% in the progesterone assay; 3.5% and 6.7% in the testosterone assay, respectively. The sample dilution ratios were adjusted appropriately to range within the standard curves.

RNA extraction and reverse transcription

Total RNA was extracted from the cells with TRIsure reagent (Bioline Inc., London, UK) according to the manufacturer's instructions. The cDNA was reverse-transcribed from RNA by using PrimeScript™ RT reagent Kit (Takara Bio Inc.). 500 ng total RNA was mixed with 25 pmol oligo dT primer, 50 pmol random hexamers, PrimeScript RT enzyme mix and reaction buffer, then incubated at 37°C for 30 min. The reverse transcriptase was inactivated by heating to 85°C for 10 sec, and cDNA products were stored at 4°C for further analysis.

Quantitative real-time PCR

Relative levels of target mRNA was examined with the QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) according to the manufacturer's instructions. Transcripts were quantified using the Fast SYBR Green Master Mix

 Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

(Thermo Fisher Scientific) in a total volume of 10 μL, containing 0.4 μM each primer. Primer pairs were designed on different exons; the primer sequences are shown in Table 1. Samples were initially heated for 20 sec at 95°C, followed by 40 cycles of 3 sec at 95°C for denaturation, and 30 sec at 60°C for annealing and extension.. Relative gene expression values were normalized with respect to those of the internal control (*Rpl19*), and presented as the fold-change compared to that of control group

Statistical analysis

Quantitative data were analyzed by two-tailed Student's t-test or one-way ANOVA followed by Duncan's method. Data were expressed as means ± SEM or SD. All statistical analyses of data were performed using Sigma Plot Software (Systat Software, San Jose, CA, USA) or Microsoft Excel (2016).

Results

hCG stimulates kisspeptin expression in Leydig cells

To address the concept that LH could trigger kisspeptin expression in Leydig cells, a dose response of hCG on primary mouse Leydig cells was performed (Figure 1). The protein level of kisspeptin precursor was significantly increased after hCG treatment. The increased StAR protein level and medium testosterone concentration represent the effectiveness of hCG treatment in the primary cells.

The cAMP/PKA/CREB signaling pathway, a downstream of LHCGR, may mediate hCG-stimulated kisspeptin expression in Leydig cell. Based on the ChIP-seq experiment, CREB is a potential transcription factor to regulate mouse *Kiss1* gene expression (Yevshin et al., 2019). Supporting with this idea, in the primary mouse Leydig cells, CREB Ser133 phosphorylation was significantly increased after hCG treatment (Figure 1A and B). To confirm the hypothesis, MA-10 cell, a mouse Leydig tumor cell line, was used for the following experiments due to good performance in cell transfections. Treatments of forskolin (Fsk), an adenylyl cyclase activator, plus 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, elevated intracellular cAMP level and triggered robust *Kiss1* expression, which is completely blocked by PKA inhibitor H89 (Figure 2A). Knockdown of *Creb1* significantly inhibits Fsk/IBMX-induced *Kiss1* expression (Figure 2B). Similar results were observed in the PKA activator 8-Br-cAMP-treating experiment, which shown that *Kiss1* level in *Creb1*-knockdowned cells was significantly lower than the control cells after stimulation (data not shown). Furthermore, at the protein level, knockdown of *Creb1* diminishes hCG-induced kisspeptin expression (Figure 2C and D). The above results suggest that *Kiss1* expression is regulated by cAMP/PKA/CREB signaling pathway in mouse Leydig cells.

 Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

KISS1R regulates basal and hCG-stimulated steroidogenesis

Before carrying out the later experiments, we tested whether serum deprivation altered cellular states and further changed the outcomes since most of steroidogenesis-related experiments were achieved in the serum-free condition for many years. As the results shown in Figure 3, serum deprivation significantly increased *Kiss1* expression but inhibited 8-Br-cAMP induction; on the other hand, *Kiss1r* mRNA level in the same experiment displayed opposite expression pattern. Since serum deprivation greatly affected gene expressions and cell responses, the following experiments were performed in the complete growth media.

To examine the direct effect of kisspeptin on steroidogenesis, we designed two experiments having different kisspeptin-exposing periods. For testing the acute effect, MA-10 cells were treated with different concentrations of mouse kisspeptin-10 for 4 hours (Figure 4A). For testing the chronic effect, a 2-day exposure, the cells were transiently transfected with mouse *Kiss1*-expressing plasmid and secreted kisspeptin continuously (Figure 4B), in view of that kisspeptin-10 would be degraded rapidly by serum proteinases in the culture medium and wasn't suitable for a long-term treatment (Takino et al., 2003, Liu et al., 2013). In both conditions, the medium progesterone concentrations did not change significantly when compared to their own control group.

In regard to the putatively autocrine action of kisspeptin, the cells may be in a steady state that they could not respond to additional kisspeptin stimulations. To break down the autocrine action, suppressing endogenous *Kiss1* or *Kiss1r* expressions by siRNAs was performed. *Kiss1r*-knockdowned MA-10 cells significantly decreased progesterone production in both basal (saline) and hCG-treated conditions (Figure 4C). However, knockdown of *Kiss1* only showed a slight effect on progesterone production. This may be caused by the technical limit of siRNA transfection that the

partial cells were not transfected and kept low-level kisspeptin in the culture media. Considering the potential effects of *Kiss1* or *Kiss1r* knockdown on cell proliferation, we tested the cell viability by sulforhodamine B assay, and the results shown no difference in all groups after siRNA transfection (data not shown). The above results indicate that KISS1R signaling is important in regulating both basal and hCG-stimulated steroidogenesis. Interestingly, the suppressive effect of *Kiss1r* knockdown on progesterone production was only found in hCG-treated but not Fsk/IBMX-treated (Figure 4C) or 8-Br-cAMP-treated (data not shown) conditions, suggesting that KISS1R signaling may regulate the steroidogenic pathway between LHCGR and adenylyl cyclase.

Lack of KISS1R does not diminish the cellular CYP11A1 and HSD3B activities

Since *Kiss1r* knockdown directly affects basal progesterone production, we assumed that lack of KISS1R function might cause steroidogenic enzyme deficiency. In the steroidogenesis-related gene expression profiles, the mRNA levels of *Lhcgr*, *Star* and *Cyp11a1* were significantly decreased in the *Kiss1r*-knockdowned MA-10 cells (Figure 5A). We had checked the *Kiss1r* siRNA sequences, which did not match with mouse *Lhcgr*, *Star* and *Cyp11a1* mRNAs by using global alignment, thus excluding the possibility of off-target effects on the interested genes by siRNA transfection. Next, to further verify steroidogenic enzyme functions in the cells, we performed the enzyme-activity assay, supplied different cholesterol-derivative substrates in the culture media and further analyzed progesterone concentrations. 22R-hydroxycholesterol (22R-OHC) is catalyzed to progesterone by the activities of CYP11A1 and HSD3B; pregnenolone is converted directly by HSD3B. As the results shown in Figure 5B, the progesterone production did not decrease in the *Kiss1r*-knockdowned cells when given the indicated substrates in either basal or

 Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

hCG-treated conditions, which demonstrates that KISS1R-deficit-induced steroidogenic deficiency did not result from insufficient activities of either CYP11A1 or HSD3B.

KISS1R maintains hCG sensitivity by regulating Lhcgr expression

Based on the previous results, the *Kiss1r*-knockdowned MA-10 cells were evidently desensitized to hCG stimulation, which was contributed to the functional failure in the steroidogenic pathway between LHCGR and adenylyl cyclase (Figure 4C). Furthermore, in the gene expression profiles, the *Lhcgr* expression in the *Kiss1r*-knockdowned cells was significantly decreased compared to control cells (Figure 5A). These evidences reveal that KISS1R functions involve the regulation of *Lhcgr* expression, and, importantly, hCG desensitization in KISS1R-deficient cells may be caused by insufficient LHCGR level. Consisting with our hypothesis, the results showed that exogenous human LHCGR (hLHCGR) expression obviously rescued the steroidogenic function from the *Kiss1r*-knockdowned cells (Figure 6). In the hCG-treated condition, both progesterone production and StAR protein level were significantly lower in *Kiss1r*-knockdowned cells than in control cells (EGFP), and these outcomes were reversed by expressing hLHCGR. However, in the saline-treated condition, the progesterone production in *Kiss1r*-knockdowned cells was not fully recovered by exogenous hLHCGR (Figure 6C), which means that other factors, such as StAR, for the basal steroidogenesis were changed due to KISS1R deficiency. Taken together, the above results suggest that functions of KISS1R signaling in Leydig cell at least include maintaining LH sensitivity through regulating *Lhcgr* expression.

354

 Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

355 **Discussion**

356 The role of kisspeptin, a crucial neuropeptide for stimulating GnRH secretion to
357 initiate puberty and control the reproductive activity, has been well-established in the
358 mammalian physiology (de Roux et al., 2003, Funes et al., 2003, Seminara et al.,
359 2003, Kirilov et al., 2013). Beyond the central neuroendocrine regulation, the other
360 functions of kisspeptin in peripheral tissues, including pancreas, bone, ovary and
361 kidney, are suggested (Yi et al., 2010, Dorfman et al., 2014, Song et al., 2014, Son et
362 al., 2018). In the current study, we propose a putative model that testicular kisspeptin
363 modulates steroidogenesis via an autocrine signaling loop on the mouse Leydig cell.
364 In this model, LH stimulates Leydig cell to generate kisspeptin, which depends on the
365 cAMP/PKA/CREB pathway. The secreted kisspeptin further acts on the same cells to
366 regulate testosterone production; this regulation involves two aspects, basal
367 steroidogenesis and gonadotropin sensitivity, through conducting *Star* and *Lhcgr*
368 expressions. In the physiological view, this autocrine action of kisspeptin may allow
369 Leydig cell to amplify and prolong LH signal, and, particularly, maintain LH
370 sensitivity.
371
372 Based on the mouse Leydig cell model, hCG and PKA activators were able to
373 induce kisspeptin expression, indicating that Leydig cell is one of the sources of
374 testicular kisspeptin production triggered by LH signal. Supporting this idea, previous
375 reports have shown that gonadotropin could stimulate *Kiss1* expression in the primary
376 mouse and goat Leydig cells (Salehi et al., 2015, Samir et al., 2018). We also
377 demonstrated that testicular *Kiss1* expression was synchronizing with *Lhcgr*
378 expression as well as testis growths (Wang et al., 2015). In addition to the postnatal
379 mouse model, the cycles of testicular development and regression in the seasonal
380 breeding animals provided another valuable model to examine this idea. Hamsters are

seasonal breeding animals, and the activity of reproductive system depends on the photoperiod. At the long photoperiod, the endocrine and spermatogenic functions of testes were active. In contrast, at the short photoperiod, these functions were suppressed; this testicular regression is a consequence of reduced release of gonadotropins (Siegel, 1985). As consisting with the concept, in the striped hamster, the testicular *Kiss1* expression was significantly decreased at the short photoperiod (Li et al., 2015). Similar observation was described in the non-mammalian model, anuran amphibian, which shown increased testicular kisspeptin protein level in the breeding season (Chianese et al., 2017).

CREB-deficient Leydig cells failed in *Kiss1* expression induced by either hCG or Fsk/IBMX treatments, suggesting that CREB is a critical transcription factor for gonadotropin-induced *Kiss1* expression (Figure 2). Supporting this concept, the significance of cAMP/PKA/CREB pathway in activating *Kiss1* gene transcription was found in other cell types, including *Kiss1* neuron and hepatocyte (Song et al., 2014, Treen et al., 2016). The novel peptide phoenixin, discovered as a potential upstream regulator of mouse *Kiss1* neuron, is able to simulate *Kiss1* expression, which was greatly suppressed by pre-treating cAMPS-Rp, a competitive antagonist of PKA (Treen et al., 2016). Apart from the neuron, hepatocytes also produce kisspeptin via activating this pathway (Song et al., 2014). The raising intracellular cAMP concentrations, induced by either glucagon or Fsk/IBMX treatments, promoted *Kiss1* expression in mouse hepatocytes. Additionally, in the luciferase reporter assay, the Fsk/IBMX-stimulated *Kiss1* transcriptional response was obviously suppressed by the mutations of two putative cAMP response element (CRE) sites on the *Kiss1* promoter. Furthermore, a prior report indicated that the disruption of CREB-regulated transcription coactivator-1 (*Crtc1*) gene resulted decreasing *Kiss1* mRNA levels in the

 Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

hypothalamic cells (Altarejos et al., 2008). These observations provide strong evidences to support that CREB acts as a key regulator to mediate *Kiss1* gene transcription.

This is the first report to describe that KISS1R signaling is crucial for maintaining gonadotropin sensitivity in the Leydig cells through regulating *Lhcgr* expression (Figure 4 and 5). This finding could explain why the male patients with loss-of-function mutations (C233R or F272S) in *KISS1R* gene show severe gonadotropin insensitivity (Semple et al., 2005, Nimri et al., 2011). In the repeated stimulation tests, most patients failed to show increased testosterone levels after short and prolonged hCG administrations, performed repeatedly in different ages from infancy to early adulthood. In the mouse model, a recent study indicated that lack of KISS1R in testis resulted insufficient testicular functions (Leon et al., 2016). The male Kiss1r$^{-/-}$Tg (aka Gpr54$^{-/-}$Tg in Leon's study) rescued mice, which were the global *Kiss1r* null mice had selective re-introduction of *Kiss1r* expression in the GnRH neurons to preserve gonadotropic axis activity, shown the incomplete growths in testes and epididymes, and the appearance of multinucleated giant cells and apoptotic germ cells in the seminiferous tubules. Interestingly, these male Kiss1r$^{-/-}$Tg rescued mice also showed lower testosterone:LH ratio, which had similar testosterone levels but approximately 2.2-fold higher LH levels compared to wild-type mouse data, suggesting that the Leyding cells in those rescued mice were desensitized to gonadotropin stimulation. Furthermore, KISS1R signaling may be important in maintaining neurotransmitter sensitivity in the hypothalamic or pituitary neurons. In the same report, *Kiss1r* null mice were extremely irresponsive to different neurotransmitter analogue stimulations on LH secretion, which was recovered obviously in Kiss1r$^{-/-}$Tg mice (Leon et al., 2016). Similar results were found in the

433 *Kiss1* null rats, which failed to elicit LH secretion after monosodium glutamate,
434 NMDA and norepinephrine administrations (Uenoyama et al., 2015). Regarding the
435 transcriptional regulations between KISS1R and LHCGR found in this study, we
436 assumed that KISS1R signaling might be mandatory for some neurotransmitter
437 receptor expressions on GnRH neuron or its downstream neurons.
438
439 The fact that serum deprivation caused a remarkable disturbance in *Kiss1*
440 expression was an unexpected finding (Figure 3). A prior report suggested that serum
441 deprivation induces strong and temporal CREB phosphorylation (Leicht et al., 2001),
442 which may explain the result of robust *Kiss1* expression. However, this outcome was
443 disrupted when combining PKA agonist treatment and serum deprivation, which
444 reveals that other factors derived from serum and their downstream signaling
445 pathways affect PKA/CREB-dependent *Kiss1* transcription. AMP-activated protein
446 kinase (AMPK), an intracellular energy sensor and signaling regulator, mediating
447 *Kiss1* expression in both *Kiss1* neuron and GT1-7 neural cell line had been reported
448 (Wen et al., 2012, Roa et al., 2018); this finding might associate with the results we
449 observed since serum contains plentiful nutrients, growth factors and hormones.
450 Importantly, in addition to CREB, the roles of numerous transcription factors
451 (Yevshin et al., 2019) on regulating *Kiss1* expression either positively or negatively
452 haven't been characterized well. Therefore, studies focused on *Kiss1* transcriptional
453 regulation and its molecular mechanism are needed.
454
455 In the past, the experiments for studying steroidogenesis were usually examined
456 in a serum-free condition because serum contains various and undetermined factors
457 that influenced steroidogenic function (Pate and Condon, 1982). However, serum
458 deprivation undeniably disrupts cellular homeostasis at least including elevated *Kiss1*

 Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

459 and reduced *Kiss1r* expressions in Leydig cells, which may account for the
460 ineffectiveness of kisspeptin treatment in our previous studies (Hsu et al., 2014, Wang
461 et al., 2015). This finding also suggests that researchers should cautiously design
462 experiments and interpret the results when applying the cell line, primary cell and
463 tissue cultures in serum-free media to study steroidogenesis.
464
465 In summary, we have shown that the cAMP/PKA/CREB signaling pathway
466 mediates gonadotropin-induced *Kiss1* gene transcription in mouse Leydig cells.
467 Regarding the putatively autocrine action of kisspeptin, our study also highlights the
468 importance of KISS1R singling in maintaining gonadotropin sensitivity and
469 steroidogenic function through regulating *Lhcgr* and the related gene expressions.
470 This study provides new insights into the pathophysiology of gonadotropin
471 insensitivity in idiopathic hypogonadotropic hypogonadism.
472
473

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Ministry of Science and Technology (grant number 106-2320-B-002-040-MY3, 2017).

Author contribution statement

MCH and CHC conceived the study and wrote the manuscript. MCH performed experiments and analyzed data. LSW and DSJ reviewed and edited the manuscript.

Acknowledgements

The authors thank Dr. Yi-Fan Jiang (School of Veterinary Medicine, National Taiwan University, Taiwan) and Dr. Hetal V. Shah (National Institute of Neurological Disorders and Stroke, National Institutes of Health, USA) for the critical reading and comments of the manuscript.

 Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

References

ALTAREJOS, J. Y., GOEBEL, N., CONKRIGHT, M. D., INOUE, H., XIE, J., ARIAS, C. M., SAWCHENKO, P. E. & MONTMINY, M. 2008. The Creb1 coactivator Crtc1 is required for energy balance and fertility. *Nat Med,* 14**,** 1112-7.

CHIANESE, R., CIARAMELLA, V., FASANO, S., PIERANTONI, R. & MECCARIELLO, R. 2017. Kisspeptin regulates steroidogenesis and spermiation in anuran amphibian. *Reproduction,* 154**,** 403-414.

CHIANESE, R., COBELLIS, G., CHIOCCARELLI, T., CIARAMELLA, V., MIGLIACCIO, M., FASANO, S., PIERANTONI, R. & MECCARIELLO, R. 2016. Kisspeptins, Estrogens and Male Fertility. *Curr Med Chem,* 23**,** 4070-4091.

CHIANESE, R., COLLEDGE, W. H., FASANO, S. & MECCARIELLO, R. 2018. Editorial: The Multiple Facets of Kisspeptin Activity in Biological Systems. *Front Endocrinol (Lausanne),* 9**,** 727.

DE ROUX, N., GENIN, E., CAREL, J. C., MATSUDA, F., CHAUSSAIN, J. L. & MILGROM, E. 2003. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci U S A,* 100**,** 10972-6.

DORFMAN, M. D.,GARCIA-RUDAZ, C.,ALDERMAN, Z.,KERR, B.,LOMNICZI, A.,DISSEN, G. A.,CASTELLANO, J. M.,GARCIA-GALIANO, D.,GAYTAN, F.,XU, B., et al. 2014. Loss of Ntrk2/Kiss1r signaling in oocytes causes premature ovarian failure. *Endocrinology,* 155**,** 3098-111.

FUNES, S., HEDRICK, J. A., VASSILEVA, G., MARKOWITZ, L., ABBONDANZO, S., GOLOVKO, A., YANG, S., MONSMA, F. J. & GUSTAFSON, E. L. 2003. The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. *Biochem Biophys Res Commun,* 312**,** 1357-63.

GONZALEZ, G. A. & MONTMINY, M. R. 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell,* 59**,** 675-80.

HAN, Y.,ZHAO, Y.,SI, W.,JIANG, X.,WU, J.,NA, R.,HAN, Y.,LI, K.,YANG, L.,E, G., et al. 2020. Temporal expression of the KISS1/GPR54 system in goats' testes and epididymides and its spatial expression in pubertal goats. *Theriogenology,* 152**,** 114-121.

HANSSON, V., SKALHEGG, B. S. & TASKEN, K. 2000. Cyclic-AMP-dependent protein kinase (PKA) in testicular cells. Cell specific expression, differential regulation and targeting of subunits of PKA. *J Steroid Biochem Mol Biol,* 73**,**

81-92.
HSU, M. C., WANG, J. Y., LEE, Y. J., JONG, D. S., TSUI, K. H. & CHIU, C. H. 2014. Kisspeptin modulates fertilization capacity of mouse spermatozoa. *Reproduction,* 147**,** 835-45.
HUSSAIN, M. A., SONG, W. J. & WOLFE, A. 2015. There is Kisspeptin - And Then There is Kisspeptin. *Trends Endocrinol Metab,* 26**,** 564-572.
KIRILOV, M., CLARKSON, J., LIU, X., ROA, J., CAMPOS, P., PORTEOUS, R., SCHUTZ, G. & HERBISON, A. E. 2013. Dependence of fertility on kisspeptin-Gpr54 signaling at the GnRH neuron. *Nat Commun,* 4**,** 2492.
KOTANI, M.,DETHEUX, M.,VANDENBOGAERDE, A.,COMMUNI, D.,VANDERWINDEN, J. M.,LE POUL, E.,BREZILLON, S.,TYLDESLEY, R.,SUAREZ-HUERTA, N.,VANDEPUT, F., et al. 2001. The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *J Biol Chem,* 276**,** 34631-6.
LEICHT, M., BRIEST, W., HOLZL, A. & ZIMMER, H. G. 2001. Serum depletion induces cell loss of rat cardiac fibroblasts and increased expression of extracellular matrix proteins in surviving cells. *Cardiovasc Res,* 52**,** 429-37.
LEON, S.,BARROSO, A.,VAZQUEZ, M. J.,GARCIA-GALIANO, D.,MANFREDI-LOZANO, M.,RUIZ-PINO, F.,HERAS, V.,ROMERO-RUIZ, A.,ROA, J.,SCHUTZ, G., et al. 2016. Direct Actions of Kisspeptins on GnRH Neurons Permit Attainment of Fertility but are Insufficient to Fully Preserve Gonadotropic Axis Activity. *Sci Rep,* 6**,** 19206.
LI, S. N., XUE, H. L., ZHANG, Q., XU, J. H., WANG, S., CHEN, L. & XU, L. X. 2015. Photoperiod regulates the differential expression of KiSS-1 and GPR54 in various tissues and sexes of striped hamster. *Genet Mol Res,* 14**,** 13894-905.
LIU, Z., REN, C., JONES, W., CHEN, P., SEMINARA, S. B., CHAN, Y. M., SMITH, N. F., COVEY, J. M., WANG, J. & CHAN, K. K. 2013. LC-MS/MS quantification of a neuropeptide fragment kisspeptin-10 (NSC 741805) and characterization of its decomposition product and pharmacokinetics in rats. *J Chromatogr B Analyt Technol Biomed Life Sci,* 926**,** 1-8.
MANNA, P. R., DYSON, M. T., EUBANK, D. W., CLARK, B. J., LALLI, E., SASSONE-CORSI, P., ZELEZNIK, A. J. & STOCCO, D. M. 2002. Regulation of steroidogenesis and the steroidogenic acute regulatory protein by a member of the cAMP response-element binding protein family. *Mol Endocrinol,* 16**,** 184-99.
MEI, H., DORAN, J., KYLE, V., YEO, S. H. & COLLEDGE, W. H. 2013. Does Kisspeptin Signaling have a Role in the Testes? *Front Endocrinol (Lausanne),* 4**,** 198.

 Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

569 MESSAGER, S.,CHATZIDAKI, E. E.,MA, D.,HENDRICK, A. G.,ZAHN,
570 D.,DIXON, J.,THRESHER, R. R.,MALINGE, I.,LOMET, D.,CARLTON, M.
571 B., et al. 2005. Kisspeptin directly stimulates gonadotropin-releasing hormone
572 release via G protein-coupled receptor 54. *Proc Natl Acad Sci U S A,* 102**,**
573 1761-6.
574 NIMRI, R., LEBENTHAL, Y., LAZAR, L., CHEVRIER, L., PHILLIP, M., BAR, M.,
575 HERNANDEZ-MORA, E., DE ROUX, N. & GAT-YABLONSKI, G. 2011. A
576 novel loss-of-function mutation in GPR54/KISS1R leads to hypogonadotropic
577 hypogonadism in a highly consanguineous family. *J Clin Endocrinol Metab,*
578 96**,** E536-45.
579 OHTAKI, T.,SHINTANI, Y.,HONDA, S.,MATSUMOTO, H.,HORI,
580 A.,KANEHASHI, K.,TERAO, Y.,KUMANO, S.,TAKATSU, Y.,MASUDA,
581 Y., et al. 2001. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a
582 G-protein-coupled receptor. *Nature,* 411**,** 613-7.
583 PATE, J. L. & CONDON, W. A. 1982. Effects of serum and lipoproteins on
584 steroidogenesis in cultured bovine luteal cells. *Mol Cell Endocrinol,* 28**,**
585 551-62.
586 PETRUCCI, L.,MARANESI, M.,VERINI SUPPLIZI, A.,DALL'AGLIO,
587 C.,MANDARA, M. T.,QUASSINTI, L.,BRAMUCCI, M.,MIANO,
588 A.,GOBBETTI, A.,CATONE, G., et al. 2020. Kisspeptin/GnRH1 system in
589 Leydig cells of horse (Equus caballus): Presence and function.
590 *Theriogenology,* 152**,** 1-7.
591 ROA, J.,BARROSO, A.,RUIZ-PINO, F.,VAZQUEZ, M. J.,SEOANE-COLLAZO,
592 P.,MARTINEZ-SANCHEZ, N.,GARCIA-GALIANO, D.,ILHAN,
593 T.,PINEDA, R.,LEON, S., et al. 2018. Metabolic regulation of female puberty
594 via hypothalamic AMPK-kisspeptin signaling. *Proc Natl Acad Sci U S A,* 115**,**
595 E10758-E10767.
596 SALEHI, S., ADESHINA, I., CHEN, H., ZIRKIN, B. R., HUSSAIN, M. A.,
597 WONDISFORD, F., WOLFE, A. & RADOVICK, S. 2015. Developmental
598 and endocrine regulation of kisspeptin expression in mouse Leydig cells.
599 *Endocrinology,* 156**,** 1514-22.
600 SAMIR, H., NAGAOKA, K. & WATANABE, G. 2018. Effect of kisspeptin
601 antagonist on goat in vitro Leydig cell steroidogenesis. *Theriogenology,* 121**,**
602 134-140.
603 SEMINARA, S. B.,MESSAGER, S.,CHATZIDAKI, E. E.,THRESHER, R.
604 R.,ACIERNO, J. S., JR.,SHAGOURY, J. K.,BO-ABBAS, Y.,KUOHUNG,
605 W.,SCHWINOF, K. M.,HENDRICK, A. G., et al. 2003. The GPR54 gene as a
606 regulator of puberty. *N Engl J Med,* 349**,** 1614-27.

SEMPLE, R. K., ACHERMANN, J. C., ELLERY, J., FAROOQI, I. S., KARET, F. E., STANHOPE, R. G., O'RAHILLY, S. & APARICIO, S. A. 2005. Two novel missense mutations in g protein-coupled receptor 54 in a patient with hypogonadotropic hypogonadism. *J Clin Endocrinol Metab,* 90**,** 1849-55.

SHARMA, A., THAVENTHIRAN, T., MINHAS, S., DHILLO, W. S. & JAYASENA, C. N. 2020. Kisspeptin and Testicular Function-Is it Necessary? *Int J Mol Sci,* 21.

SIEGEL, H. I. 1985. The Hamster Reproduction and Behavior. Boston, MA: Springer US : Imprint : Springer,.

SON, H. E., KIM, K. M., KIM, E. J. & JANG, W. G. 2018. Kisspeptin-10 (KP-10) stimulates osteoblast differentiation through GPR54-mediated regulation of BMP2 expression and activation. *Sci Rep,* 8**,** 2134.

SONG, W. J.,MONDAL, P.,WOLFE, A.,ALONSO, L. C.,STAMATERIS, R.,ONG, B. W.,LIM, O. C.,YANG, K. S.,RADOVICK, S.,NOVAIRA, H. J., et al. 2014. Glucagon regulates hepatic kisspeptin to impair insulin secretion. *Cell Metab,* 19**,** 667-81.

TAKINO, T., KOSHIKAWA, N., MIYAMORI, H., TANAKA, M., SASAKI, T., OKADA, Y., SEIKI, M. & SATO, H. 2003. Cleavage of metastasis suppressor gene product KiSS-1 protein/metastin by matrix metalloproteinases. *Oncogene,* 22**,** 4617-26.

TREEN, A. K., LUO, V. & BELSHAM, D. D. 2016. Phoenixin Activates Immortalized GnRH and Kisspeptin Neurons Through the Novel Receptor GPR173. *Mol Endocrinol,* 30**,** 872-88.

UENOYAMA, Y.,NAKAMURA, S.,HAYAKAWA, Y.,IKEGAMI, K.,WATANABE, Y.,DEURA, C.,MINABE, S.,TOMIKAWA, J.,GOTO, T.,IEDA, N., et al. 2015. Lack of pulse and surge modes and glutamatergic stimulation of luteinising hormone release in Kiss1 knockout rats. *J Neuroendocrinol,* 27**,** 187-97.

WAHAB, F., ATIKA, B., SHAHAB, M. & BEHR, R. 2016. Kisspeptin signalling in the physiology and pathophysiology of the urogenital system. *Nat Rev Urol,* 13**,** 21-32.

WANG, J. Y., HSU, M. C., TSENG, T. H., WU, L. S., YANG, K. T. & CHIU, C. H. 2015. Kisspeptin expression in mouse Leydig cells correlates with age. *J Chin Med Assoc,* 78**,** 249-57.

WEN, J. P., LIU, C., BI, W. K., HU, Y. T., CHEN, Q., HUANG, H., LIANG, J. X., LI, L. T., LIN, L. X. & CHEN, G. 2012. Adiponectin inhibits KISS1 gene transcription through AMPK and specificity protein-1 in the hypothalamic GT1-7 neurons. *J Endocrinol,* 214**,** 177-89.

 Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

645 WU, L. S., SHEU, S. Y., HUANG, C. C., CHIU, C. H., HUANG, J. C., YANG, J. R.,
646 LIAN, W. X., LAI, C. H., CHEN, Y. P. & LIN, J. H. 2000. Ginseng flowers
647 stimulate progesterone production from bovine luteal cells. *Am J Chin Med,*
648 28**,** 371-7.
649 YEVSHIN, I., SHARIPOV, R., KOLMYKOV, S., KONDRAKHIN, Y. &
650 KOLPAKOV, F. 2019. GTRD: a database on gene transcription
651 regulation-2019 update. *Nucleic Acids Res,* 47**,** D100-D105.
652 YI, T., TAN, K., CHO, S. G., WANG, Y., LUO, J., ZHANG, W., LI, D. & LIU, M.
653 2010. Regulation of embryonic kidney branching morphogenesis and
654 glomerular development by KISS1 receptor (Gpr54) through NFAT2- and
655 Sp1-mediated Bmp7 expression. *J Biol Chem,* 285**,** 17811-20.
656

Figure Legends

Figure 1. hCG stimulates kisspeptin expression in mouse Leydig cells. Primary mouse Leydig cells were treated with the different concentrations of hCG for 8 hours, and then the samples were collected for analyzing the specific protein levels and steroidogenesis. (A) Representative immunoblots of kisspeptin, StAR, and phospho-CREB (Ser133) from the hCG-treated Leydig cells are shown. (B) Densitometric analyses of Western blotting data from panel A. GAPDH and total CREB were used as the loading controls. (C) At the end of treatment, testosterone concentrations from the culture media were measured. Data are represented as means ± SEM (n = 5). Bars with different letters are significantly different by using Duncan's Method ($p < 0.05$).

Figure 2. The regulation of kisspeptin expression involves cAMP/PKA/CREB pathway in Leydig cells. (A) MA-10 cells, mouse Leydig tumor cell line, were pre-treated with 50 μM H89 for 1 hour, and then treated with 50 μM Forskolin (Fsk)/ 100 μM IBMX for further 8 hours. The *Kiss1* mRNA level was measured by real-time PCR. For the relative comparison of real-time PCR data, *Rpl19* was used as the internal controls. (B) MA-10 cells were firstly transfected with *Creb1* siRNA for 48 hours, and then treated with Fsk/ IBMX for 8 hours. The *Kiss1* and *Creb1* mRNA levels were measured by real-time PCR. (C) MA-10 cells were transfected with *Creb1* siRNA. After 48 hours, the cells were treated with 10 mIU/mL hCG for 4 hours and cell lysates were analyzed by immunoblots (D) Densitometric analyses of immunoblotting data of kisspeptin from panel C. Data are represented as means ± SEM (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

 Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

683 **Figure 3. Serum deprivation disrupts gene expressions and cell responses in**
684 **MA-10 cells.** The cells were seeded and cultured in the complete growth medium for
685 2 days. For the serum deprivation test, the media were replaced with fresh complete
686 growth or serum-free media containing DMSO or 100 μM 8-Br-cAMP as indicated,
687 and the cells were cultured for a further 8 hours. The *Kiss1* and *Kiss1r* mRNA levels
688 were measured by real-time PCR. For the relative comparison of real-time PCR data,
689 *Rpl19* was used as the internal controls. Data are represented as means ± SEM (n = 3).
690 $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.
691
692 **Figure 4. KISS1R regulates the basal and hCG-stimulated steroidogenesis.** To
693 investigate the steroidogenic regulations of kisspeptin and KISS1R on MA-10 cells,
694 the post-treated media from the following experiments were analyzed for
695 progesterone concentrations by using ELISA. (A) MA-10 cells were treated with
696 different concentrations of mouse kisspeptin-10 (Kp-10) for 4 hours. (B) MA-10 cells
697 were transiently transfected with pcDNA3.1(+)-EGFP (EGFP), or
698 pcDNA3.1(+)-mKiss1-T2A-EGFP (Kisspeptin OE) plasmids, or added only with
699 transfection reagent (control). The basal steroidogenesis in different groups were
700 examined post 2-day transfection. (C) MA-10 cells were transiently transfected with
701 the indicated siRNAs. After 2 days, the cells were treated with saline, 100 mIU/mL
702 hCG, or 50 μM Fsk/ 100 μM IBMX for 4 hours. Data are represented as means ± SD
703 (n = 4). Bars with different letters are significantly different by using Duncan's
704 Method ($p < 0.05$). $*p < 0.05$, $**p < 0.01$. NS, not significant.
705
706 **Figure 5. Lack of KISS1R does not diminish the cellular CYP11A1 and HSD3B**
707 **activities.** (A) MA-10 cells were transiently transfected with the *Kiss1r* siRNA. After
708 2 days, the steroidogenesis-related gene expressions were analyzed by real-time PCR.

Rpl19 was used as the internal controls. Data are represented as means ± SEM (n = 3). (B) MA-10 cells were transiently transfected with the *Kiss1r* siRNA. After 2 days, the cells were treated with saline or 100 mIU/mL hCG, combined with different cholesterol-derivative substrates, 10 μg/mL 22R-hydroxycholesterol (22R-OHC) or 1 μg/mL pregnenolone (P5), or 0.1% ethanol (solvent). The media were collected after 4-hour treatment and analyzed by ELISA. Data are represented as means ± SD (n = 5). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. NS, not significant.

Figure 6. Exogenous expression of human luteinizing hormone receptor (hLHCGR) rescues steroidogenesis on *Kiss1r*-knockdowned MA-10 cells. (A) MA-10 cells were transiently transfected with the *Kiss1r* siRNA, pcDNA3.1(+)-EGFP (EGFP, transfection control) or pcDNA3.1(+)-hLHCGR (hLHCGR) plasmids as indicated. After 48 hours, the cells were treated with 100 mIU/mL hCG for 4 hours and cell lysates were analyzed by immunoblots (B) Densitometric analyses of immunoblotting images from panel A. Data are represented as means ± SEM (n = 3) (C) The cells were treated as same as panel A description, and the post-treated media were analyzed for progesterone concentrations by ELISA. Data are represented as means ± SD (n = 5). *$p < 0.05$, ***$p < 0.001$. NS, not significant.

Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

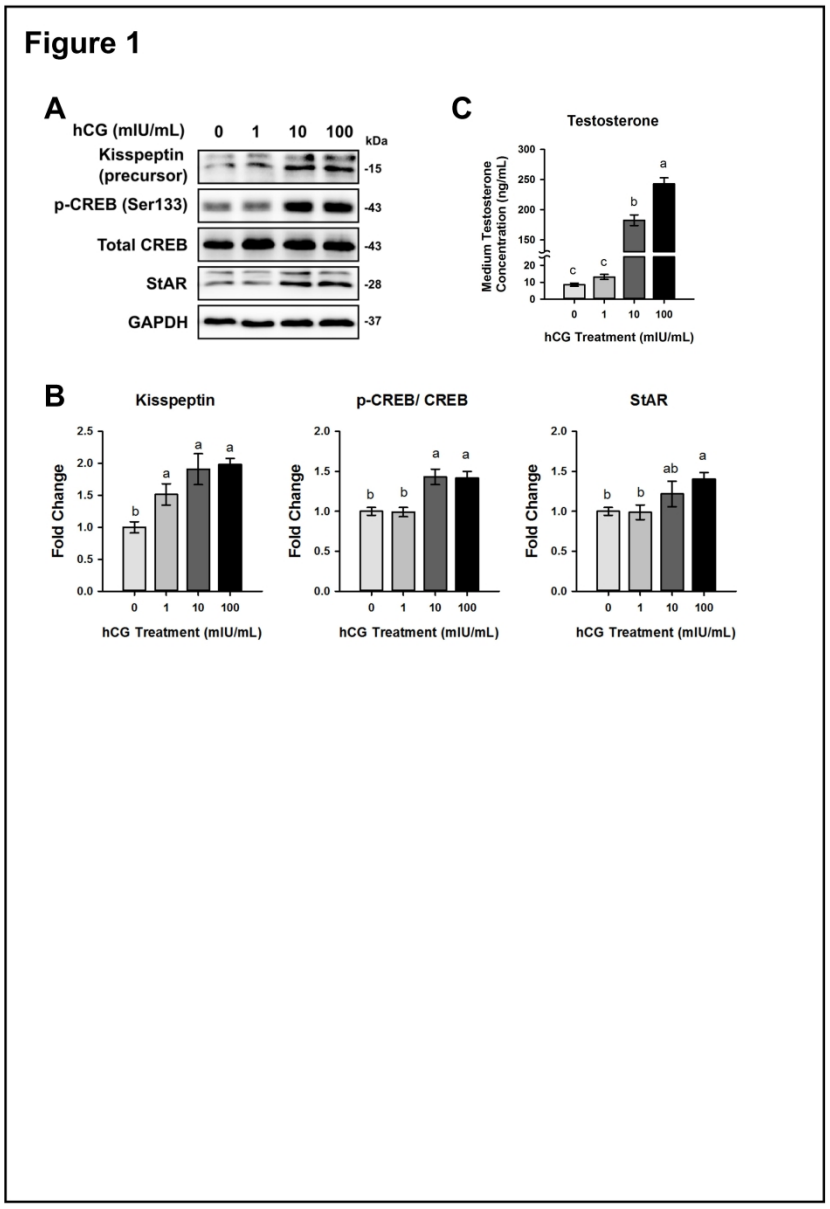


Figure 1. hCG stimulates kisspeptin expression in mouse Leydig cells.

190x278mm (600 x 600 DPI)

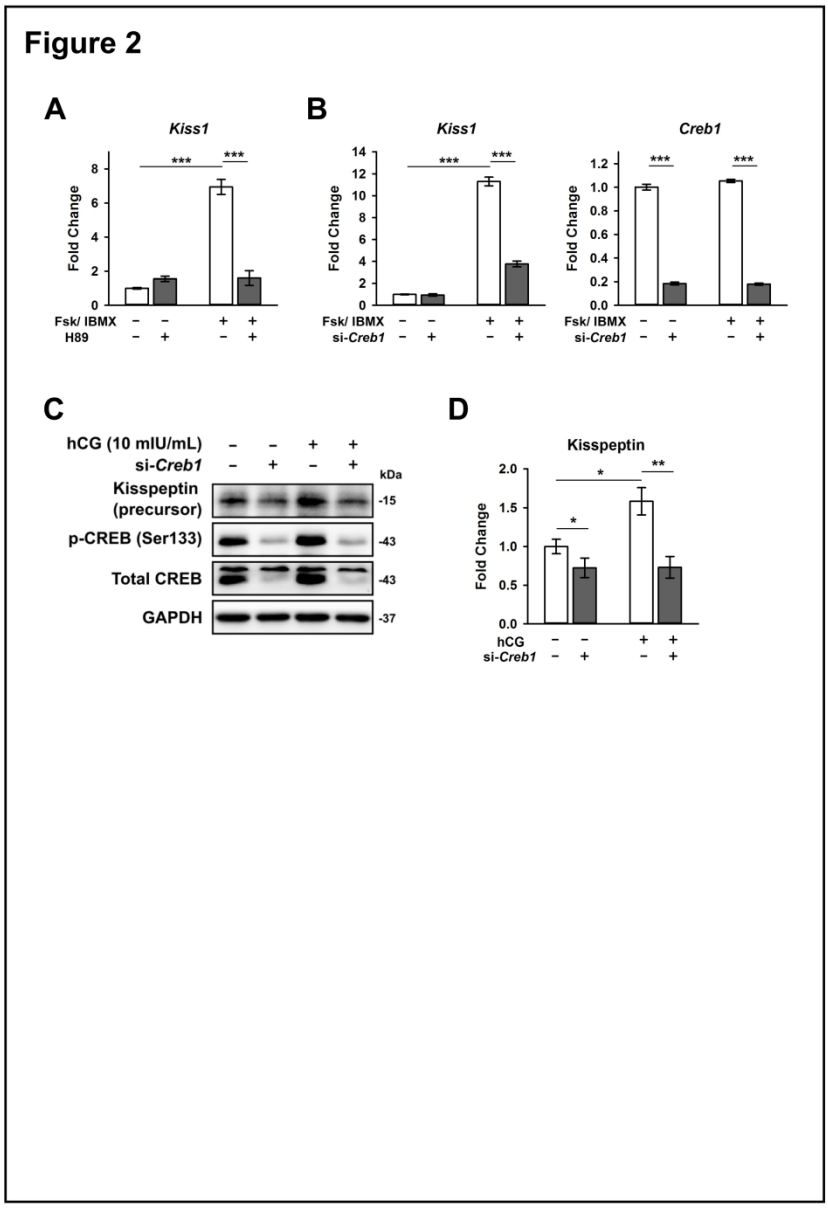


Figure 2. The regulation of kisspeptin expression involves cAMP/PKA/CREB pathway in Leydig cells.

190x278mm (600 x 600 DPI)

Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

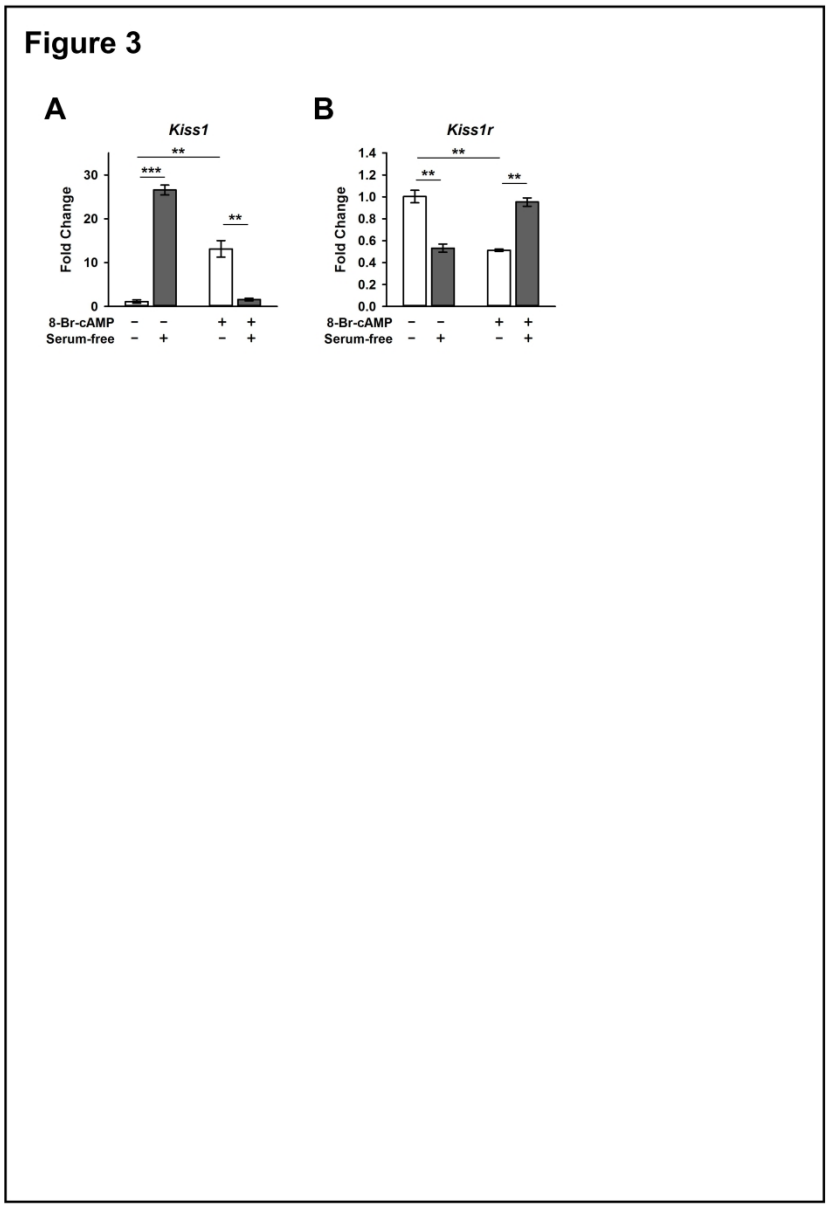


Figure 3. Serum deprivation disrupts gene expressions and cell responses in MA-10 cells.

190x278mm (600 x 600 DPI)

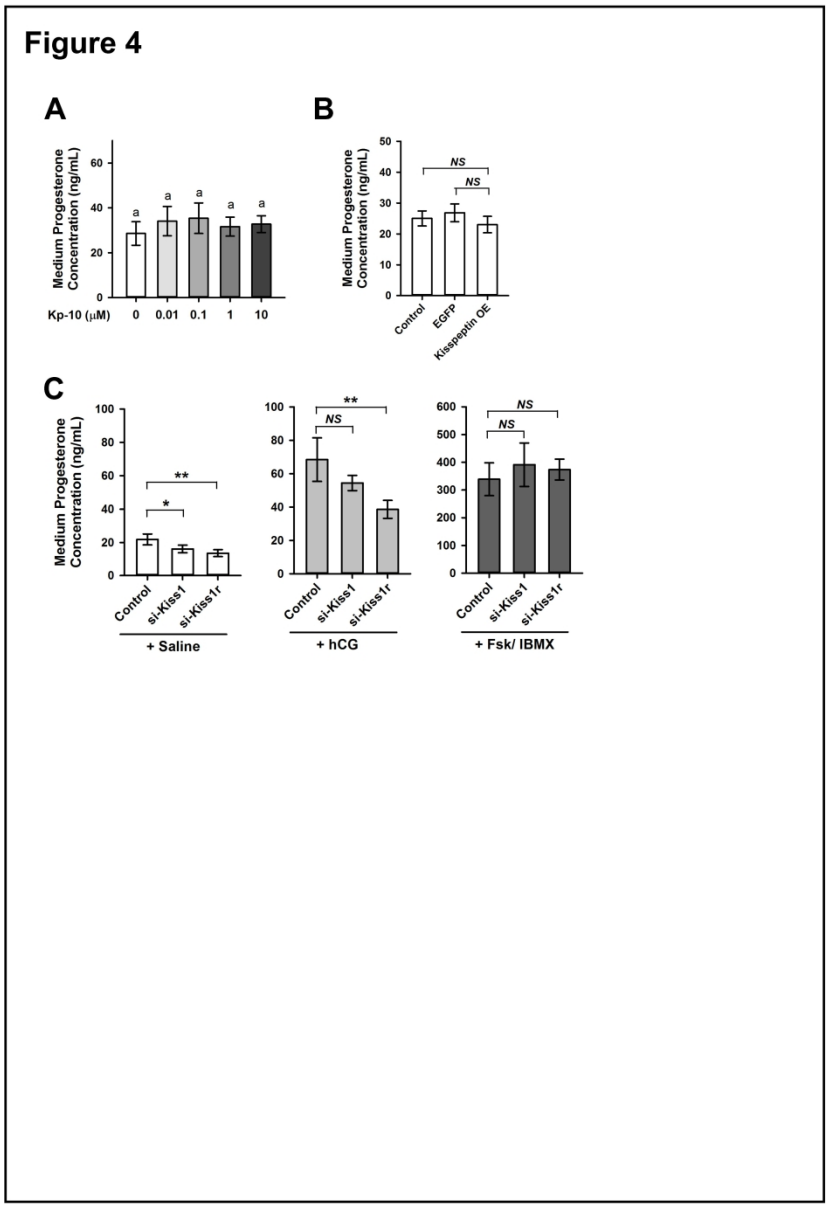


Figure 4. KISS1R regulates the basal and hCG-stimulated steroidogenesis.

190x278mm (600 x 600 DPI)

Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

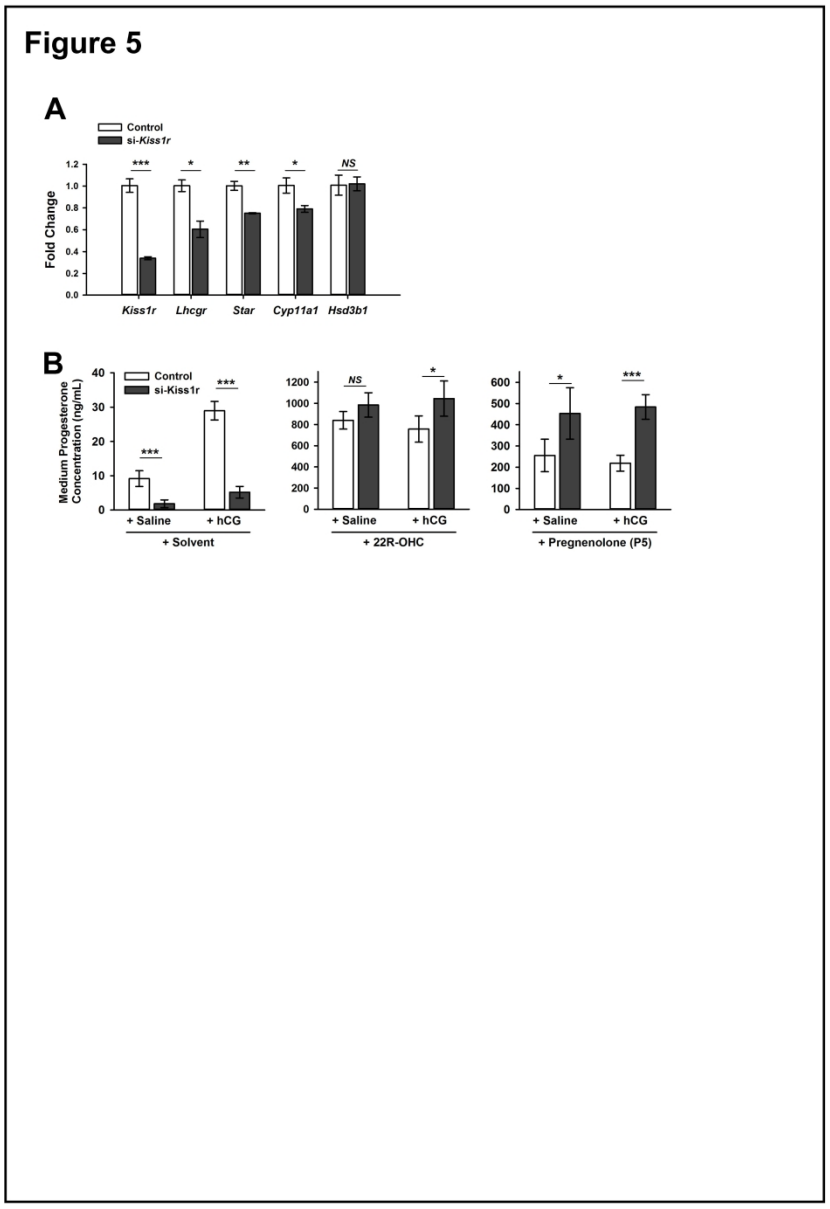


Figure 5. Lack of KISS1R does not diminish the cellular CYP11A1 and HSD3B activities.

190x278mm (600 x 600 DPI)

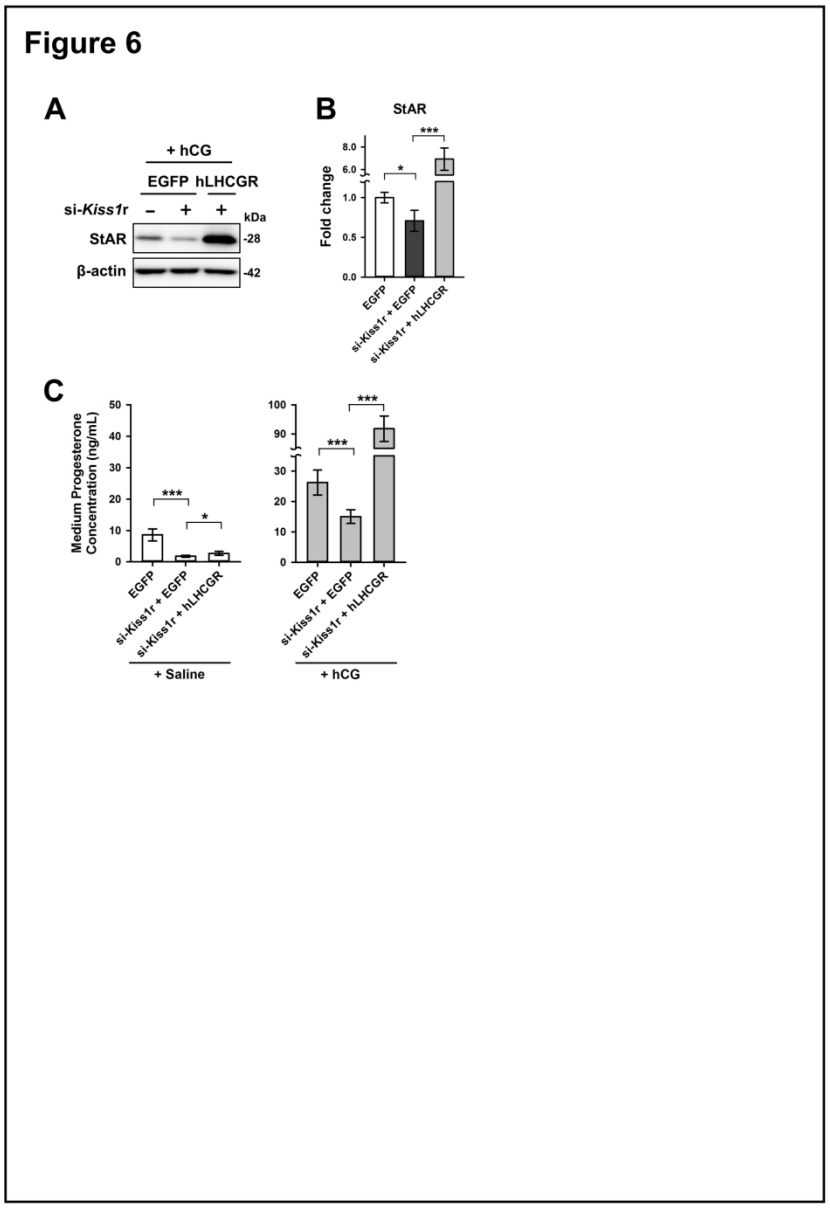


Figure 6. Exogenous expression of human luteinizing hormone receptor (hLHCGR) rescues steroidogenesis on Kiss1r-knockdowned MA-10 cells.

190x278mm (300 x 300 DPI)

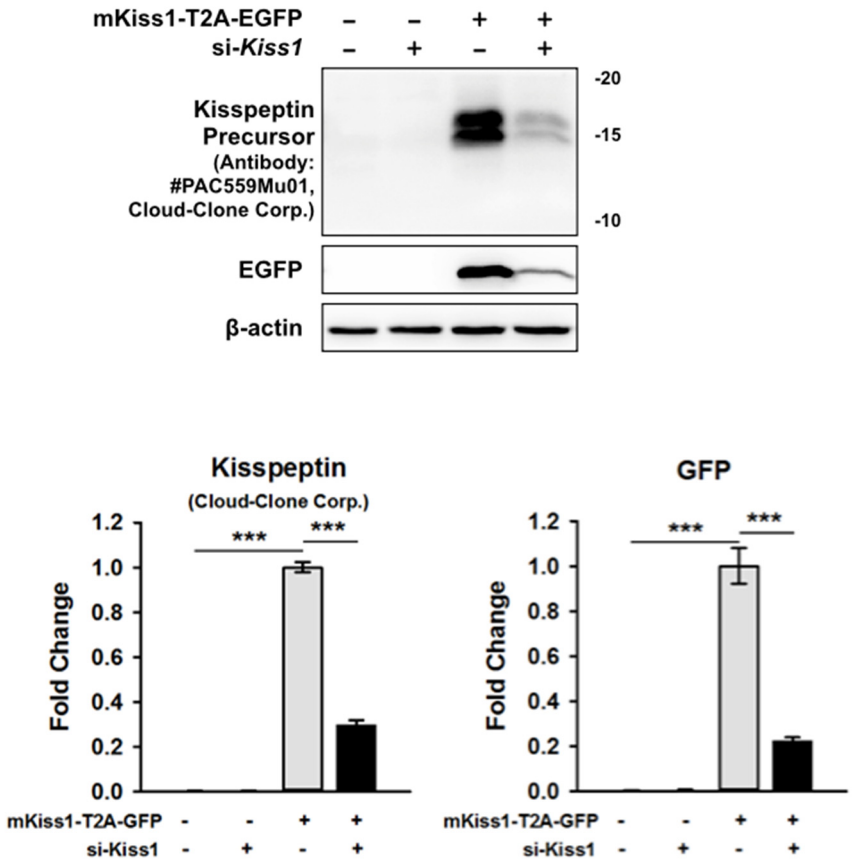
 Accepted Manuscript published as REP-20-0328.R2. Accepted for publication: 03-Sep-2020

Table 1 Primers used in quantitative real-time PCR analysis.

Target Gene	**Primer Sequence** (5' to 3')		**Product Size (bp)**	**Accession Number**
	Forward	Reverse		
Kiss1	TGCTGCTTCTCCTCTGTGTCGC	CAGGCTTGCTCTCTGCATACCGC	139	NM_178260.3
Kiss1r	GTGCAAATTCGTCAACTACATCC	AGCGGGAACACAGTCACATAC	103	NM_053244.5
Rpl19	GCTCTTTCCTTTCGCTGCTGC	CAGTCACAGGCTTGCGGATGAT	200	NM_009078.2
Creb1	CAGTGCCAACCCCCATTTAC	ACCCCATCCGTACCATTGTT	97	NM_001037726.1
Lhcgr	GCCCGACTATCTCTCACCTATC	CCTTTCCAGGGAATCACTCTGA	111	NM_013582.2
Star	AGCATGTTCCTCGCTACGTT	GCACAGCTTGGTGCCTTAATC	88	NM_011485.4
Cyp11a1	TGGCACACAGAAAATCCATTACC	TTGGGGTCCACGATGTAAACT	108	NM_019779.3
Hsd3b1	TTTGCTCTCTCAGTTGTGACCA	GCCTGCTTCGTGACCATATTTATT	134	NM_008293.3

Supplemental Information

Supplemental Figure 1.



Supplemental Figure 1. The validation of anti-mouse kisspeptin antibodies

To evaluate the specificity of the commercial anti-mouse kisspeptin antibodies, #PAC559Mu01 (Cloud-Clone Corp.), the protein levels in *Kiss1*-overexpressed or knockdowned cells were analyzed by using Western blotting. The MA-10 cells were transfected with pcDNA3.1(+) mKiss1-T2A-GFP and *kiss1* siRNA as indicated. Representative immunoblots and densitometric results of kisspeptin precursor and GFP in MA-10 cells are shown. For densitometric analyses, β-actin was used as the loading controls. Data are expressed as mean ± SEM (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.