KISS1R signaling modulates gonadotropin sensitivity in mouse Leydig cell

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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 first 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 StAR 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.

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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, Messenger *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 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 AMP (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 StAR protein (StAR) (Gonzalez & 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 have 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 unresponsive 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 whether the LH-induced kisspeptin expression depended on cAMP/PKA/CREB signaling pathway. Next, we investigated the possible regulations of kisspeptin and *KISS1R* on steroidogenesis by manipulating cellular *Kiss1* or *Kiss1r* expressions endogenously and exogenously.

Materials and methods

Reagents, compounds and antibodies

Recombinant human chorionic gonadotropin (hCG, C1063) and gelatin (G1890) were purchased from Sigma-Aldrich. 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 kisspeptin-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. Lipofectamine RNAiMAX, Lipofectamine 3000, pcDNA3.1(+) vector, Restore Stripping Buffer and goat anti-rabbit IgG HRP antibody (#31460) were purchased from Thermo Fisher Scientific. Anti-kisspeptin antibody (PAC559Mu01) was purchased from Cloud-Clone Corp. (Houston, USA) and the antibody validation was shown in Supplementary Fig. 1 (see section on [supplementary materials](#) given at the end of this article). Anti-CREB (#9197), anti-phospho-CREB(Ser133) (#9198), anti-StAR (#8449), and anti-GAPDH (#2118)

antibodies were purchased from Cell Signaling Technology, Inc. Anti- β -actin antibody (sc-47778) and goat anti-mouse IgG HRP antibody (sc-2005) were purchased from Santa Cruz Biotechnology. The chemicals used in medium and buffer preparations without specific mention were purchased from Sigma-Aldrich or Merck Millipore.

Animals

Male imprinting control region (ICR) mice, purchased from the Laboratory Animal Center, College of Medicine, National Taiwan University, were maintained in a 12 h light: 12 h darkness cycle at $25 \pm 2^\circ\text{C}$, 50–70% relative humidity 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 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 five to ten 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 2×10^5 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; #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 h 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 1.6×10^5 cells/well for Western blotting and progesterone releasing assay, or on 12-well culture plate with 2.5×10^5 cells/well for real-time PCR analysis. After 24–48 h post-seeding, the cells were treated with the different indicated materials for 4 or 8 h 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, 10 μM 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 h. The ligated plasmid was transferred to DH5 α competent cell ECOSTM 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*, subcloned from LHCGR-Tango plasmid (#66417, Addgene, Watertown, USA).

Western blotting

The cultured cells were rinsed with cold PBS once and lysed with 1 $\times$ Laemmli buffer containing 50 mM dithiothreitol on the ice, and further heated at 98°C for 10 min to achieve protein denaturation. The cell lysates were then subjected to 10% or 13% Tris–Glycine SDS-PAGE electrophoresis, and then transferred to PVDF membranes. The membrane was blocked with 5% non-fat milk for 1 h and then incubated with primary antibody in TBS containing 0.1% Tween-20 (TBST) containing 1% BSA at 4°C overnight. After washing in TBST, the membrane was incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody in TBST with 5% non-fat milk. After washing in TBST three times, signals were detected by ECL (GE Healthcare) using a ChemiDocTM Imaging System (Bio-Rad Laboratories, Inc.). For detection of internal control protein, the same membrane was incubated with stripping buffer for 20 min at room temperature, and reprobed with internal control primary antibody. The protein levels were determined by obtaining the signal density in a defined area using Image Lab version 6.0 (Bio-Rad Laboratories, Inc.) or ImageJ version 1.49b (NIH).

Enzyme-linked immunosorbent assay (ELISA)

Medium progesterone and testosterone concentrations were determined using the direct competitive enzyme immunoassay (Wu *et al.* 2000). Briefly, 50 μ L diluted sample aliquot and 150 μ L diluted HRP-conjugated progesterone solution (Fitzgerald Industries International, Acton, MA, USA) were added to microtiter plates, which had 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 20–40 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 20–40 min by using substrate solution, 0.1 M pH 6.0 phosphate buffer containing 3.7 mM o-phenylenediamine and 0.03% H₂O₂. Reaction was stopped by the addition of 50 μ L 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 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 TRIreagent (Bioline Inc., London, UK) according to the manufacturer's instructions. The cDNA was reverse-transcribed from RNA by using PrimeScriptTM 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 s, and cDNA products were stored at 4°C for further analysis.

Quantitative real-time PCR

Relative levels of target mRNA were 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 (Thermo Fisher Scientific) in a total volume of 10 μ L, containing 0.4 μ M each primer. Primer pairs were designed on different exons; the primer sequences are shown in Table 1. Samples were initially heated for 20 s at 95°C, followed by 40 cycles of 3 s at 95°C for denaturation, and 30 s at 60°C for annealing and extension. Relative gene expression values were normalized with respect to those of the internal control (*Rp19*), 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 $\pm$ S.E.M. or S.D. 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

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

Target gene	Primer Sequence (5'–3')		Product size (bp)	Accession number
	Forward	Reverse		
<i>Kiss1</i>	TGCTGCTTCTCTGTGTGCGC	CAGGCTTGCTCTGTGCATACCGC	139	NM_178260.3
<i>Kiss1r</i>	GTGCAAATTCGTCACACTACATCC	AGCGGGAACACAGTCACATAC	103	NM_053244.5
<i>Rpl19</i>	GCTCTTTCCTTTCGCTGCTGC	CAGTCACAGGCTTGCGGATGAT	200	NM_009078.2
<i>Creb1</i>	CAGTGCCAACCCCCATTAC	ACCCCATCCGTACCAATTGTT	97	NM_001037726.1
<i>Lhcgr</i>	GCCCCACTATCTCTCACCTATC	CCTTCCAGGGAATCACTCTGA	111	NM_013582.2
<i>Star</i>	AGCATGTTCTCGCTACGTT	GCACAGCTTGGTGCCTTAATC	88	NM_011485.4
<i>Cyp11a1</i>	TGGCACACAGAAAATCCATTACC	TTGGGGTCCACGATGTAAACT	108	NM_019779.3
<i>Hsd3b1</i>	TTTGCTCTCTCAGTTGTGACCA	GCCTGCTCGTGACCATATTATT	134	NM_008293.3

primary mouse Leydig cells was performed (Fig. 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 (Fig. 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

(Fig. 2A). Knockdown of *Creb1* significantly inhibits Fsk/IBMX-induced *Kiss1* expression (Fig. 2B). Similar results were observed in the PKA activator 8-Br-cAMP-treating experiment, which shown that *Kiss1* level in *Creb1*-knockdown 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 (Fig. 2C and D). The previously described results suggest that *Kiss1* expression is regulated by cAMP/PKA/CREB signaling pathway in mouse Leydig cells.

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 Fig. 3, serum deprivation significantly increased *Kiss1* expression but inhibited 8-Br-cAMP induction;

Figure 1 hCG stimulates kisspeptin expression in mouse Leydig cells. Primary mouse Leydig cells were treated with the different concentrations of hCG for 8 h, 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 $\pm$ S.E.M. ($n=5$). 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 μ M H89 for 1 h, and then treated with 50 μ M Forskolin (Fsk)/100 μ M IBMX for further 8 h. 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 first transfected with *Creb1* siRNA for 48 h, and then treated with Fsk/IBMX for 8 h. The *Kiss1* and *Creb1* mRNA levels were measured by real-time PCR. (C) MA-10 cells were transfected with *Creb1* siRNA. After 48 h, the cells were treated with 10 mIU/mL hCG for 4 h and cell lysates were analyzed by immunoblots (D) Densitometric analyses of immunoblotting data of kisspeptin from panel C. Data are represented as means $\pm$ S.E.M. ($n=3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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 h (Fig. 4A). For testing the chronic effect, a 2-day exposure, the cells were transiently transfected with mouse *Kiss1*-expressing plasmid and secreted kisspeptin continuously (Fig. 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.*

Figure 3 Serum deprivation disrupts gene expressions and cell responses in MA-10 cells. The cells were seeded and cultured in the complete growth medium for 2 days. For the serum deprivation test, the media were replaced with fresh complete growth or serum-free media containing DMSO or 100 μ M 8-Br-cAMP as indicated, and the cells were cultured for a further 8 h. The *Kiss1* (A) and *Kiss1r* (B) mRNA levels were measured by real-time PCR. For the relative comparison of real-time PCR data, *Rpl19* was used as the internal controls. Data are represented as means $\pm$ S.E.M. ($n=3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 4 KISS1R regulates the basal and hCG-stimulated steroidogenesis. To investigate the steroidogenic regulations of kisspeptin and KISS1R on MA-10 cells, the post-treated media from the following experiments were analyzed for progesterone concentrations by using ELISA. (A) MA-10 cells were treated with different concentrations of mouse kisspeptin-10 (Kp-10) for 4 h. (B) MA-10 cells were transiently transfected with pcDNA3.1(+)-EGFP (EGFP), or pcDNA3.1(+)-mKiss1-T2A-EGFP (Kisspeptin OE) plasmids, or added only with transfection reagent (control). The basal steroidogenesis in different groups were examined post-2-day transfection. (C) MA-10 cells were transiently transfected with the indicated siRNAs. After 2 days, the cells were treated with saline, 100 mIU/mL hCG, or 50 μ M Fsk/100 μ M IBMX for 4 h. Data are represented as means $\pm$ S.D. ($n=4$). Bars with different letters are significantly different by using Duncan's method ($P < 0.05$). * $P < 0.05$, ** $P < 0.01$. NS, not significant.

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*-knockdown MA-10 cells significantly decreased progesterone production in both basal (saline) and hCG-treated conditions (Fig. 4C). However, knockdown of *Kiss1* only showed a slight effect on progesterone production. This may be caused by the technical limitations 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 previously described 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 (Fig. 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*-knockdown MA-10 cells (Fig. 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 Fig. 5B, the progesterone production did not decrease in the *Kiss1r*-knockdown cells when given the indicated substrates in either basal or hCG-treated conditions, which demonstrates that KISS1R-deficit-induced steroidogenic deficiency did not result from insufficient activities of either CYP11A1 or HSD3B.

Figure 5 Lack of KISS1R does not diminish the cellular CYP11A1 and HSD3B activities. (A) MA-10 cells were transiently transfected with the *Kiss1r* siRNA. After 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 $\pm$ S.E.M. ($n=3$). (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 μ g/mL 22R-hydroxycholesterol (22R-OHC) or 1 μ g/mL pregnenolone (P5), or 0.1% ethanol (solvent). The media were collected after 4-h treatment and analyzed by ELISA. Data are represented as means $\pm$ S.D. ($n=5$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. NS, not significant.

KISS1R maintains hCG sensitivity by regulating *Lhcgr* expression

Based on the previous results, the *Kiss1r*-knockdown 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 (Fig. 4C). Furthermore, in the gene expression profiles, the *Lhcgr* expression in the *Kiss1r*-knockdown cells was significantly decreased compared to control cells (Fig. 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. Consistent with our hypothesis, the results showed that exogenous human LHCGR (hLHCGR) expression obviously rescued the steroidogenic function from the *Kiss1r*-knockdown cells (Fig. 6). In the hCG-treated condition, both progesterone production and StAR protein level were significantly lower in *Kiss1r*-knockdown 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*-knockdown cells was not fully recovered by exogenous hLHCGR (Fig. 6C), which means that other factors, such as StAR, for the basal steroidogenesis were changed due to KISS1R deficiency. Taken together, the previously described results suggest that functions of KISS1R signaling in Leydig cell at least include maintaining LH sensitivity through regulating *Lhcgr* expression.

Figure 6 Exogenous expression of human luteinizing hormone receptor (hLHCGR) rescues steroidogenesis on *Kiss1r*-knockdown 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 h, the cells were treated with 100 mIU/mL hCG for 4 h and cell lysates were analyzed by immunoblots. (B) Densitometric analyses of immunoblotting images from panel A. Data are represented as means $\pm$ S.E.M. ($n=3$). (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 $\pm$ S.D. ($n=5$). * $P < 0.05$, *** $P < 0.001$. NS, not significant.

Discussion

The role of kisspeptin, a crucial neuropeptide for stimulating GnRH secretion to initiate puberty and control the reproductive activity, has been well-established in the mammalian physiology (de Roux *et al.* 2003, Funes *et al.* 2003, Seminara *et al.* 2003, Kirilov *et al.* 2013). Beyond the central neuroendocrine regulation, the other functions of kisspeptin in peripheral tissues, including pancreas, bone, ovary and kidney, are suggested (Yi *et al.* 2010, Dorfman *et al.* 2014, Song *et al.* 2014, Son *et al.* 2018). In the current study, we propose a putative model that testicular kisspeptin modulates steroidogenesis via an autocrine signaling loop on the mouse Leydig cell. In this model, LH stimulates Leydig cell to generate

kisspeptin, which depends on the cAMP/PKA/CREB pathway. The secreted kisspeptin further acts on the same cells to regulate testosterone production; this regulation involves two aspects, basal steroidogenesis and gonadotropin sensitivity, through conducting *Star* and *Lhcgr* expressions. In the physiological view, this autocrine action of kisspeptin may allow Leydig cell to amplify and prolong LH signal, and, particularly, maintain LH sensitivity.

Based on the mouse Leydig cell model, hCG and PKA activators were able to induce kisspeptin expression, indicating that Leydig cell is one of the sources of testicular kisspeptin production triggered by LH signal. Supporting this idea, previous reports have shown that gonadotropin could stimulate *Kiss1* expression in the primary mouse and goat Leydig cells (Salehi *et al.* 2015, Samir *et al.* 2018). We also demonstrated that testicular *Kiss1* expression was synchronizing with *Lhcgr* expression as well as testis growths (Wang *et al.* 2015). In addition to the postnatal mouse model, the cycles of testicular development and regression in the seasonal 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 the reduced release of gonadotropins (Siegel, 1985). Consistent with this concept, in the striped hamster, the testicular *Kiss1* expression was significantly decreased at the short photoperiod (Li *et al.* 2015). A similar observation was described in the non-mammalian model, anuran amphibian, which showed 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 (Fig. 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 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 (Figs 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 WT mouse data, suggesting that the Leydig 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 analog stimulations on LH secretion, which was recovered obviously in *Kiss1r*^{-/-}*Tg* mice (Leon et al. 2016). Similar results were found in the *Kiss1* null rats, which failed to elicit LH secretion after monosodium glutamate, NMDA and norepinephrine administrations (Uenoyama et al. 2015). Regarding the transcriptional regulations between KISS1R and LHCGR found in this study, we assumed that KISS1R signaling might be mandatory for some neurotransmitter receptor expressions on GnRH neuron or its downstream neurons.

The fact that serum deprivation caused a remarkable disturbance in *Kiss1* expression was an unexpected finding (Fig. 3). A prior report suggested that serum deprivation induces strong and temporal CREB phosphorylation (Leicht et al. 2001), which may explain the result of robust *Kiss1* expression. However, this outcome was disrupted when combining PKA agonist treatment and serum deprivation, which reveals that other factors derived from serum and their downstream signaling pathways affect PKA/CREB-dependent *Kiss1* transcription. AMP-activated protein kinase (AMPK),

an intracellular energy sensor and signaling regulator, mediating *Kiss1* expression in both *Kiss1* neuron and GT1-7 neural cell line had been reported (Wen et al. 2012, Roa et al. 2018); this finding might associate with the results we observed since serum contains plentiful nutrients, growth factors and hormones. Importantly, in addition to CREB, the roles of numerous transcription factors (Yevshin et al. 2019) on regulating *Kiss1* expression either positively or negatively haven't been characterized well. Therefore, studies focused on *Kiss1* transcriptional regulation and its molecular mechanism are needed.

In the past, the experiments for studying steroidogenesis were usually examined in a serum-free condition because serum contains various and undetermined factors that influenced steroidogenic function (Pate & Condon 1982). However, serum deprivation undeniably disrupts cellular homeostasis at least including elevated *Kiss1* and reduced *Kiss1r* expressions in Leydig cells, which may account for the ineffectiveness of kisspeptin treatment in our previous studies (Hsu et al. 2014, Wang et al. 2015). This finding also suggests that researchers should cautiously design experiments and interpret the results when applying the cell line, primary cell and tissue cultures in serum-free media to study steroidogenesis.

In summary, we have shown that the cAMP/PKA/CREB signaling pathway mediates gonadotropin-induced *Kiss1* gene transcription in mouse Leydig cells. Regarding the putatively autocrine action of kisspeptin, our study also highlights the importance of KISS1R signaling in maintaining gonadotropin sensitivity and steroidogenic function through regulating *Lhcgr* and the related gene expressions. This study provides new insights into the pathophysiology of gonadotropin insensitivity in idiopathic hypogonadotropic hypogonadism.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0328>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M-C H and C-H C conceived the study and wrote the manuscript. M-C H performed experiments and analyzed data. L-S W and D-S J reviewed and edited the manuscript.

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