Role of kisspeptin on cell proliferation and steroidogenesis in luteal cells in vitro and in vivo

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Abstract

Background: Kisspeptin (KISS1) and kisspeptin receptor (KISS1R) are essential gatekeepers of the reproductive system. The functions of KISS1 and KISS1R in corpus luteal cells remain ambiguous. The objective was to observe normal physiologic functions of corpus luteal cells in vivo and clarify the functions of KISS1 in vitro.

Methods: We conducted an in vivo observation of cellular patterns as well as the levels of steroidogenic enzymes and KISS1/KISS1R in corpus luteal cells obtained from female crossbred Taiwan native goats in the estrous cycle; the observation was performed using hematoxylin and eosin and immunohistochemistry staining. Subsequently, we used kisspeptin-10 (Kp-10) to stimulate temperature sensitive-caprine luteal cell line (ts-CLC-D) cells to investigate the progesterone (P4) levels, steroidogenic messenger RNA (mRNA)/protein levels, cell survival rate, intracellular Ca^{2+} concentration, and cell proliferation-related mRNA/protein levels in the mitogen-activated protein kinase pathway in vitro by applying immunofluorescence staining, Western blotting, 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide assay, and real-time polymerase chain reaction.

Results: We observed the presence of proteins and mRNAs for *STAR, CYP11A1, HSD3B, KISS1*, and *KISS1R* in the corpus luteal cells from goats in vivo. In vitro, the addition of Kp-10 reduced the P4 levels ($p < 0.01$) and increased cell proliferation ($p < 0.05$) of the ts-CLC-D cells. Furthermore, we found that the levels of proteins and mRNA for *STAR*, *CYP11A1*, and *HSD3B* decreased significantly when Kp-10 was added ($p < 0.05$). However, adding Kp-10 did not affect the mRNA levels for *PLCG2, DAG1, PRKCA, KRAS, RAF1, MAP2K1, MAP2K2, MAPK3, MAPK1,* and *MAPK14*.

Conclusion: We determined that KISS1 could affect the P4 levels, steroidogenesis, and cell proliferation in luteal cells. However, further research is required to clarify how KISS1 regulates proliferation and steroid production in luteal cells.

Keywords: Cell proliferation; Corpus luteum; Kisspeptins; Mitogen-activated protein kinase pathway; Progesterone; Steroidogenesis

1. INTRODUCTION

Reproduction is a crucial aspect of the biology of species. The reproductive physiology of mammals is mainly mediated by the hypothalamic-pituitary-gonadal axis.[1] Gonadotropin Releasing Hormone (GnRH) nerve cells located in the hypothalamus are regulated by peptides secreted by other nerve cells, including RFamide peptides and neuropeptides. Kisspeptin (KISS1) is a member of the RFamide peptide family and is secreted by KISS1 nerve cells. The secreted KISS1 is transmitted to GnRH nerve cells across a synapse. When KISS1 and kisspeptin receptor (KISS1R) are combined, they can synergistically regulate the production and secretion of GnRH.[2] Loss of KISS1 and KISS1R function would prevent mammals from reaching sexual maturity or would render them infertile. Therefore, KISS1 and KISS1R are crucial in the reproductive physiology of mammals.[3]

Studies have indicated that ovaries also express KISS1 and KISS1R genes and proteins.[4–8] KISS1 and KISS1R are expressed in follicular membrane cells (theca cells), large luteal cells, interstitial cells, and ovarian epidermal cells (ovarian surface epithelium) in human, marmoset, and rat ovaries. Levels of *Kiss1* messenger RNA (mRNA) in rat and hamster ovaries peaks before and after ovulation and levels of *Kiss1* mRNA in rat eggs are stimulated by gonadotropin. Furthermore, follicular granulosa cells begin to express KISS1 and KISS1R after luteinization in humans, marmosets, and rats. Accordingly, KISS1 and KISS1R appear to mediate reproductive processes and have certain functions in luteal cells.[9–11]

Follicular cells transform into luteal cells after ovulation and enter a luteal phase. Luteal cells proliferate rapidly during the luteal phase.[12] The steroidogenic acute regulatory

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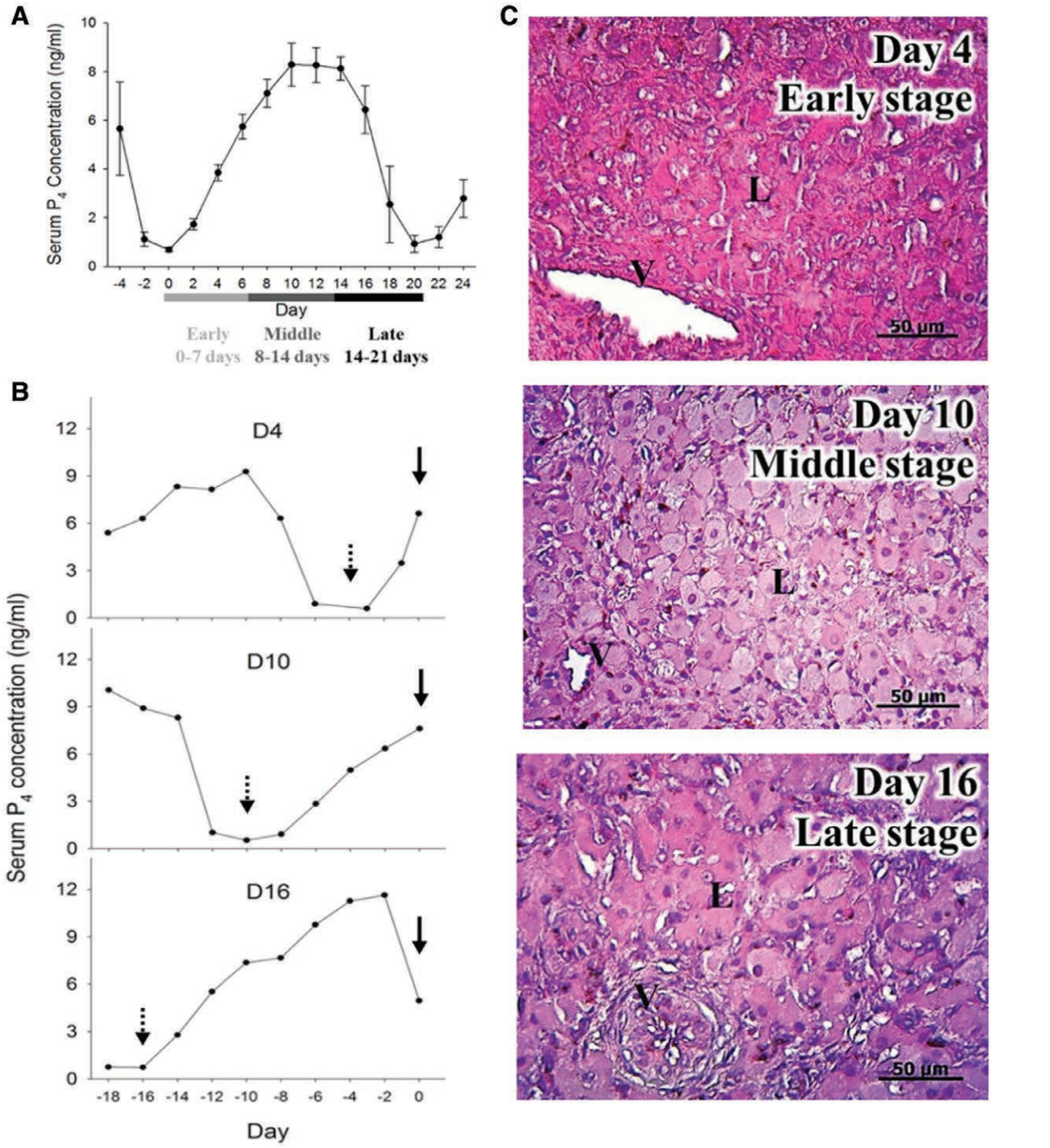

Fig. 1 A, Changes in goat serum progesterone (P4) levels (ng/mL) during the estrous cycle (n ≥ 4). B, Samples were collected during three stages of the estrous cycle. For each goat, the dotted arrow indicates the estrus, and the solid arrow indicates the time of sample collection. C, Photomicrographs with hematoxylin and eosin stain reveal the corpus luteum (CL) morphology collected during three stages of the estrous cycle in vivo. CL cells were obtained during the early (day [D] 4), middle (day 10), and late stages (day 16). L = luteal cell; V = blood vessel.

protein (STAR),[13–16] cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1),[17,18] and 3β-hydroxylated steroid dehydrogenase (HSD3B) that regulate the synthesis of steroid hormones steroids are required for the progesterone (P4) production.[19]

The combination of KISS1 and KISS1R activates the G protein on the cell membrane and activates downstream pathways; for example, such a combination alters intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) and activates the mitogen-activated protein kinase (MAPK) pathway, which in turn alters cell function.[20,21] Luteinization is accompanied by changes in the cell cycle and steroid production and engenders KISS1 and KISS1R expression levels. However, whether KISS1 regulates the functions of luteal cells is unclear.

To resolve this uncertainty, we conducted this study using corpus luteal cells collected from female goats to observe normal

physiologic functions in vivo. We then performed experiments on the temperature sensitive-caprine luteal cell line (ts-CLC-D cell line) in vitro to clarify the effect of KISS1 on luteal cell functions.

2. METHODS

We conducted an in vivo observation of cellular patterns, steroidogenic mRNA/protein levels, and KISS1/KISS1R levels for corpus luteal cells obtained from female crossbred Taiwan native goats in the estrous cycle. Subsequently, we used kisspeptin-10 (Kp-10) to stimulate the ts-CLC-D cells to investigate the P4 levels, steroidogenic proteins, cell survival rates, cell proliferation–related proteins, $[Ca^{2+}]I$, and associated proteins in the MAPK pathway in vitro.

2.1. Animals and tissue preparation

2.1.1. Animals

We purchased female crossbred Taiwanese native goats "(12~18 months old)" from National Taiwan University. The detailed information is available in Appendix 1 (http://links.lww.com/JCMA/A72).

2.1.2. Goat corpus luteum

The experimental animals were divided into three phases according to the development of the corpus luteum in the estrus cycle (preluteal phase on days 1-7, middle luteal phase on days 8-14, and end luteal phase on day 15-21). The female goats were fasted 24 hours before the operation, and drinking water was adequately supplied. During the operation, we used Rompun (0.1–0.2 mL/kg) and Keraset (0.03-0.04 mL/kg) with an intramuscular injection for sedation. During the operation, nitrous oxide (N_2O:O_2 = 1:2) and anesthetic fluothane were used for anesthesia. First, midline laparotomy about 10 cm in length was performed. After entering the peritoneum, the ovary was resected with a scalpel and quickly placed in ice bath PBS. Then we took out the corpus luteum tissues with storage in formalin (10% formaldehyde/PBS).

2.1.3. Cell line

A caprine luteal cell line (ts-CLC-D) has been established in our laboratory and validated for studies on steroidogenesis regulation.[22–24] The detailed information is available in Appendix 1 (http://links.lww.com/JCMA/A72).

2.2. RNA extraction and quantitative real-time polymerase chain reaction

We extracted total RNA from the cultured ts-CLC-D cells by using the TRIsure reagent (BIO-38032; Bioline, Cincinnati, Ohio, USA) following the manufacturer's instruction. After RNA extraction, the PrimeScript RT reagent kit (Takara Bio, Mountain View, California, USA) was used for first-strand complementary DNA synthesis. To quantify mRNA levels, real-time polymerase chain reaction (PCR) was performed in a QuantStudio 3 system (Applied Biosystems, Waltham, Massachusetts, USA) using the Fast SYBR Green Master Mix (Applied Biosystems) with specific primer pairs, as listed in Appendix Table 1 (http://links.lww.com/JCMA/A72).

2.3. Immunoblot analysis

The detailed information is available in Appendix 1 (http://links.lww.com/JCMA/A72).

2.4. Immunohistochemistry staining

The detailed information is available in Appendix 1 (http://links.lww.com/JCMA/A72).

2.5. Immunofluorescence staining

The detailed information is available in Appendix 1 (http://links.lww.com/JCMA/A72).

2.6. P4 assay

Antibodies for the enzyme immunoassay of the P4 have been developed in our laboratory.[22,25] All samples and standards used in this study were coincubated with horseradish peroxidase–conjugated P4 in antibody-coated 96-well plates. Signal development was achieved through the addition of 2.2 mM o-phenylenediamine as the horseradish peroxidase substrate. The reaction was stopped by the addition of 50 µL of sulfuric acid. The absorbency of the samples, read using a dual-wavelength reader (Dynatech, Goyang-si, Gyeonggi-do, Republic of Korea) (490/630 nm), was compared with the absorbency derived from the P4 standard curve.

2.7. XTT assay

For the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay, ts-CLC-D cells with or without Kp-10 treatment were cultured in 96-well culture plates. The cells were washed and then incubated with 1.5 µM XTT, 33 nM phenazine methosulfate, and M199 powder at 40°C for 4 hours. The optical density of each sample was assessed at 450 nm using a spectrophotometer. Data are present as multiples of the control.

2.8. Intracellular free Ca^{2+} measurement

Changes in intracellular free $[Ca^{2+}]i$ were measured with Fluo-4 (Invitrogen, Carlsbad, California, USA) in 96-well black plates using a Synergy H1 Hybrid Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT, USA) at 37°C. (The detailed information in Appendix 1 (http://links.lww.com/JCMA/A72).

2.9. Statistical analysis

Each group of tests was repeated at least three times to increase the reliability of the results. Results were presented as mean ± SE. Data were analyzed using the Student's *t* test and one-way analysis of variance with Tukey's post hoc test. Two-sided $p < 0.05$ were considered to indicate a statistically significant difference. All data were analyzed using SigmaPlot 13 (v13.0.0.83; Systat Software, Inc, San Jose, California, USA).

3. RESULTS

3.1. Histological changes of corpus luteum in the estrous cycle in vivo

As the estrous cycle progressed, the serum P4 levels increased from the basal value to a peak value of approximately 8 ng/mL on days 9 and 10 and then decreased after day 15, continuing to decrease (<1 ng/mL) to the basal value (Fig. 1A). We obtained corpora lutea from the ovaries of the female goats on day 4 (early period), day 10 (midperiod), and day 16 (late period) of the estrous cycle (Fig. 1B). Each corpus luteum was sectioned and stained with hematoxylin and eosin (Fig. 1C). The results revealed that in the preluteal phase, cells were less tightly connected compared with those in other phases and that the volume of the corpus luteum was smaller with a larger blood vessel distribution than those of other cells. In the middle phase, the intercellular connections were tighter with less cell space than those in the other phases, and the volume of the corpus luteum was larger than those of other cells; moreover, the blood vessels in the tissues were relatively small. Finally, in the late phase, the intratissue structure contained numerous vascular endothelial cells, and the cell gaps were relatively empty; the proportion of luteal cells was considerably reduced.

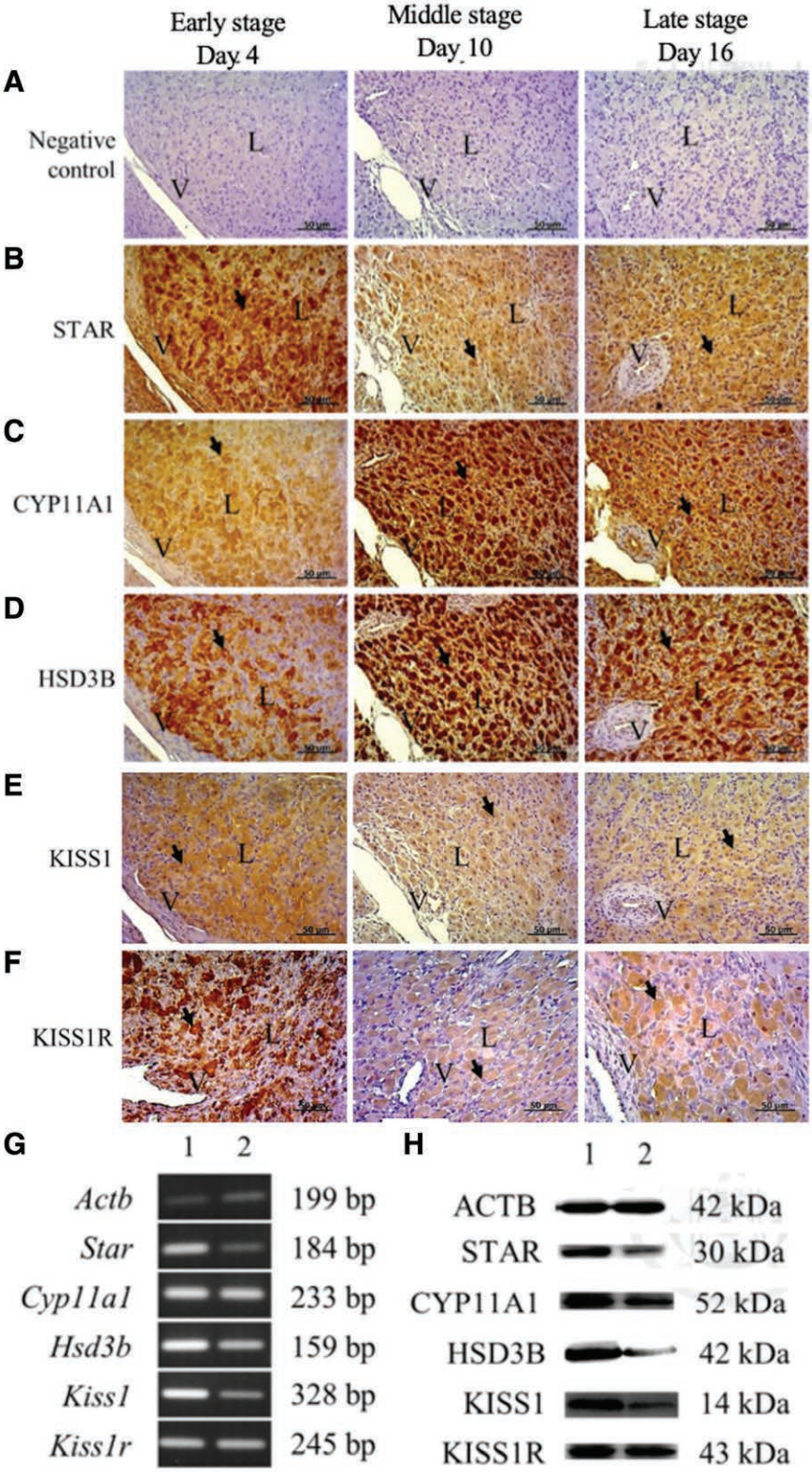

Fig. 2 Immunohistochemistry staining of caprine corpus luteum (CL) in early (day 4), middle (day 10), and late (day 16) stages of the estrous cycle in vivo. Negative control sections (A) were stained without the primary antibody. Markers of (B) steroidogenic acute regulatory protein (STAR), (C) cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1), (D) 3β-hydroxylated steroid dehydrogenase (HSD3B), (E) kisspeptin (KISS1), and (F) KISS1R were stained in brown (arrows). Representative (G) real-time polymerase chain reaction and (H) Western blotting for the gene expression and protein levels for STAR, CYP11A1, HSD3B, KISS1, and KISS1R in (1) the caprine CL cells collected on day 12 of the estrous cycle in vivo and (2) ts-CLC-D cells in vitro. The sequences of all primers are listed in Appendix Table 1 (http://links.lww.com/JCMA/A72). Actb = beta-actin; KISS1R = kisspeptin receptor; L = luteal cells; ts-CLC-D = indicates temperature sensitive-caprine luteal cell line; V = blood vessel.

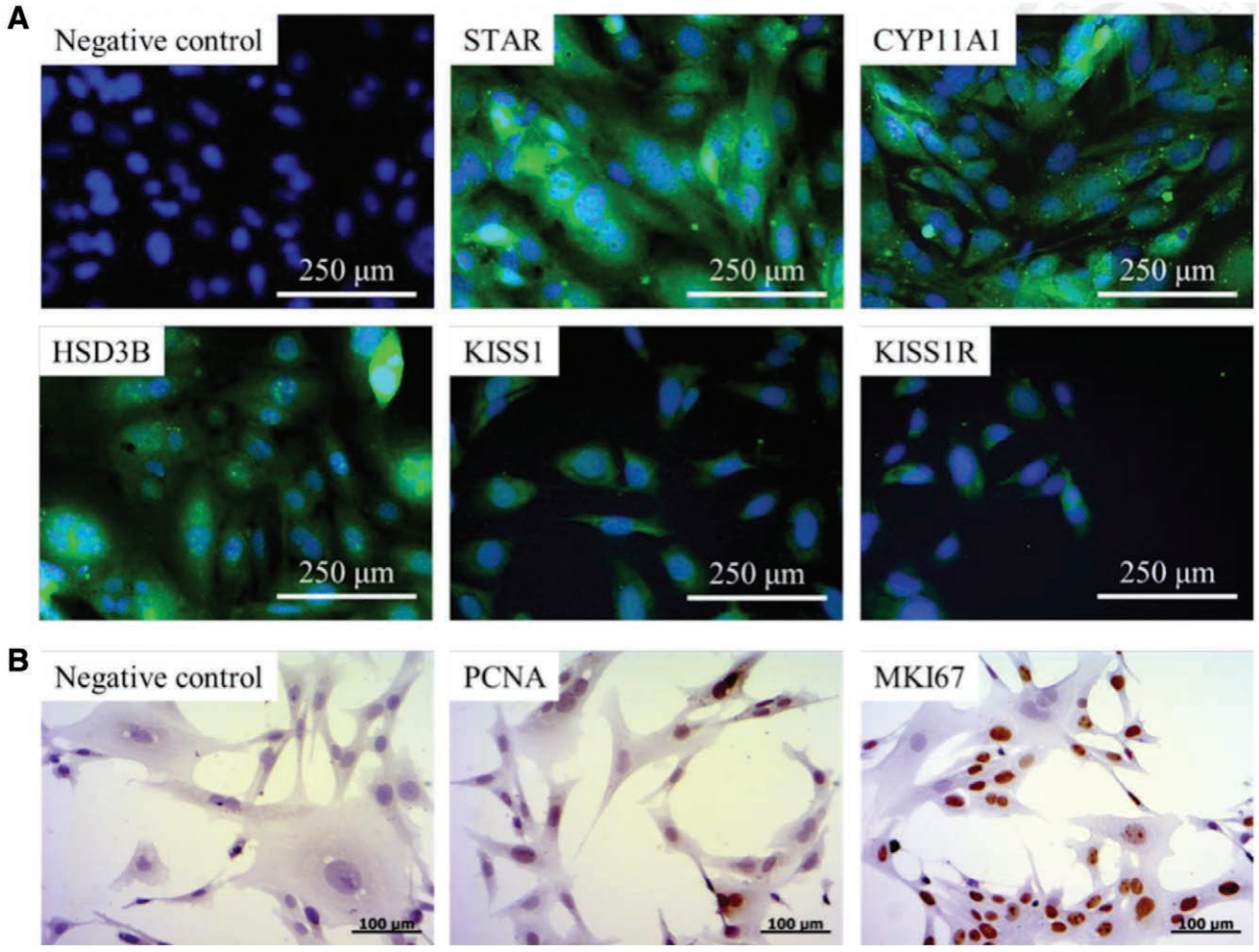

Fig. 3 Localizations of steroidogenic acute regulatory protein (STAR), cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1), 3β-hydroxylated steroid dehydrogenase (HSD3B), kisspeptin (KISS1), kisspeptin receptor (KISS1R), proliferating cell nuclear antigen (PCNA), and marker of proliferation Ki-67 (MKI67) in temperature sensitive-caprine luteal cell line (ts-CLC-D cells) were determined through (A) immunofluorescence and (B) immunocytochemistry staining. A, In the immunofluorescence staining results, green indicates the protein and blue indicates the nucleus. STAR, CYP11A1, HSD3B, KISS1, and KISS1R were stained with Dylight 488–conjugated secondary antibodies (Thermo Scientific; SA5-10038) (green). The cell nucleus was stained with Hoechst 33342 (blue). B, In the immunocytochemical staining results, brown indicates the target proteins. The negative control section was incubated without primary antibodies.

3.2. KISS1 and KISS1R levels in corpora lutea in the estrous cycle in vitro and in vivo

We used hematoxylin and eosin stain for negative control (Fig. 2A) immunohistochemistry staining to detect the "presence" of KISS1, KISS1R, and steroidogenic proteins (STAR, CYP11A1, and HSD3B) involved in the P4 production in the early, middle, and late stages of the estrous cycle in vivo. We observed that KISS1, KISS1R, STAR, CYP11A1, and HSD3B were expressed in steroid-producing corpus luteal cells. The steroidogenic proteins were not present in the vascular endothelial cells or connective tissues (Fig. 2B–D). KISS1 and KISS1R were expressed in the cytoplasm of corpus luteal cells with steroid production ability, but they were not observed in vascular endothelial cells or connective tissues (Fig. 2E, F). Moreover, we tested corpus luteal tissues from caprine in vivo on day 12 of the estrous cycle (number 1) and from ts-CLC-D cells in vitro (number 2) by using real-time PCR and Western blotting on day 12 to investigate the presence of STAR, CYP11A1, HSD3B, KISS1, and KISS1R. We determined that STAR, CYP11A1, HSD3B, KISS1, and KISS1R were exhibited at the gene expression and protein levels (Fig. 2G, H).

3.3. Protein expressions of STAR, CYP11A1, HSD3B, KISS1, KISS1R, PCNA, and MKI67 in ts-CLC-D cells in vitro

We have established a ts-CLC-D cell line in our laboratory.[24] We used fluorescence immunostaining and immunocytochemical staining to clarify the functions of steroidogenic proteins, KISS1, KISS1R, and cell proliferation–related proteins. We found STAR, CYP11A1, HSD3B, KISS1, and KISS1R presence in the cytoplasm but observed proliferating cell nuclear antigen (PCNA) and marker of proliferation Ki-67 (MKI67) presence in the nucleus (Fig. 3).

3.4. Effects of Kp-10 on the P4 levels, survival rate, gene expression, and protein levels in ts-CLC-D cells in vitro

We treated ts-CLC-D cells with different concentrations of Kp-10 (0.5, 1, 5, 10, and 50 μM) to observe the P4 levels, survival rate, and cell proliferation. The results indicated that adding 5, 10, and 50 μM Kp-10 to the cell medium and incubating for 24 hours significantly reduced the P4 levels ($p < 0.01$; Fig. 4A) and significantly increased the survival rate ($p < 0.05$; Fig. 4B) in ts-CLC-D cells. We used real-time PCR to analyze

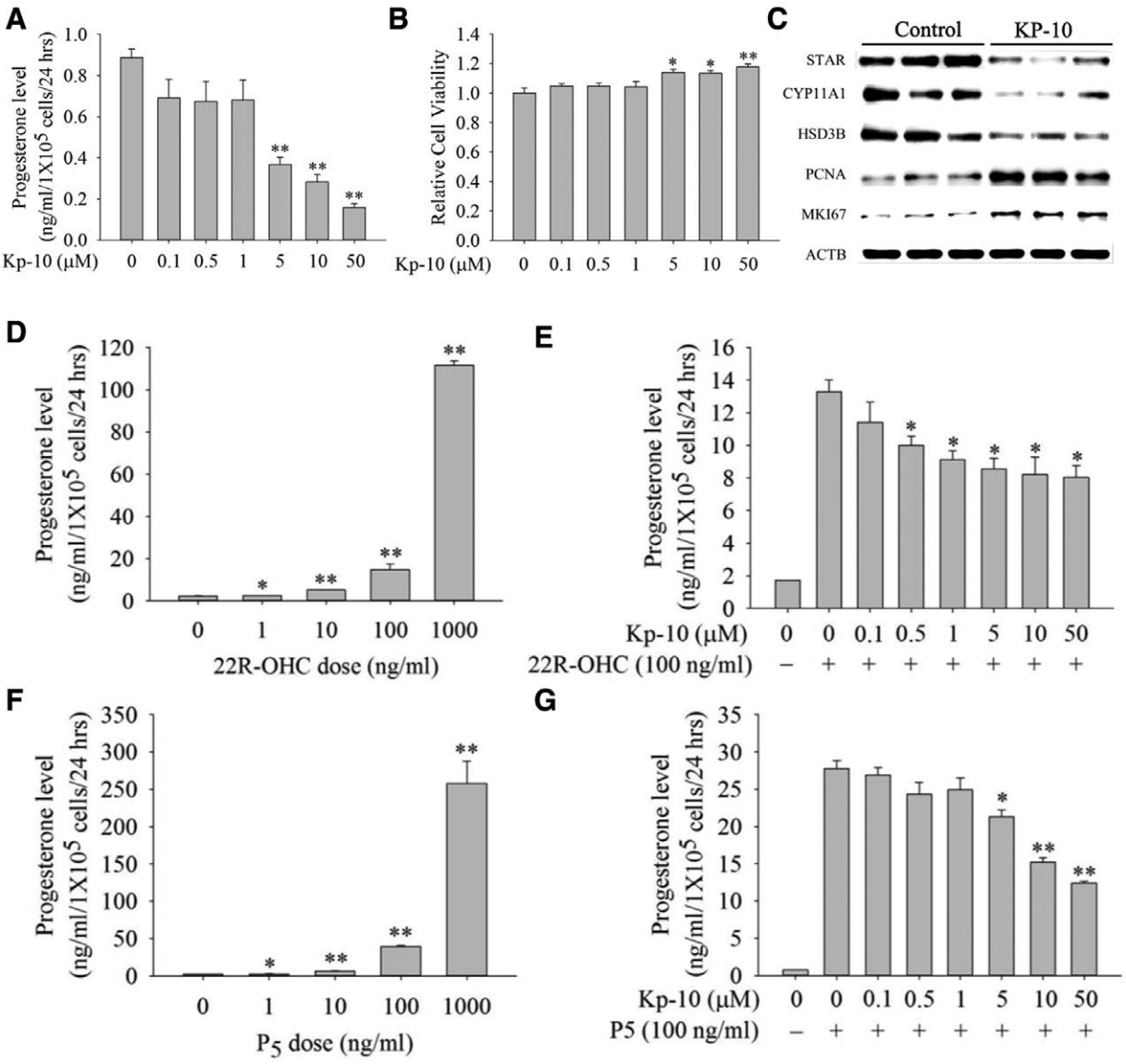

Fig. 4 A, The levels of progesterone (P4) were significantly reduced by the addition of different doses of kisspeptin-10 (Kp-10) to temperature sensitive-caprine luteal cell line (ts-CLC-D) cells. B, ts-CLC-D cells were treated with Kp-10 for 24 h, and cell viability was assessed through an XTT assay. The values for each bar represent mean ± SE (n ≥ 3). * $p < 0.05$, ** $p < 0.01$ vs 0 μM Kp-10. C, Adding 10 μM Kp-10 to ts-CLC-D cells suppressed the levels of steroidogenic proteins (steroidogenic acute regulatory protein [STAR], cytochrome P450 cholesterol side-chain cleavage enzyme [CYP11A1], 3β-hydroxylated steroid dehydrogenase [HSD3B]) and increased the levels of cell proliferation marker proteins (PCNA, MKI67), as determined in Western blotting. D and F, The P4 levels increased significantly under relatively high doses of 22R-hydroxycholesterol (22R-OHC) or pregnenolone (P5). * $p < 0.05$, ** $p < 0.01$ vs 0 ng/mL 22R-OHC or P5. E and G, The P4 levels decreased significantly under cotreatment with Kp-10 and either 22R-OHC (100 ng/mL) or P5 (100 ng/mL) in ts-CLC-D cells. * $p < 0.05$, ** $p < 0.01$ vs 0 μM Kp-10 with 100 ng/mL 22R-OHC or P5. Values for each bar represent mean ± SE (n ≥ 3). ACTB = beta-actin; MPKI67 = marker of proliferation Ki-67; PCNA = proliferating cell nuclear antigen.

gene expression and applied Western blotting to detect protein levels in ts-CLC-D cells cultured with 10 μM Kp-10 for 24 hours. The results revealed that the levels of mRNA for *STAR*, *CYP11A1*, and *HSD3B* (Fig. 5A–C) and protein (Figs. 4B and 5F–H) levels of STAR, CYP11A1, and HSD3B decreased significantly when Kp-10 was added ($p < 0.05$). We observed no significant changes in the levels of mRNA for *PCNA* and *MKI67* when Kp-10 was added (Fig. 5D, E). The protein levels of PCNA and MKI67 increased significantly when cells were treated with 10 μM Kp-10 ($p < 0.05$; Figs. 4C and 5I, J).

We added 22R-hydroxycholesterol (22R-OHC) or pregnenolone (P5) to the ts-CLC-D cells to provide the matrix required for the P4 production to indirectly detect the activity of the P4-related enzymes. We found that the P4 levels were positively correlated with the addition of 22R-OHC or P5 addition doses ($p < 0.05$; Fig. 4D, F). To facilitate P4 detection, we used 100 ng/mL 22R-OHC or P5 in the subsequent experiments. The results demonstrated that adding 22R-OHC and cotreating the ts-CLC-D cells with Kp-10 (0.5, 1, 5, 10, and 50 μM) for 24 hours significantly reduced the P4 levels ($p < 0.05$; Fig. 4E). Furthermore, cotreating the ts-CLC-D cells with Kp-10 (5, 10, and 50 μM) and P5 for 24 hours reduced the P4 levels ($p < 0.01$; Fig. 4G).

3.5. Effects of Kp-10 in ts-CLC-D cells on the intracellular calcium concentration and mRNA levels in the MAPK pathway

We treated ts-CLC-D cells with 10 μM Kp-10 to observe the effect of KISS1 on $[Ca^{2+}]i$ in the cytoplasm and levels of

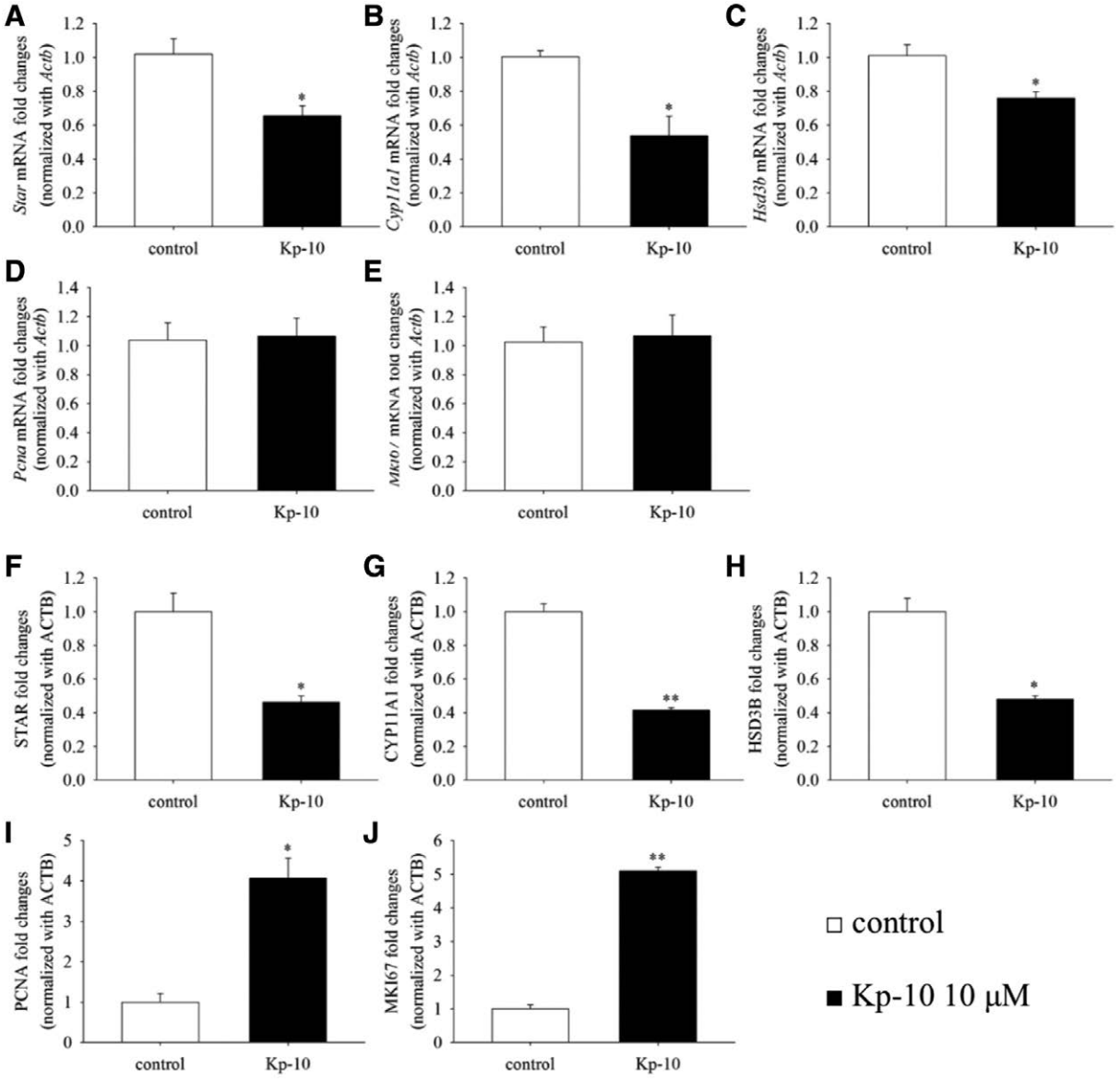

Fig. 5 Real-time polymerase chain reaction analyses revealed the suppressive effect of kisspeptin-10 (Kp-10) (10 μM) on the levels of messenger RNA (mRNA) for *STAR, CYP11A1,* and *HSD3B* (A–C) but indicated no significant changes in the levels of mRNA for *PCNA* and *MKI67* (cell proliferation markers) (D and E). The sequences of all primers are listed in Appendix Table 1 (http://links.lww.com/JCMA/A72). Values each bar indicate mean ± SE (n ≥ 3), * $p < 0.05$. Western blotting revealed that adding Kp-10 (10 μM) reduced steroidogenic acute regulatory protein (STAR), cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A1), and 3β-hydroxylated steroid dehydrogenase (HSD3B) protein levels (F–H) and increased PCNA and MKI67 protein levels (I and J). Values for each bar indicate mean ± SE (n ≥ 6), * $p < 0.05$ and ** $p < 0.01$. ACTB = beta-actin; MPKI67 = marker of proliferation Ki-67; PCNA = proliferating cell nuclear antigen.

mRNAs in the MAPK pathway. Initially, we used calcium-free media (Fig. 6A) to observe if Kp-10 would alter changes in $[Ca^{2+}]i$. Then, we used culture media contained 2 mM Ca^{2+} (Fig. 6B) to prove the hypothesis. The results showed that Kp-10 engendered changes in $[Ca^{2+}]i$ in the cytoplasm regardless of whether the cell culture medium contained Ca^{2+} (Fig. 6A, B). However, when the culture medium contained 2 mM Ca^{2+} (the normal culture medium), KISS1 induced "a more marked" increase in $[Ca^{2+}]i$ fluctuations in the cytoplasm (Fig. 6B). Besides, quantitative real-time PCR analysis did not reveal significant changes in levels of mRNA for *PLCG2, DAG1, PRKCA, KRAS, RAF1, MAP2K1, MAP2K2, MAPK3, MAPK1,* and *MAPK14* in the MAPK pathway after treatment with 10 μM Kp-10 (Fig. 6C).

4. DISCUSSION

After follicular ovulation, follicular theca cells luteinize into small luteal cells, and follicular granulosa cells luteinize into large luteal cells, thus forming a corpus luteum.[26] P4, which is transported to target tissues, is synthesized from cholesterol by both large and small luteal cells upon luteal maturation.[27] The P4 level of luteal cells reaches a peak in the middle estrous cycle.[28] Moreover, the number of corpus luteal cells decreases markedly in the late stage, as does the P4 level.[29] Large and small luteal cells could be distinguished through staining.[30] Therefore, we obtained large and small luteal cells from goats and distinguished them through staining; the large luteal cells were lightly stained with a low nuclear–cytoplasmic ratio, whereas the small luteal cells were more deeply stained with a high nuclear–cytoplasmic

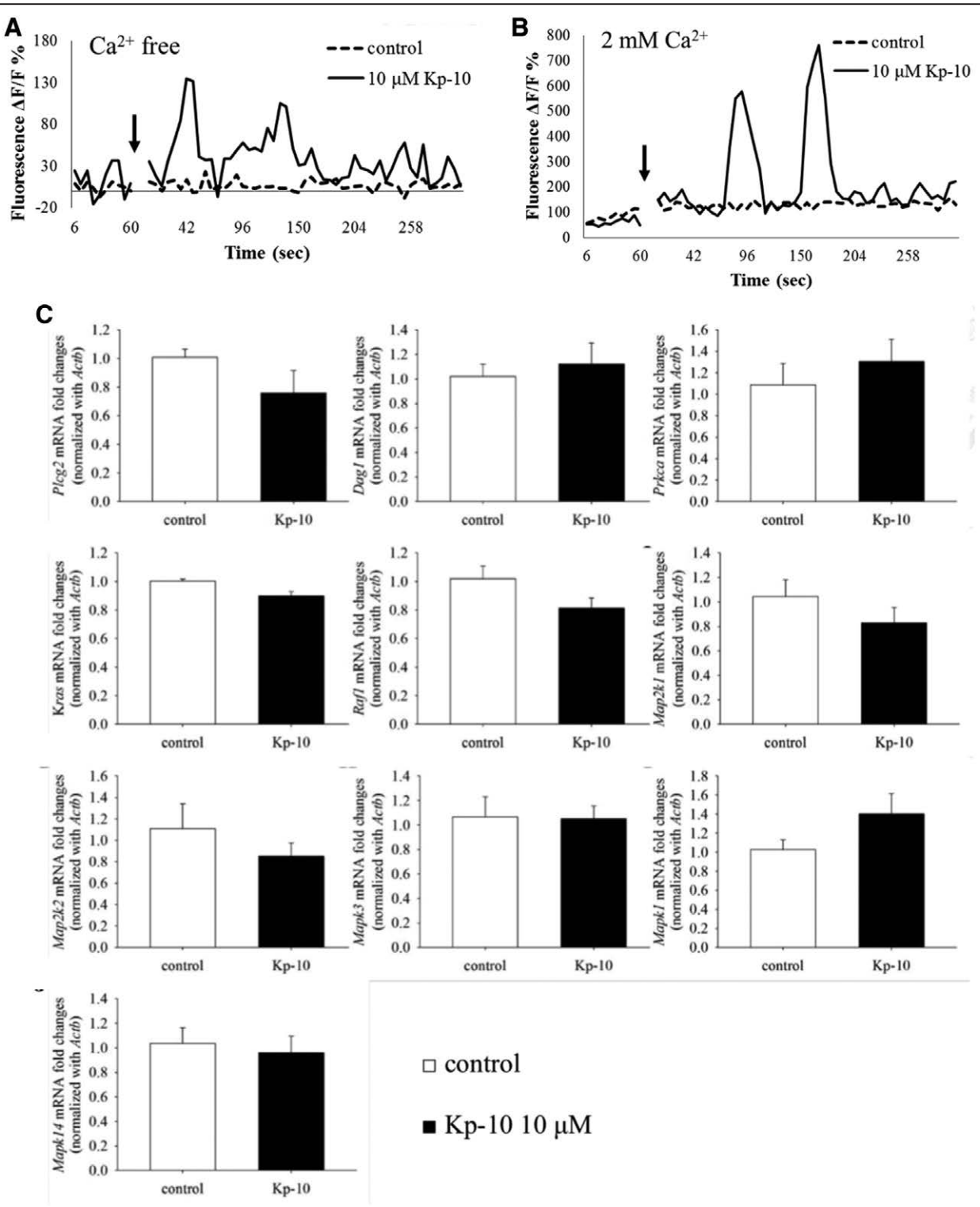

Fig. 6 A and B, Changes in [Ca^{2+}]i in the cytoplasm of temperature sensitive-caprine luteal cell line (ts-CLC-D) cells treated with 10 μM kisspeptin-10 (Kp-10). The buffer contained 5 mM KCl, 120 mM NaCl, 20 mM HEPES, pH 7.4, and penicillin/streptomycin, in addition to containing or not containing 2 mM Ca^{2+}. After a 60-s baseline was collected, Kp-10 (10 μM) and the vehicle were added (arrow). The arrow above the diagram indicated that we collected a 60-s baseline data before the addition of Kp-10 (10 μM) and the vehicle. That is, we recorded the changes in [Ca2+]i in the cytoplasm before and after ts-CLC-D cells were treated with 10 μM Kp-10. C, Effects of Kp-10 (10 μM) on the levels of messenger RNA (mRNA) for *PLC/DAG/PKC/RAS/RAF/MAPK* pathway in ts-CLC-D cells were analyzed through real-time PCR. Values for each bar indicate mean ± SE (n ≥ 3). The sequences of all primers are listed in Appendix Table 1 (http://links.lww.com/JCMA/A72).

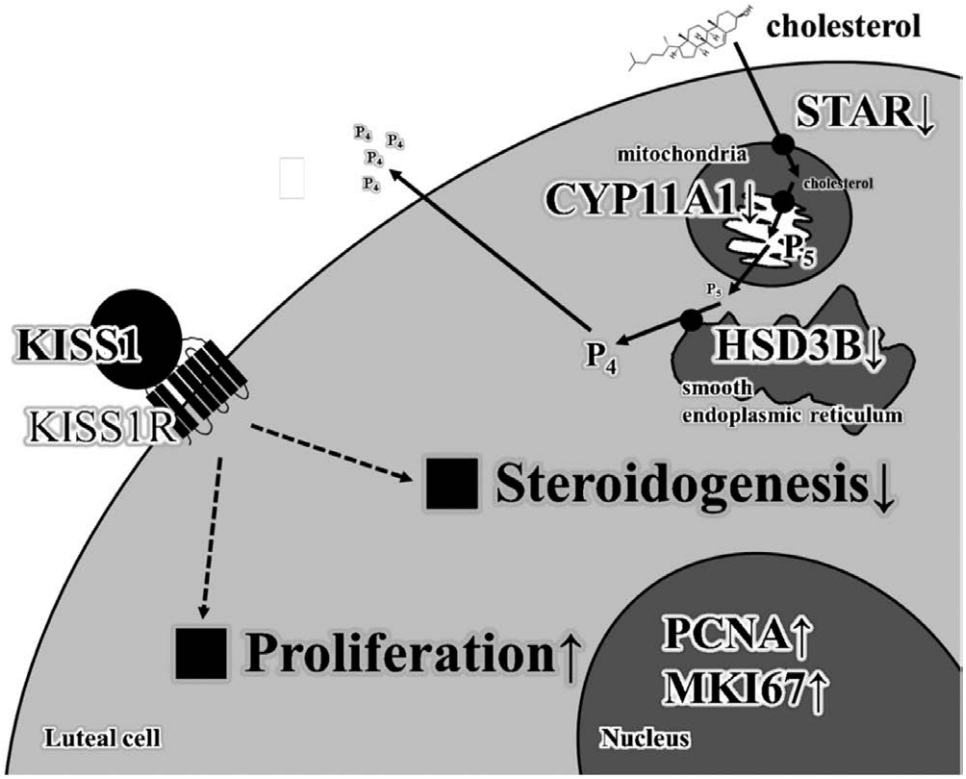

Fig. 7 Schematic of signaling pathways recruited following KISS1R activation by kisspeptin (KISS1) in luteal cells. P4 = progesterone; CYP11A1 = cytochrome P450 cholesterol side-chain cleavage enzyme; HDS3B = 3β-hydroxylated steroid dehydrogenase; KISS1R = kisspeptin receptor; MPKI67 = marker of proliferation Ki-67; PCNA = proliferating cell nuclear antigen; STAR = steroidogenic acute regulatory protein.

ratio. Our results showed the relationship between luteal histology and the P4 secretion in the early, middle, and late estrous cycle was consistent with previous studies.[28,29]

The production of a corpus luteum and P4 differed markedly in the early, middle, and late stages of the estrous cycle. The P4 production required steroidogenic proteins. We determined that steroidogenic proteins were present only in corpus luteal tissues from the goats; they were not exhibited in vascular endothelial cells or connective tissues. We infer that *STAR* and *CYP11A1* transcription might be suppressed and regulated in the early stage of corpus luteum development, thus reducing protein levels.[31]

Previous studies have reported that in human, marmoset, and rat ovaries, theca cells did not express KISS1 and KISS1R, but granulosa cells began to express KISS1 and KISS1R after luteinization.[9–11] We also identified KISS1, KISS1R, and steroidogenic proteins in corpus luteal cells. However, additional samples are required to clarify the differences in protein levels between small and large luteal cells in the estrous cycle. Previous studies have demonstrated that KISS1 and KISS1R levels increased substantially after the luteinization of granulosa cells.[9–11] Nevertheless, the mechanisms underlying this increase remain ambiguous. Although we identified mRNA and protein levels for STAR, CYP11A1, HSD3B, KISS1, and KISS1R in the middle stage of the estrous cycle, we could not clarify the difference in levels between KISS1/KISS1R and steroidogenic proteins because semiquantitative analyses could not be performed with small sample sizes.

We used a ts-CLC-D cell line instead of primary luteal cells of goats in our in vitro experiments because both cell types could produce the P4 normally and exhibit mRNA and proteins of STAR, CYP11A1, HSD3B, KISS1, and KISS1R, as validated in the present and previous studies.[23,24]

In this study, we used Kp-10 in place of KISS1 as a reagent because it has a similar biological activity to KISS1.[20] We discovered that Kp-10 inhibited the P4 levels in the ts-CLC-D cells. This phenomenon occurred in the presence of a 22R-OHC and P5 matrix. We performed real-time PCR and Western blotting to understand the mechanism through which Kp-10 inhibits the P4 levels. We determined that Kp-10 concentrations above 10 μM suppressed the mRNA and protein levels of STAR, CYP11A1, and HSD3B. Furthermore, the addition of the matrix (22R-OHC or P5) could indirectly reflect the enzyme activities of CYP11A1 and HSD3B. However, a previous study revealed that adding 80 nM Kp-10 increased the levels of P4 and mRNA for *Star* and *Cyp11a1* in the primary luteal cells of rats,[32] which is inconsistent with our findings. This inconsistency may be attributed to differences in the species, Kp-10 dosages, or cell culture methods between the two studies. Therefore, additional experiments are required to confirm the effects of Kp-10 on luteal cells.

To clarify whether Kp-10 could reduce the P4 levels and survival rate of ts-CLC-D cells, we assessed the cell proliferation and survival rate of the ts-CLC-D cells in the addition of Kp-10. Our results indicate that Kp-10 increased the survival rate of the ts-CLC-D cells. We infer that Kp-10 may have suppressed the P4 levels of the ts-CLC-D cells by inhibiting P4 production rather than promoting cell death. Moreover, we conducted an XTT assay, in which mitochondrial "dehydrogenase" activity was applied to reverse the cell survival rate and indicates whether mitochondrial functions are damaged in cells.[33] We found that Kp-10 increased the protein levels of PCNA and MKI67. Hence, we speculate that KISS1 may increase cell proliferation by increasing the transcription and translation of cell proliferation–related proteins.

A combination of KISS1 and KISS1R could activate the membranous G protein and activate downstream signaling pathways; for example, it could engender changes in [Ca^{2+}]

i and the MAPK pathway, thus altering cell functions.[20] We analyzed whether Kp-10-induced changes in [Ca^{2+}]i or the MAPK pathway would engender a reduction in the P4 levels and an increase in ts-CLC-D cell survival. Our results show that changes in [Ca^{2+}]i occurred regardless of whether extracellular Ca^{2+} was present. However, the presence of extracellular Ca^{2+} substantially increased changes in intracellular calcium concentrations ([Ca^{2+}]i). Our results demonstrate that changes in [Ca^{2+}]i might have resulted from the release of Ca^{2+} in the endoplasmic reticulum. Furthermore, extracellular Ca^{2+} could enhance changes in [Ca^{2+}]i through the entry of extracellular Ca^{2+}. We further assessed whether Kp-10 would regulate the mRNA levels in the MAPK pathway. The results indicate that Kp-10 did not affect the mRNA levels for *PLCG2, DAG1, PRKCA, KRAS, RAF1, MAP2K1, MAP2K2, MAPK3, MAPK1,* and *MAPK14*. Therefore, our results demonstrate that mRNAs levels in the MAPK pathway were not affected by Kp-10. Previous research reported that adding Kp-10 and human chorionic gonadotropin (hCG) increased extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation in luteal cells.[32] They investigated the effects that the luteal cells of rats treated with kisspeptin with or without hCG and found that the luteal cells cotreated with kisspeptin and hCG would increase ERK1/2 phosphorylation compared with the group treated with hCG alone. Furthermore, treatment with kisspeptin alone did not affect P38 MAPK and protein kinase B, PKB phosphorylation under the same experimental conditions. Our results indicate that the levels of mRNA for *Erk1/2* were not affected by treatment with kisspeptin alone, which is consistent with the result of the previous research.

In conclusion, we found that the P4 levels decreased in ts-CLC-D cells in the presence of KISS1. However, we determined that adding Kp-10 increased the survival rate of the ts-CLC-D cells. Although there were no significant changes in the levels of mRNA for *PLCG2, DAG1, PRKCA, KRAS, RAF1, MAP2K1, MAP2K2, MAPK3, MAPK1,* and *MAPK14*, adding Kp-10 would alter [Ca^{2+}]i, regardless of whether extracellular Ca^{2+} was present. In our study, we discovered that KISS1 "could" affect the P4 levels, steroidogenesis, and cell proliferation in luteal cells (Fig. 7). However, further research is required to clarify "how" KISS1 regulates cell proliferation and steroid production in luteal cells.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://doi.org/10.1097/JCMA.0000000000000264.

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