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Expression of KiSS-1 in Rat Ovary: Putative Local Regulator of Ovulation?

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Kisspeptins, the products of KiSS-1 gene, and their receptor, GPR54, have recently emerged as essential gatekeepers of reproduction, mainly through regulation of GnRH secretion at the hypothalamus. However, the profound hypogonadotropism linked to GPR54 inactivation is likely to mask additional functions of this system at other levels of the gonadal axis, in which expression of KiSS-1 and GPR54 has been preliminarily reported. We describe herein the expression of KiSS-1 gene and kisspeptin immunoreactivity (IR) in rat ovary and evaluate its developmental and hormonal regulation. KiSS-1 and GPR54 mRNAs were persistently detected in adult ovary along estrous cycle. Yet, contrary to GPR54, ovarian KiSS-1 levels fluctuated in a cyclic-dependent manner, with a robust increase in the afternoon of proestrus, *i.e.* preceding ovulation. In addition, kisspeptin-IR was observed in rat ovary, with strong signals in theca layers of growing follicles, corpora lutea, and interstitial gland, compartments in which modest GPR54-IR was also detected. Interestingly, the rise in ovarian KiSS-1 mRNA at proestrus was prevented by blockade of preovulatory gonadotropin surge and restored by replacement with human chorionic gonadotropin as superagonist of LH. In addition, immature ovaries showed low to negligible levels of KiSS-1 mRNA, which were significantly enhanced by gonadotropin priming. In summary, we present novel evidence for the developmental and hormonally regulated expression of the KiSS-1 gene, and the presence of kisspeptin-IR, in rat ovary. The ability of the LH surge to timely induce ovarian expression of KiSS-1 at the preovulatory period strongly suggests a previously unsuspected role of locally produced kisspeptin in the control of ovulation. (*Endocrinology* 147: 4852–4862, 2006)

KISSPEPTINS WERE originally identified as the products of the metastasis suppressor gene KiSS-1 (1–3). These are structurally related peptides (that include metastin and kisspeptin-10) that share their C-terminal region containing an Arg-Phe-NH_2 motif, distinctive of the RF-amide peptide family. Kisspeptins are ligands of the previously orphan G protein-coupled receptor GPR54, which was initially identified in the rat on the basis of its partial sequence similarity to galanin receptors (4). The first biological function assigned to kisspeptins was metastasis suppression because metastin was demonstrated to display a potent antimetastasis activity in several tumors, such as breast carcinoma and melanoma (1, 5–7), and loss of KiSS-1 gene expression was linked to tumor progression and metastasis in different malignancies (8, 9). Thereafter additional biological roles, such as regulation of trophoblast invasion (10), have been suggested for kisspeptin. However, our knowledge of KiSS-1 function was recently revolutionized by the seminal observations of de Roux *et al.* (11) and Seminara *et al.* (12), who independently reported that deletions and inactivating mutations in the gene encoding GPR54 are associated with hypogonadotropic hypogonadism in humans and mice. Phenotypic analyses of these models, as well as subsequent physiological and pharmacological studies, have set the contention that the KiSS-1/GPR54 system is primarily acting at the hypothalamus, in which kisspeptins operate as essential gatekeepers of GnRH function (13, 14). Nonetheless, although central KiSS-1 has been proven as an indispensable element in the cascade of signals controlling reproduction, additional potential actions of kisspeptins at other levels of the gonadal axis remain ill defined.

The ovary is a complex endocrine organ responsible for oocyte production and hormonogenesis (15). The cellular structures supporting those functions are arranged into several tissue compartments including ovarian follicles at different stages of development, corpora lutea, ovarian stroma, and hilus cells (15, 16). The hallmark of ovarian function is the cyclic release of fertilizable oocytes at ovulation. This complex process is the end point of follicular growth, development, and selection, and it is triggered by the preovulatory LH surge, which initiates the sequential activation of a series of events including maturation of the oocyte, expansion of the cumulus, rupture of the follicle wall, and final release of the cumulus-oocyte complex to the periovarian space.

Rupture of the follicular apex at ovulation requires active tissue remodeling and intensive proteolytic degradation of the extracellular matrix in several tissue compartments of the ovary, such as granulosa and theca layers, tunica albuginea,

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Abbreviations: GnRH-ANT, Antagonist of GnRH; GPR54, G protein-coupled receptor 54; hCG, human chorionic gonadotropin; IR, immunoreactivity; PMS-G, pregnant mare serum gonadotropin; MMP-9, matrix metalloproteinase-9.

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and ovarian surface epithelium. In this phenomenon, several classes of proteolytic enzymes, such as the plasminogen activator-plasmin and matrix metalloproteinase (MMP) systems, as well as the progesterone-induced A disintegrin and metalloproteinase with thrombospondin-like repeats-1 (ADAMTS) and cathepsin L proteases, have been involved (17, 18). Of note, ovarian proteolytic activity is highly timely and spatially regulated to selectively target follicular breakdown at the apex during the periovulatory period and prevent uncontrolled proteolysis of the remaining ovarian tissue. Thus, protease inhibitors such as plasminogen activator inhibitors and tissue inhibitors of MMPs are also concomitantly expressed in the ovary during ovulation (19, 20). Yet the mechanism and signals ultimately responsible for time and spatial targeting of follicular rupture at the apex remain unknown. Similarly, the cellular sources for the proteolytic and antiproteolytic factors operating at the ovary are yet to be fully unraveled.

Among peripheral tissues, preliminary evidence recently suggested that KiSS-1 and GPR54 genes are prominently expressed in rat ovary (21), yet, their pattern of expression and potential roles at this site have not been explored. Notably, the mechanism whereby kisspeptin prevents tumor metastasis and, eventually, uncontrolled trophoblast invasion likely involves inhibition of proteases, such as MMP-9 (22), which has been implicated in tissue remodeling and follicular rupture (23). This raises the intriguing possibility that, in addition to its essential role in the hypothalamic control of preovulatory LH surge (24), locally produced kisspeptin might directly contribute to the regulation of ovulation. To provide a basis for such a hypothesis, we report herein the first detailed characterization of the expression of the KiSS-1 gene and kisspeptin immunoreactivity in the rat ovary, and provide evidence for its developmental and hormonally regulated pattern of expression.

Materials and Methods

Animals and drugs

Wistar female rats bred in the vivarium of the University of Córdoba were used. The day the litters were born was considered as d 1 of age. The animals were maintained under constant conditions of light (14 h of light, from 0700 h) and temperature (22 C) and were weaned at d 21 of age in groups of four rats per cage with free access to pelleted food and tap water. Experimental procedures were approved by the Córdoba University Ethical Committee for animal experimentation and were conducted in accordance with the European Union normative for care and use of experimental animals. Human chorionic gonadotropin (hCG) and pregnant mare serum gonadotropin (PMS-G) were purchased from Sigma (St. Louis, MO). The potent antagonist of GnRH (GnRH-ANT) Org 30276 (Ac-D-pClPhe-D-pClPhe-D-Trp-Ser-Tyr-D-Arg-Leu-Arg-Pro-D-Ala-NH_2CH_3COOH) was generously supplied by Organon (Oss, The Netherlands).

Experimental designs

In experiment 1, expression of the genes encoding kisspeptin and their putative receptor, GPR54, were explored in the cyclic rat ovary. To this end, adult virgin female rats, weighing 235 ± 17 g, were monitored for estrous cyclicity by daily vaginal cytology. Only rats with at least two consecutive regular 4-d estrous cycles were used in the subsequent expression studies. Groups of cyclic females, at estrus, metestrus, or diestrus-1, diestrus-2, and proestrus were used as ovarian donors. For each phase, tissue sampling was conducted between 0900 and 1000 h. However, to target specific changes in expression of KiSS-1 and GPR54 genes associated with ovulation, sampling of ovarian tissue was also conducted at additional time points along the proestrus-to-estrus transition: proestrus at 1800 h, proestrus at 2100 h, and estrus at 0200 h. Ovarian tissue sampling was conducted immediately after decapitation of the experimental animals, when the tissues were snap frozen in liquid nitrogen and stored at −80 C until used for RNA analysis. Each group was composed of at least five animals.

Based in our initial observation of ovarian expression of KiSS-1 gene, in experiment 2 we aimed to define the presence and pattern of kisspeptin immunoreactivity (IR) in the cyclic rat ovary. To this end, cyclic virgin female rats, checked for vaginal cyclicity as indicated in experiment 1, were used. Groups of females (n = 4) were sampled at 1000 h estrus, metestrus, diestrus, and proestrus. An additional sampling was set at 2100 h of proestrus. In addition, in selected ovarian samples, the presence of GPR54 immunostaining was also screened. Ovaries were excised immediately after decapitation of experimental animals and processed for kisspeptin or GPR54 immunohistochemistry, as described below.

In the next series of experiments, the hormonal regulation of KiSS-1 gene expression in the adult rat ovary was explored. Because a prominent increase in KiSS-1 mRNA levels was observed in the afternoon of proestrus, this phase of the cycle was selectively targeted. In experiment 3, groups of cyclic virgin female rats (n = 5) received, at 1200 h of proestrus, a single ip injection of an effective dose of GnRH-ANT (5 mg/kg) because this regimen is known to prevent the preovulatory rise of gonadotropins and ovulation (25). Vehicle-injected animals served as controls. Ovarian samples were obtained, upon decapitation of the animals, at 1300 and 1800 h of proestrus (*i.e.* 1 and 6 h after administration of GnRH-ANT). Immediately after sampling, the tissues were frozen in liquid nitrogen and stored at −80 C until used for RNA analysis.

Because results from *e*xperiment 3 suggested that the preovulatory gonadotropin surge is responsible for the increase in ovarian KiSS-1 mRNA levels at the afternoon of proestrus, the specific contribution of LH (as major driving signal for ovulation) to this phenomenon was evaluated. To this end, in experiment 4, groups of cyclic female rats (n = 5–6) were injected at 1200 h with GnRH-ANT (5 mg/kg) and an effective dose of hCG (25 IU/rat) as superagonist of LH. Animals injected with vehicle served as controls. Ovarian samples were obtained, upon decapitation of the animals, at 1300, 1500, and 1800 h of proestrus and 1200 h of next estrus (*i.e.* 1, 3, 6, and 24 h after administration of GnRH-ANT and hCG). Immediately after sampling, the tissues were frozen in liquid nitrogen and stored at −80 C until used for RNA analysis.

In the final set of experiments, developmental changes in the ovarian expression of KiSS-1 gene were explored. Thus, in experiment 5, groups of immature female rats (n = 6) were used for sampling of ovarian tissue at the following postnatal ages: 20, 23, 26, 29, and 32 d. In addition, a group of cyclic females at metestrus were sampled for reference purposes. All tissue samples were obtained between 900 and 1000 h, immediately after decapitation of the animals, when ovaries were excised, snap frozen in liquid nitrogen, and stored at −80 C until use for RNA analysis. Because results from this experiment evidenced that expression of KiSS-1 in the immature ovary is low to negligible, we sought to determine whether such expression can be induced by hCG, as demonstrated in the adult ovary. To this end, in experiment 6, immature female rats (n = 10/group) were subjected to a standard protocol of gonadotropin priming, consisting of a single sc injection of PMS-G (10 IU/rat) on postnatal d 22, followed 48 h later by injection of hCG (10 IU/rat), in keeping with previous references (26). The animals were decapitated 3 h after injection of hCG, except for a subset (n = 3–4) of immature rats, which were killed 24 h after hCG treatment (*i.e.* on d 25 postpartum) to check for successful ovulation induction. At the end of the experimental procedures, the ovaries were excised and snap frozen in liquid nitrogen. Vehicle-injected animals served as controls.

RNA analysis by semiquantitative RT-PCR

Total RNA was isolated from ovarian samples using the single-step, acid guanidinium thiocyanate-phenol-chloroform extraction method. Ovarian expression of KiSS-1 and GPR54 mRNAs was assessed by RT-PCR, optimized for semiquantitative detection, using previously defined primer pairs and conditions (13, 27). As internal control for reverse transcription and reaction efficiency, amplification of a 290-bp fragment of L19 ribosomal protein mRNA was carried out in parallel in each sample (28). PCRs consisted in a first denaturing cycle at 97 C for

5 min, followed by a variable number of cycles of amplification defined by denaturation at 96 C for 30 sec, annealing for 30 sec, and extension at 72 C for 1 min. A final extension cycle of 72 C for 15 min was included. Annealing temperature was adjusted for each target and primer pair: 62.5 C for KiSS-1 and GPR54 and 58 C for RP-L19 transcripts. Based in our previous optimization curves testing different numbers of PCR cycles to select exponential amplification conditions, 34 and 24 PCR cycles were chosen for semiquantitative analysis of specific targets (KiSS-1 and GPR54) and RP-L19, respectively. Specificity of PCR products was confirmed by direct sequencing (Central Sequencing Service, University of Cordoba, Cordoba, Spain). Quantification of intensity of RT-PCR signals was carried out by densitometric scanning using an image analysis system (1-D Manager, TDI Ltd., Madrid, Spain), and values of the specific targets were normalized to those of internal controls to express arbitrary units of relative expression. In all assays, liquid controls and reactions without reverse transcription resulted in negative amplification.

Kisspeptin and GPR54 immunohistochemistry

Rat ovarian tissue was collected from adult cyclic female rats at different stages of the estrous cycle, as described in experiment 2. In addition, human placental samples from gestational month 3 (generously supplied from the archives of the Pathology Department of the University of Cordoba) and rat placental tissue (obtained at d 13.5 of pregnancy) were used as positive control for kisspeptin immunolabeling because human and rodent placenta has been previously proven to express kisspeptin-IR (10, 21). Ovarian samples were fixed overnight in neutral-buffered formalin (4%) and routinely processed for paraffin embedding, in keeping with previous references (29). For kisspeptin immunohistochemistry, ovarian sections (5 μm thick) were cut and placed on poly-L-lysine-coated slides and, after dewaxing in xylene and rehydration in graded ethanol series, were incubated in 2% hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase. After washing in PBS, the sections were blocked with normal serum and incubated overnight with the primary antihuman kisspeptin-13 (4–13) antibody (Bachem AG, Bubendorf, Switzerland). Of note, this antibody potentially recognizes all forms of kisspeptins sharing the common 10 amino acid terminal region. Final dilution of the antibody was 1:800. The sections were then processed according to the avidin-biotin-peroxidase complex technique as previously described (28). Immunohistochemical controls included omission of the primary antikisspeptin antibody (negative control) and the use of sections of rat and human placenta as positive control. As additional control for the specificity of the primary antibody, immunohistochemical reactions were carried out in slices of placental and ovarian tissue after preabsorption of the antiserum overnight at 4 C with 1 μg/ml of mouse kisspeptin [KiSS-1 (112–121)-NH_2; Phoenix Pharmaceuticals Ltd., Belmont, CA], a procedure that completely abolished kisspeptin immunolabeling. Tissue sections were counterstained with hematoxylin following standard procedures.

In addition, the presence of GPR54 immunoreactivity in ovarian tissue was analyzed using commercially available antimouse GPR54 antiserum [anti-GPR54 (375–396) antibody; Phoenix Pharmaceuticals] at a final dilution of 1:300 and standard immunohistochemical procedures, as described above. Of note, due to the constitutive pattern of GPR54 mRNA expression in the rat ovary along the estrus cycle (experiment 1), GPR54 immunoreactivity was selectively studied in a limited set of ovarian samples, including those at the preovulatory period (proestrus) and during the luteal phase (diestrus). Omission of the primary anti-GPR54 antibody was used as negative control, whereas sections of rat and human placenta served as positive controls Tissue sections were counterstained with hematoxylin following standard procedures.

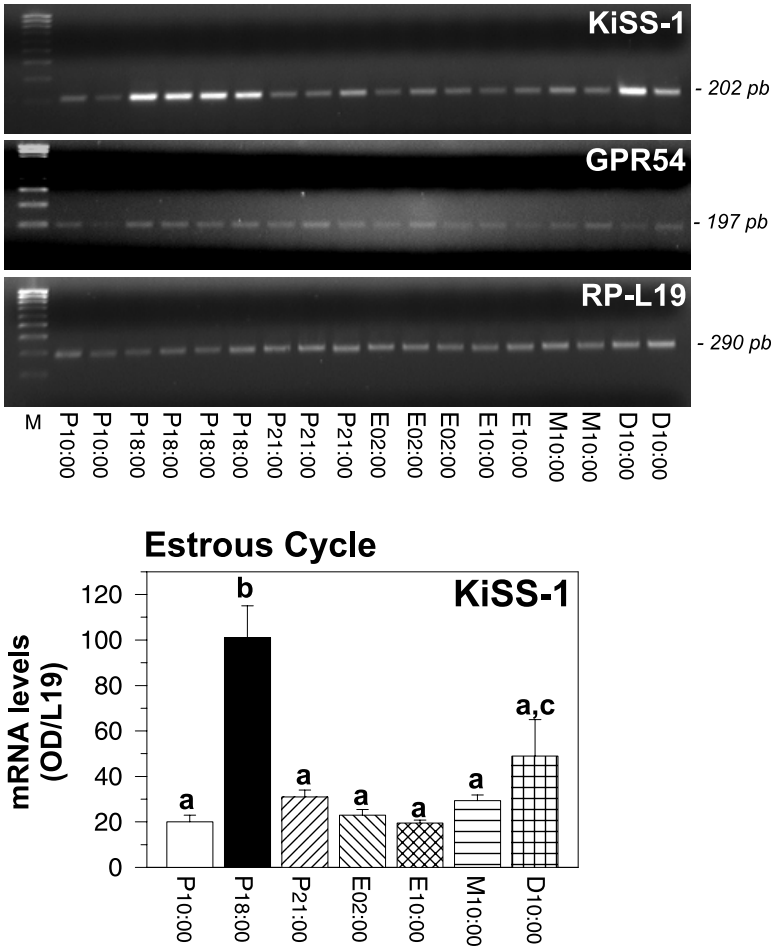

FIG. 1. Profiles of expression of KiSS-1 and GPR54 genes in adult cyclic rat ovaries at different stages of the estrous cycle. For each phase, at least two representative independent samples, obtained at 1000 h of proestrus (P), estrus (E), metestrus (M), and diestrus (D), are presented. In addition, to characterize in more detail potential changes in the proestrus-to-estrus transition, additional samples, obtained at 1800 and 2100 h of proestrus and 0200 h of estrus, are also shown. Parallel amplification of L-19 ribosomal protein mRNA served as internal control. In the *lower panels*, semiquantitative values of KiSS-1 mRNA levels are the mean ± SEM of at least four to six independent determinations. Groups with *different superscript letters* are statistically different ($P < 0.05$; ANOVA followed by Student-Newman-Keuls multiple range test).

Presentation of data and statistics

Semiquantitative RT-PCR analyses were carried out in duplicate from at least four independent RNA samples of each experimental group. For generation of RNA samples, individual ovarian specimens were used. Semiquantitative data are presented as mean ± SEM. Results were analyzed for statistically significant differences using one- or two-way ANOVA, followed by Student-Newman-Keuls multiple range test (SigmaStat 2.0; Jandel Corp., San Rafael, CA). $P \leq 0.05$ was considered significant.

Results

Expression of KiSS-1 and GPR54 genes in the cyclic rat ovary

Preliminary published data (21), and our initial screening assays, suggested expression of KiSS-1 and GPR54 genes in the adult rat ovary. On this basis, the expression profiles of these mRNAs were thoroughly explored in rat ovary along the estrous cycle. Semiquantitative RT-PCR assays were conducted in ovarian samples obtained in the morning (1000 h) of each of the stages of the cycle: estrus, metestrus, diestrus, and proestrus. In addition to target potential changes in expression around ovulation, the proestrus-to-estrus transition was analyzed in further detail by assessing KiSS-1 and GPR54 mRNA levels at intermediate time points: 1800 and 2100 h of proestrus evening, and 0200 h of estrus. Our analyses revealed that KiSS-1 and GPR54 genes are expressed in rat ovarian tissue throughout the estrous cycle. However, whereas relative mRNA levels of GPR54 remained low and did not significantly fluctuate along the cycle, ovarian expression of KiSS-1 mRNA showed a distinctive stage-specific pattern of expression. Thus, low levels were detected at the morning of proestrus, which sharply increased by the afternoon of that phase, with maximal levels at 1800 h proestrus. Expression of KiSS-1 mRNA abruptly decreased thereafter and returned to moderate to low levels throughout the estrus cycle, except for a transient modest increase observed at diestrus (Fig. 1).

Kisspeptin and GPR54 immunoreactivity in the cyclic ovary and control (placental) tissue

Gene expression analyses were complemented by immunohistochemical assays, aiming to provide evidence for the expression and pattern of distribution of kisspeptin IR in adult rat ovarian tissue. To this end, an antiserum raised against human kisspeptin-10, able to recognize all forms of kisspeptin sharing the 10-amino-acid terminal region, was used. As control for the immunohistochemical procedure, human and rat placental tissues, as proven sources of kisspeptin-IR (10, 21), were used in parallel. As shown in Fig. 2A, strong cytoplasmic immunostaining for kisspeptin was present in the villi of 3-month-old human placental samples. Immunolabeled cells corresponded to the syncytiotrophoblast, whereas the cytotrophoblast and the villous stroma were negative, in keeping with previous references (10). Kisspeptin IR was also detected in the cytoplasm of trophoblastic giant cells of 13.5-d-old rat placenta (Fig. 2C), in good agreement with previous references (21). Yet immunostaining was fainter and more irregular than in human placental tissue. In both human and rat control samples, preadsorption of the primary antibody with kisspeptin-10 abolished IR, thus proving specificity of reaction (Fig. 2, B and D).

In the cyclic rat ovary, strong kisspeptin-IR was detected in several compartments of the tissue, including growing and preovulatory follicles, corpora lutea and interstitial gland. Yet kisspeptin immunolabeling showed a cell- and stage-

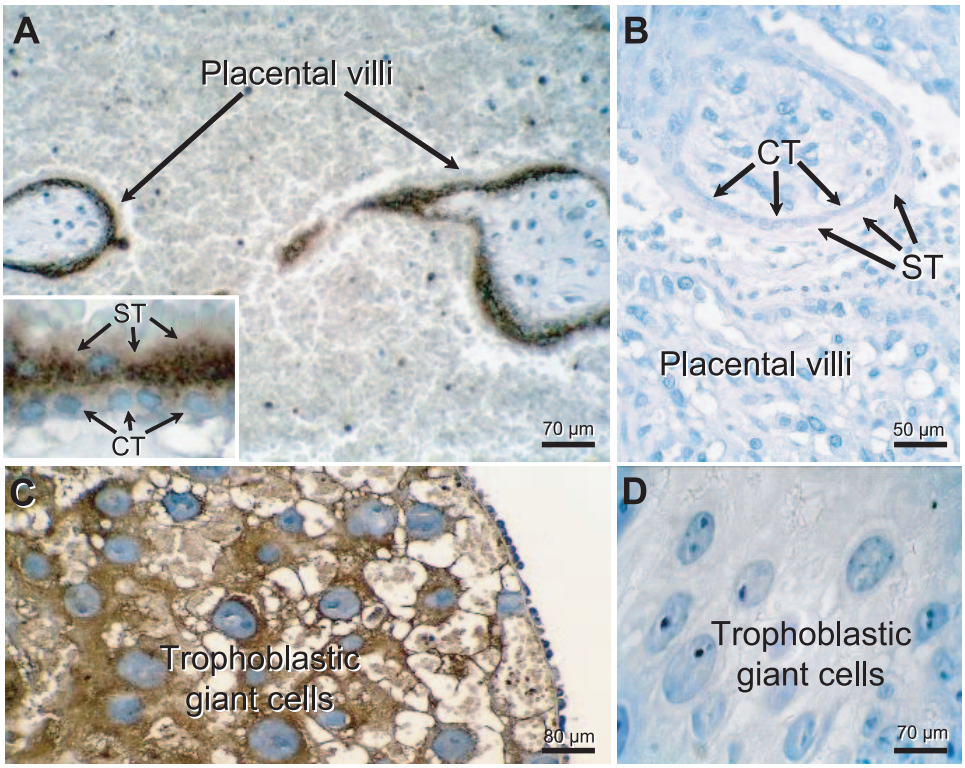

FIG. 2. Validation of the immunohistochemical procedures with the polyclonal antiserum against human kisspeptin-13 (4–13), *i.e.* kisspeptin-10, using human and rat placenta as positive control tissues. Sections from human (from 3-month gestation) and rat (from 13.5-d pregnancy) placentas were immunostained with the specific primary antibody and counterstained with hematoxylin. In human placenta (A), strong cytoplasmic staining was present in the syncytiotrophoblast (ST), whereas kisspeptin-IR was absent in the cytotrophoblast (CT) and villous stroma. In rat placenta (C), cytoplasmic immunostaining was also detected in the giant trophoblast cells, yet IR was fainter than in human sections. Preadsorption of the primary antibody with kisspeptin-10 totally abolished immunostaining in human and rat tissues, used as negative controls (B and D).

specific pattern of distribution along the estrous cycle. Thus, in growing and preovulatory follicles, the theca cells were intensely immunostained, whereas granulosa cells were totally negative from early estrus to early proestrus (Fig. 3, A and B). On late proestrus, however, kisspeptin-IR began to appear at the basal cells of the granulosa layer of preovulatory follicles (Fig. 3C). In addition, immunostaining for kisspeptin was also apparent in oocyte cytoplasm. After ovulation, kisspeptin-IR was clearly detected in theca-lutein cells, whereas fainter but detectable immunolabeling was also observed in the granulosa-lutein cells of newly formed corpora lutea (Fig. 4A). Of note, the intensity of kisspeptin-IR apparently increased in parallel to corpus luteum development. Thus, kisspeptin immunostaining was intense in the corpus luteum of the current cycle in proestrus (see Fig. 3B), whereas it progressively decreased in regressing corpora lutea (Fig. 4B), in which kisspeptin-IR was confined to the remaining scattered steroidogenic cells present in corpus luteum remnants (Fig. 3B). In addition, interstitial steroidogenic cells showed strong kisspeptin immunolabeling throughout the estrous cycle (Fig. 3, A and C). Finally, the ovarian surface epithelium also showed clear-cut immunostaining for kisspeptin (Fig. 4B).

In addition to kisspeptin, the presence GPR54-IR was also screened in rat ovarian tissue using a commercially available antimouse GPR54 antiserum. However, due to the apparently constitutive pattern of expression of GPR54 mRNA along the cycle, this analysis was selectively conducted in a subset of ovarian samples, including those at the preovulatory period (proestrus) and luteal phase (diestrus). As positive controls, human and rat placental tissues were used. Unambiguous staining for GPR54 was detected in the villi of human placenta, in which cells from cytotrophoblast and syncytiotrophoblast were immunolabeled, the stroma being negative (Fig. 5, A and B). Likewise, faint but detectable GPR54-IR was observed in trophoblastic giant cells of 13.5-d-old rat placenta (data not shown). In the cyclic rat ovary, GPR54 immunostaining was detected in the theca layer of

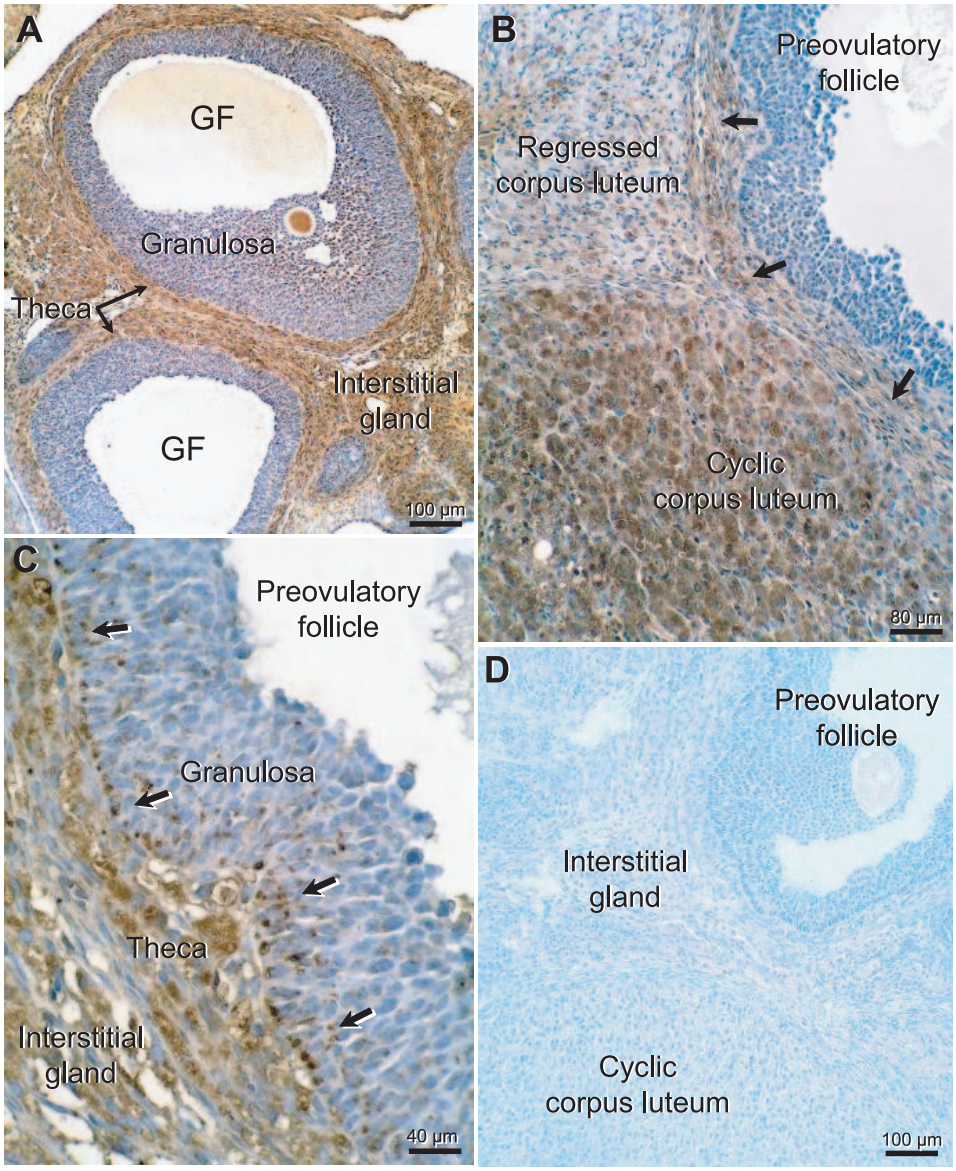

FIG. 3. Kisspeptin-IR in sections from cyclic rat ovaries from early diestrus (A), early proestrus (B and D), and late proestrus (C). Strong cytoplasmic immunostaining was present in the theca layer of growing (GF) and preovulatory follicles (A–C); oocytes also showed apparent kisspeptin-IR (A). In addition, immunolabeling was also detected in interstitial gland cells (A and C) and steroidogenic cells of the corpus luteum of the current cycle (B), whereas kisspeptin-IR was nearly absent in regressing corpus luteum remnants (B). Granulosa cells were negative for kisspeptin immunostaining throughout follicular development (A and B). Yet kisspeptin-IR appeared at the basal zone of preovulatory follicles on late proestrus (C, *arrows*). Immunostaining was absent in negative control sections (D).

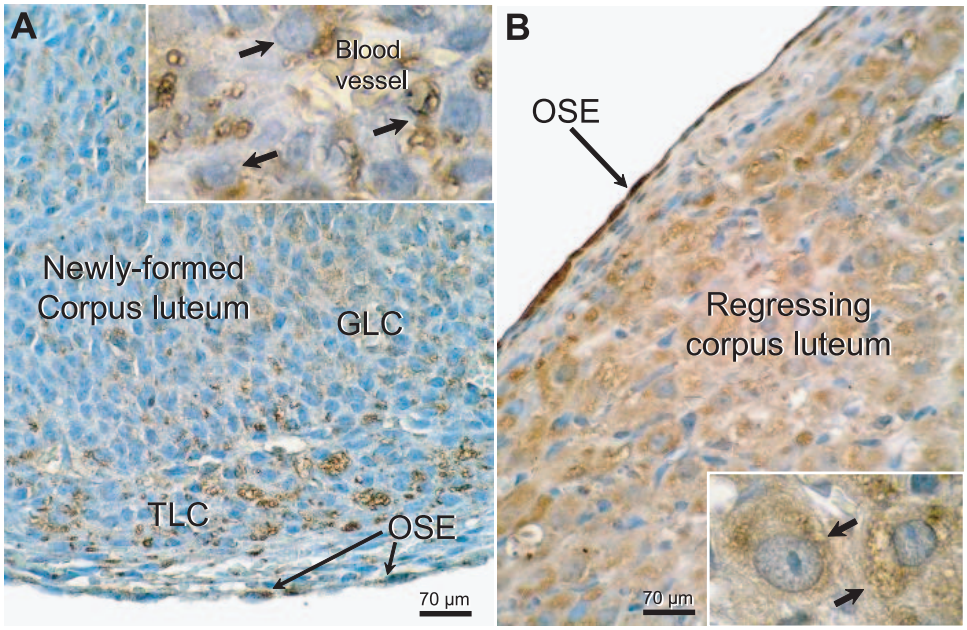

FIG. 4. Kisspeptin-IR in newly formed (A) and regressing (B) corpora lutea at early estrus. Newly formed corpora lutea showed intense immunostaining in theca-lutein cells (TLC) and weaker but increasing kisspeptin-IR in granulosa-lutein cells (GLC; *arrows* in *inset*). In regressing corpora lutea of the previous cycle, immunostaining was present in remaining steroidogenic cells (*arrows* in *inset*). Strong kisspeptin-IR was also detected in the ovarian surface epithelium (OSE).

preovulatory follicles, whereas granulosa cells were negative (Fig. 5C). In addition, GPR54-IR was also observed in the corpus luteum, with positive staining being detected in morphologically discernible steroidogenic luteal cells (Fig. 5D). GPR54 immunolabeling was also noticed at the interstitial compartment (gland) of the rat ovary.

The preovulatory LH surge elicits ovarian KiSS-1 mRNA expression at proestrus

Our initial RNA analysis evidenced a sharp increase in KiSS-1 gene expression preceding ovulation. Thus, we sought to determine whether such a rise in KiSS-1 mRNA levels was driven by the preovulatory surge of gonadotropins. As first approach, we assayed KiSS-1 mRNA in ovarian samples at different time intervals after administration of a potent GnRH antagonist at proestrus, a protocol that is known to block the preovulatory rise of LH and prevent ovulation (25, 29). As shown in Fig. 6, whereas controls injected with vehicle showed the expected increase in relative KiSS-1 mRNA levels at 1800 h on proestrus, injection of an effective dose of GnRH-ANT totally prevented such a rise, therefore suggesting that this phenomenon is gonadotropin dependent. To further explore the hormonal signals involved, female rats injected with GnRH-ANT at proestrus were cotreated with an effective dose of hCG (25 IU/rat) as superagonist of LH, and ovarian samples were obtained at

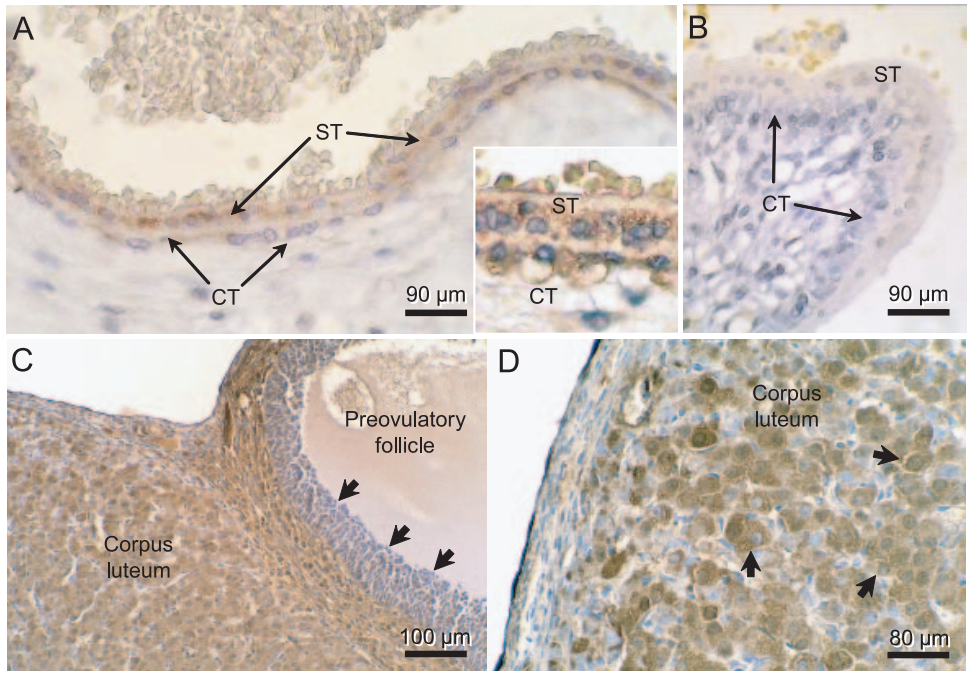

FIG. 5. GPR54-IR in control (human) placental tissue and cyclic rat ovary. Sections from human placenta were immunostained with the specific primary antibody and counterstained with hematoxylin (A). Staining for GPR54 was detected in the syncytiotrophoblast (ST) and cytotrophoblast (CT), denoted by *long arrows*, whereas the villous stroma was negative. As negative control, omission of the primary antibody totally abolished immunostaining in placental tissues (B). In the cyclic rat ovary, discernible GPR54-IR was detected in the theca layer of preovulatory follicles, whereas granulosa cells (indicated by *arrowheads*) were negative (C). In addition, GPR54-IR was also observed in the corpus luteum in morphologically discernible steroidogenic luteal cells (D, *arrows*).

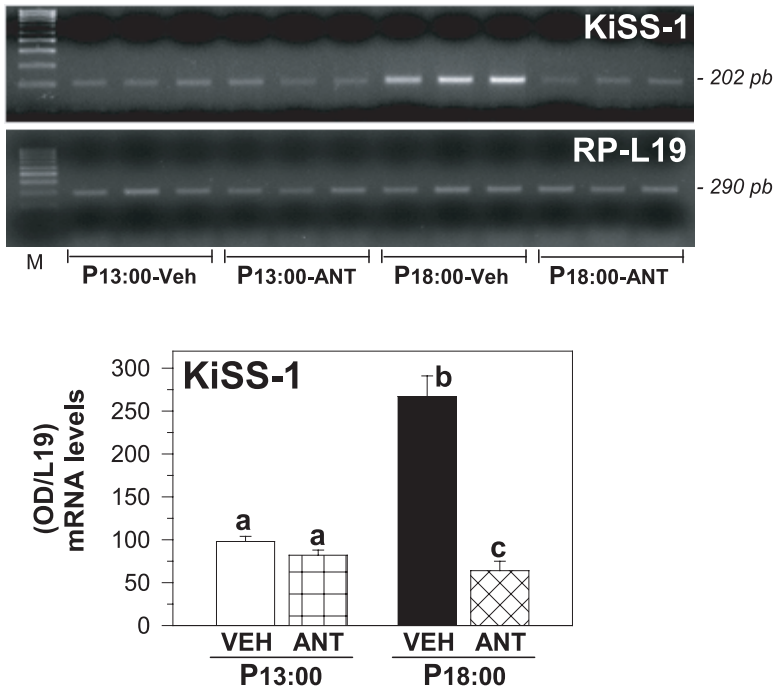

FIG. 6. Expression levels of KiSS-1 mRNA in ovaries from rats treated with an effective dose of GnRH-ANT (5 mg/kg) or vehicle (Veh) at 1200 h on proestrus (P) and sampled at 1 h (1300 h) and 6 h (1800 h) after treatment. For each group, three representative independent samples are presented. Parallel amplification of L-19 ribosomal protein mRNA served as internal control. In the *lower panels*, semiquantitative values of KiSS-1 mRNA levels are the mean ± SEM of at least five independent determinations. Groups with *different superscript letters* are statistically different ($P < 0.05$; ANOVA followed by Student-Newman-Keuls multiple range test).

different time intervals (from 1–24 h) after treatment. Vehicle-injected animals, serving as controls, showed low, unchanged levels of KiSS-1 mRNA along the study period; thus, representative values at 3 h after injection are shown in Fig. 7. In contrast, hCG administration evoked a significantly increase in relative KiSS-1 mRNA levels over control values, which peaked at 3 h and progressively declined thereafter (Fig. 7).

Negligible expression of KiSS-1 gene in the immature ovary and induction by PMS-G and hCG

Collectively considered, previous data evidenced that KiSS-1 is expressed in cyclic rat ovary, and its mRNA levels dramatically increase preceding ovulation under the control of pituitary LH. In this context, we found it relevant to define the profile of expression and potential hormonal (gonadotropin) regulation of KiSS-1 gene in the immature ovary. First, we assayed KiSS-1 mRNA levels in ovarian tissue from juvenile to peripubertal female rats. Our data showed that, in contrast to clearly detectable expression in adult ovarian tissue at metestrus (taken for reference purposes), relative levels of KiSS-1 mRNA were very low along the period of prepubertal maturation studied, with faint expression between d 20 and 26 postpartum and virtually negligible levels on d 29 and 32 of age (Fig. 8). Second, we investigated whether (low) expression of KiSS-1 gene in the immature ovary could be enhanced by a standard protocol of gonadotropin priming, known to elicit ovulation in the prepubertal female (26). Immature rats (d 22 postpartum) were injected with an effective dose of PMS-G to induce follicular maturation followed 48 h later by an effective dose of hCG to trigger ovulation, assessment of KiSS-1 mRNA levels being conducted 3 h after hCG injection. As shown in Fig. 9, whereas ovarian expression of KiSS-1 in 24-d-old females injected with vehicle remained barely detectable (in keeping with our previous results), gonadotropin priming by PMS-G evoked a significant increase in ovarian KiSS-1 mRNA levels that was further enhanced by injection of an ovulatory dose of hCG.

Discussion

Compelling evidence has now defined the indispensable role of the KiSS-1/GPR54 system in the control of development and function of male and female reproductive axis. On the basis of genetic, molecular, and pharmacological approaches, this essential function is assumed to be primarily (if not exclusively) conducted at central hypothalamic levels, in which KiSS-1 neurons have been proposed as gatekeepers of the GnRH system (30). Although this contention is now undisputed, a conspicuous finding is that expression of KiSS-1 and/or GPR54 genes has been reported in different peripheral tissues, including placenta and other reproductive organs, such as the gonads (1, 21), for which the potential functions of this system remain mostly unexplored. Based on preliminary evidence showing prominent expression of KiSS-1 gene (and to a lesser extent of GPR54) in the rat ovary (21), in the present study, we aimed to define the profile of ovarian KiSS-1 gene expression and kisspeptin-IR and identify its potential regulation during development and by key hormonal factors as a mean to initiate characterization of the putative role(s), if any, of locally produced KiSS-1 in ovarian physiology.

Our analyses conclusively demonstrated, for the first time, that the genes encoding kisspeptin and its putative receptor GPR54 are persistently expressed in the adult rat ovary throughout the different phases of the estrous cycle. More interestingly, our current data evidenced that the profiles of ovarian expression of both targets are clearly distinct. Thus, whereas GPR54 mRNA levels remained low along the cycle, without significant fluctuations, KiSS-1 mRNA sharply in-

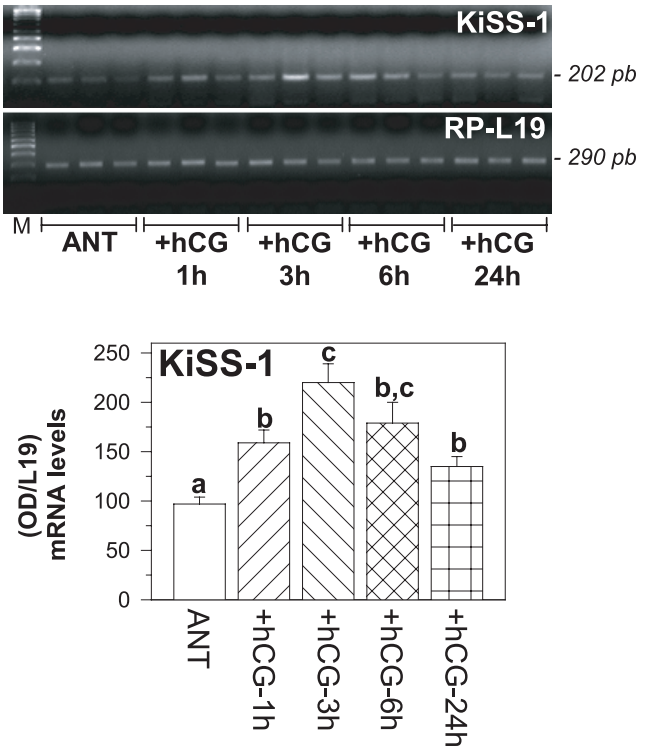

FIG. 7. Expression levels of KiSS-1 mRNA in ovaries from rats treated with an effective dose of GnRH-ANT (5 mg/kg) at 1200 h on proestrus and coinjected with hCG (25 IU/rat) or vehicle. Ovarian samples were obtained at 1, 3, 6, and 24 h after hCG or vehicle injection. Animals treated with GnRH-ANT alone showed unchanged KiSS-1 mRNA levels along the study period; three representative samples obtained 3 h after injection of the antagonist (ANT) are presented. In addition, for each time point after hCG treatment, three representative independent samples are presented. Parallel amplification of L-19 ribosomal protein mRNA served as internal control. In the *lower panels*, semiquantitative values of KiSS-1 mRNA levels are the mean ± SEM of at least five independent determinations. Groups with *different superscript letters* are statistically different ($P < 0.05$; ANOVA followed by Student-Newman-Keuls multiple range test).

creased in the ovary at 1800 h on proestrus and abruptly decreased thereafter; only a moderate increase in KiSS-1 mRNA (of much lower magnitude) was also detected on diestrus. Of note, this sharp rise in ovarian KiSS-1 mRNA at proestrus appeared tightly coincident with the preovulatory surge of gonadotropins, responsible for triggering ovulation, which in our animal stock takes place from approximately 1600 h proestrus onward (our personal observation). This timely regulated profile of expression, in the context of the proposed role of kisspeptin in the control of proteases, such as MMP-9, previously involved in the ovulatory process (23), is strongly suggestive of a potential, previously unsuspected role of locally derived KiSS-1 in the direct control of ovulation (see below). Yet persistent expression of KiSS-1 gene at other phases of the ovarian cycle pointed out that, unlike other factors such as cyclooxygenase-2 and the epidermal growth factor family member epiregulin (see Ref. 31), kisspeptin might also be involved in ovarian functions other than ovulation.

Such a contention (*i.e.* potential role of KiSS-1 in ovulation as well as in other ovarian functions) appeared to be in good agreement with our immunohistochemical analyses, which demonstrated specific kisspeptin-IR in different tissue compartments of the cyclic ovary, including the theca layer of growing and preovulatory follicles, theca- and granulosa-lutein cells of the corpus luteum, and the interstitial gland as well as the ovarian surface epithelium and, apparently, the oocyte. Of particular note, follicular kisspeptin immunostaining showed a striking cell- and stage-specific pattern of distribution along the estrous cycle, with granulosa cells being totally negative for kisspeptin-IR from early estrus to early proestrus. However, in late proestrus, kisspeptin signal began to appear also at the basal cells of the granulosa layer of preovulatory follicles. This phenomenon grossly coincides with the observed rise in KiSS-1 mRNA levels at the time of the preovulatory LH surge, yet its functional relevance remains to be determined. In addition, clear-cut kisspeptin-IR was detected at the corpus luteum in the cytoplasm of steroidogenic cells of theca and granulosa origin. Such luteal expression of kisspeptin-IR may reflect the observed moderate increase in KiSS-1 mRNA levels during the diestrus phase. Considering the proven functional roles of kisspeptins in other cellular backgrounds, it is tempting to propose that expression of kisspeptin at this site might be related to the intense tissue remodeling during formation and/or regression of corpora lutea. Alternatively, locally produced kisspeptin might contribute to regulation of hormone secretion by steroidogenic luteal (and eventually, interstitial) cells, which are LH/chorionic gonadotropin responsive. Finally, the presence of kisspeptin in the oocyte, and its eventual functional relevance, merit further investigation, which is presently in progress at our laboratory.

Mandatory for direct biological actions of kisspeptin, the expression of GPR54 gene (albeit at apparently low levels) and the presence of GPR54-like IR were observed in the cyclic rat ovary. However, in contrast to KiSS-1, relative levels of GPR54 mRNA appeared rather constitutively detected along the estrous cycle. This fact prevented us to conduct detailed immunohistochemical analyses of GPR54 at all phases of the ovarian cycle. Moreover, the incomplete homology (~68%) between the mouse and rat GPR54 sequences used for generation of the (mouse) antiserum used in this study brings some caution in the interpretation of our immunohistochemical results. Nonetheless, although it is assumed that additional localization data (by means of *in situ* hybridization and/or highly specific anti-GPR54 antisera, when available) would be ideally required to fully unravel the precise distribution of GPR54, our current mRNA and protein results are highly suggestive of the presence of kisspeptin receptors in the rat ovary, with a pattern of distribution that is grossly coincident with that of the ligand itself. These observations support the plausibility of direct kisspeptin effects at those sites of the ovary. Furthermore, potential indirect actions of kisspeptins at other ovarian compartments (via intermediate cells expressing GPR54) cannot be excluded on the basis of our present data.

The salient expression of KiSS-1 gene (and kisspeptin-IR) during the preovulatory period prompted us to study in more detail the hormonal factors controlling ovarian KiSS-1 mRNA levels during the proestrus-to-estrus transition. Blockade of the preovulatory surge of gonadotropins by

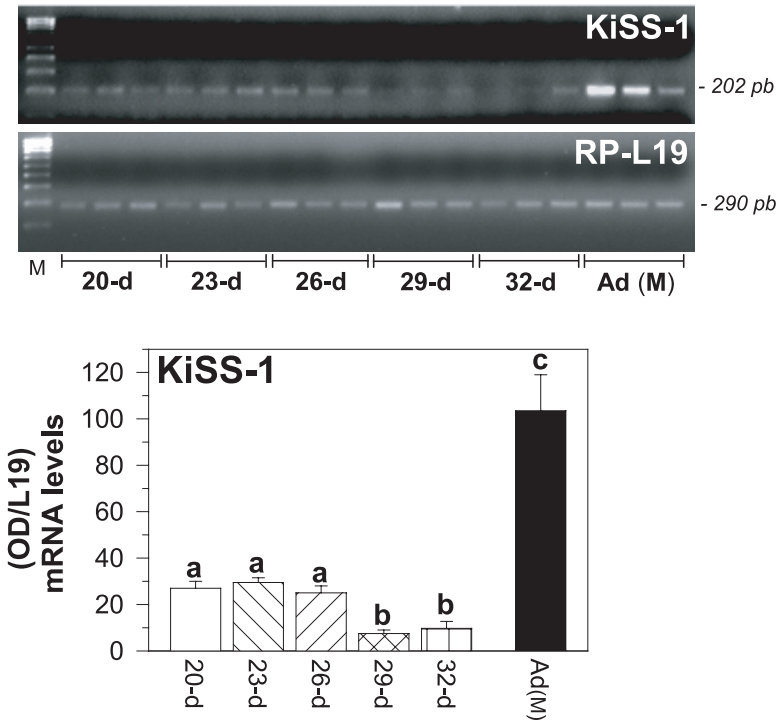

FIG. 8. Profile of expression of KiSS-1 gene in immature rat ovaries at different age points of prepubertal maturation. For each age point, three representative independent samples are presented: 20-, 23-, 26-, 29-, and 32-d-old female rats. For reference purposes, triplicate samples from adult cyclic ovaries at metestrus (M) are also shown. Parallel amplification of L-19 ribosomal protein mRNA served as internal control. In the *lower panels*, semiquantitative values of KiSS-1 mRNA levels are the mean ± SEM of at least six independent determinations. Groups with *different superscript letters* are statistically different ($P < 0.05$; ANOVA followed by Student-Newman-Keuls multiple range test).

means of administration of GnRH-ANT, in a regimen known to prevent ovulation (25, 29), was able to totally block the rise in KiSS-1 mRNA levels at 1800 h on proestrus. In turn, exogenous administration of hCG (as superagonist of LH) to GnRH-ANT-treated females elicited a significant increase in ovarian KiSS-1 mRNA, which reached maximal levels 3 h after hCG injection. These data indicate that, among other biochemical and cellular events leading to ovulation, the rise in serum LH levels during proestrous afternoon drives a transient increase in ovarian expression of KiSS-1 gene. Yet whether this is a direct LH action on KiSS-1-expressing cells or it stems from LH-induced release of intermediate factors awaits to be unveiled. Likewise, the hierarchical role of such a phenomenon (LH-evoked KiSS-1 expression) in the cascade of processes linked to ovulation cannot be ascertained on the basis of our current data.

Further evidence for the potential involvement of KiSS-1 in the local control of ovulation was provided by our studies in immature female rats. These expression analyses indicated that during the prepubertal period, relative mRNA levels of KiSS-1 in the rat ovary were low, whereas they significantly increased in the transition to the reproductive (cyclic) state after puberty. Of note, such a rise in KiSS-1 expression was apparently preceded by a significant suppression of KiSS-1 mRNA to virtually undetectable levels immediately before puberty onset (*i.e.* between d 29 and 32 postpartum); a phenomenon whose functional relevance merits further investigation. Interestingly, implementation of a standard procedure for gonadotropin priming and induction of ovulation by administration of PMS-G and hCG to immature females not only resulted in successful oocyte release (data not shown) but also evoked a significant elevation of ovarian KiSS-1 mRNA levels, whose magnitude was maximal after combined PMS-G and hCG treatment. Altogether, these findings demonstrate that the immature ovary is capable to respond to appropriate gonadotropin priming with an increase in KiSS-1 gene expression preceding ovulation (as is apparently the case in adult rats), therefore suggesting that locally produced KiSS-1 might be involved in not only the ovulatory process of adult cycling rats but also the induction of ovulation in immature females.

The questions arising from our present data are to what extent ovarian KiSS-1 might play a role, distinct to that of central kisspeptin, in the tuning of ovulation and the physiological relevance of that role, if any. Notably, the possibility of potential peripheral actions of kisspeptin in the control of the reproductive axis has remained largely neglected, mostly due to the fact that gonadotropin replacement appeared sufficient to rescue the profound hypogonadal state of GPR54 null mouse models. Indeed, *GPR54*$^{-/-}$ female mice were induced to ovulate after standard gonadotropin priming (12), suggesting that KiSS-1 signaling at the ovary is not absolutely mandatory for ovulation. Whereas this contention is not disputed by our present data, our results strongly suggest that kisspeptin might be involved in the local modulation of some aspects of the ovulatory process. In this sense, it is to be noted that the qualitative and quantitative characteristics of ovulation in gonadotropin-primed GPR54 null mice have not been apparently explored in detail (see Ref. 12). Thus, it remains possible that, despite successful oocyte release after PMS-G and hCG treatment, gonadotropin-primed *GPR54*$^{-/-}$ females might bear subtle (qualitative and/or quantitative) alterations in ovulation.

A similar situation may apply also for the potential role of KiSS-1 in placental physiology because, despite a wealth of experimental evidence having been presented suggesting a role of kisspeptin in trophoblast invasion (10, 21), placental formation in fetal and gonadotropin-replaced maternal

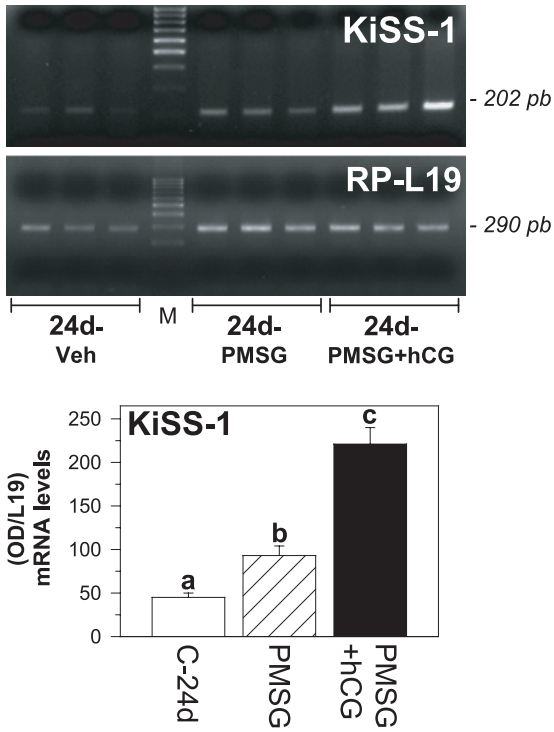

FIG. 9. Ability of gonadotropin priming to elicit KiSS-1 mRNA expression in the immature rat ovary. Immature (22 d old) female rats were treated with a single dose of PMS-G alone or PMS-G followed 48 h later by administration of an ovulatory dose of hCG; ovaries were sampled 3 h after the last injection. Pair-aged females treated with vehicle (Veh) alone served as controls (C-24d). For each group, three representative independent samples are presented. Parallel amplification of L-19 ribosomal protein mRNA served as internal control. In the *lower panels*, semiquantitative values of KiSS-1 mRNA levels are the mean ± SEM of at least six independent determinations. Groups with *different superscript letters* are statistically different ($P < 0.05$; ANOVA followed by Student-Newman-Keuls multiple range test).

GPR54 null mutants appeared to be globally preserved. Overall, the concurrent analysis of the previously assigned functional roles of kisspeptin in terms of inhibition of protease (MMP) activity (1, 22), and the temporal pattern of ovarian expression of KiSS-1 gene preceding ovulation reported here, lead us to hypothesize that, whereas not strictly indispensable for oocyte release, ovarian kisspeptin may participate in the local regulation of some facets of the ovulatory process. These may include the selective targeting of ovarian rupture to the follicular apex at the time of ovulation, *i.e.* the prevention of uncontrolled breakdown of the follicular wall except for the apex, which is a timely regulated phenomenon essential for proper delivery of the oocyte into the periovarian space. Similarly, ovarian kisspeptin may contribute to the local control of extensive tissue remodeling around ovulation (and luteinization). Such hypotheses would be fully compatible with the grossly preserved ovulation observed in gonadotropin-primed GRP54 null mice. Nonetheless, additional experimental work on the characterization of ovulation in this model would be needed to validate (or refute) such possibilities.

In conclusion, we report herein the profiles of KiSS-1 gene expression and kisspeptin-IR in the adult cyclic ovary and provide novel evidence for its developmental (negligible before puberty) and hormonal regulation. Overall, the demonstration that LH/chorionic gonadotropin is able to elicit a consistent increase in ovarian KiSS-1 mRNA levels preceding ovulation, in both adult cyclic rats and gonadotropin-primed immature females, is highly suggestive of a previously unsuspected role of locally produced kisspeptin in the direct control of the ovulatory process. Whereas the essential role of hypothalamic KiSS-1 system in the central control of female reproductive axis remains unquestioned, our present data add complexity to our knowledge of the potential mode of action of kisspeptin at different levels of the gonadal axis, a phenomenon whose physiological relevance warrants further investigation.

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References

1. **Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, Terao Y, Kumano S, Takatsu Y, Masuda Y, Ishibashi Y, Watanabe T, Asada M, Yamada T, Suenaga M, Fujino C, Usuki S, Kurokawa T, Onda H, Nishimura O, Fujino M** 2001 Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G protein-coupled receptor. Nature 411:613–617
2. **Muir AI, Chamberlain L, Elshourbagy NA, Michalovich D, Moore DJ, Calamari A, Szekeres PG, Sarau HM, Chambers JK, Murdock P, Steplewski K, Shabon U, Miller JE, Middleton SE, Darker JG, Larminie CGC, Wilson S, Bergsma DJ, Emson P, Faull R, Philpott KL, Harrison DC** 2001 AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. J Biol Chem 276:28969–28975
3. **Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, Brezillon S, Tyldesley R, Suarez-Huerta N, Vandeput F, Blanpain C, Schiffmann SN, Vassart G, Parmentier M** 2001 The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. J Biol Chem 276:34631–34636
4. **Lee DK, Nguyen T, O'Neill GP, Chang R, Liu Y, Howard AD, Coulombe N, Tan CP, Tang-Nguyen AT, George SR, O'Dowd BF** 1999 Discovery of a receptor related to the galanin receptors. FEBS Lett 446:103–107
5. **Ringel MD, Hardy E, Bernet VJ, Burch HB, Schuppert F, Burman KD, Saji M** 2002 Metastin receptor is overexpressed in papillary thyroid cancer and activates MAP kinase in thyroid cancer cells. J Clin Endocrinol Metab 87: 2399–2402
6. **Lee JH, Welch DR** 1997 Suppression of metastasis in human breast carcinoma MDA-MB-435 cells after transfection with the metastasis suppressor gene, KiSS-1. Cancer Res 57:2384–2387
7. **Masui T, Doi R, Mori T, Toyoda E, Koizumi M, Kami K, Ito D, Peiper SC, Broach JR, Oishi S, Niida A, Fujii N, Imamura M** 2004 Metastin and its variant forms suppress migration of pancreatic cancer cells. Biochem Biophys Res Commun 315:85–92
8. **Ikeguchi M, Yamaguchi K, Kaibara N** 2004 Clinical significance of the loss of KiSS-1 and orphan G-protein-coupled receptor (hOT7T175) gene expression in esophageal squamous cell carcinoma. Clin Cancer Res 10:1379–1383
9. **Sanchez-Carbayo M, Capodieci P, Cordon-Cardo C** 2003 Tumor suppressor role of KiSS-1 in bladder cancer: loss of KiSS-1 expression is associated with bladder cancer progression and clinical outcome. Am J Pathol 162:609–617
10. **Bilban M, Ghaffari-Tabrizi N, Hintermann E, Bauer S, Molzer S, Zoratti C, Malli R, Sharabi A, Hiden U, Graier W, Knofler M, Andreae F, Wagner O,**

Quaranta V, Desoye G 2004 Kisspeptin-10, a KiSS-1/metastin-derived decapeptide, is a physiological invasion inhibitor of primary human trophoblasts. J Cell Sci 117:1319–1328
11. **de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E** 2003 Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Natl Acad Sci USA 100:10972–10976
12. **Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley WF, Aparicio SA, Colledge WH** 2003 The GPR54 gene as a regulator of puberty. N Engl J Med 349:1614–1627
13. **Navarro VM, Castellano JM, Fernandez-Fernandez R, Barreiro ML, Roa J, Sanchez-Criado JE, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M** 2004 Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor GPR54 in rat hypothalamus and potent LH releasing activity of KiSS-1 peptide. Endocrinology 145:4565–4574
14. **Seminara SB** 2005 Metastin and its G protein-coupled receptor, GPR54: critical pathway modulating GnRH secretion. Front Neuroendocrinol 26:131–138
15. **Yao HH, Bahr JM** 1998 The ovary—overview. In: Knobil E, Neill JD, eds. Encyclopedia of reproduction. San Diego: Academic Press; 590–597
16. **Greenwald GS, Roy SK** 1994 Follicular development and its control. In: Knobil E, Neill JD, eds. The physiology of reproduction. New York: Raven Press; 629–724
17. **Ny T, Wahlberg P, Brändström IJM** 2002 Matrix remodeling in the ovary: regulation and functional role of the plasminogen activator and matrix metalloproteinase systems. Mol Cell Endocrinol 187:29–38
18. **Robker RL, Russell DL, Espey LL, Lydon JP, O'Malley BW, Richards JS** 2000 Progesterone-regulated genes in the ovulation process: ADAMTS-1 and cathepsin L proteases. Proc Natl Acad Sci USA 97:4689–4694
19. **Curry Jr TE, Osteen KG** 2001 Cyclic changes in the matrix metalloproteinase system in the ovary and uterus. Biol Reprod 64:1285–1296
20. **Young KA, Stouffer RL** 2004 Gonadotropin and steroid regulation of matrix metalloproteinases and their endogenous tissue inhibitors in the developed corpus luteum of the rhesus monkey during the menstrual cycle. Biol Reprod 70:244–252
21. **Terao Y, Kumano S, Takatsu Y, Hattori M, Nishimura A, Ohtaki T, Shintani Y** 2004 Expression of KiSS-1, a metastasis suppressor gene, in trophoblast giant cells of the rat placenta. Biochim Biophys Acta 1678:102–110
22. **Yan C, Wang H, Boyd DD** 2001 KiSS-1 represses 92-kDa type IV collagenase expression by down-regulating NF-κB binding to the promoter as a consequence of IκBα-induced block of p65/p50 nuclear translocation. J Biol Chem 276:1164–1172
23. **Curry Jr TE, Song L, Wheeler SE** 2001 Cellular localization of gelatinases and tissue inhibitors of metalloproteinases during follicular growth, ovulation, and early luteal formation in the rat. Biol Reprod 65:855–865
24. **Kinoshita M, Tsukamura H, Adachi S, Matsui H, Uenoyama Y, Iwata K, Yamada S, Inoue K, Ohtaki T, Matsumoto H, Maeda K** 2005 Involvement of central metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. Endocrinology 146:4431–4436
25. **Nekola MV, Coy DH** 1985 Direct and indirect inhibition of ovulation in rats by an antagonist of luteinizing hormone-releasing hormone. Endocrinology 116:756–760
26. **Yoshioka S, Fujii S, Richards JS, Espey LL** 2002 Gonadotropin-induced expression of pancreatitis-associated protein-III mRNA in the rat ovary at the time of ovulation. J Endocrinol 174:485–492
27. **Castellano JM, Navarro VM, Fernandez-Fernandez R, Nogueiras R, Tovar S, Roa J, Vazquez MJ, Vigo E, Casanueva FF, Aguilar E, Pinilla L, Dieguez C, Tena-Sempere M** 2005 Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptin in undernutrition. Endocrinology 146:3917–3925
28. **Tena-Sempere M, Barreiro ML, Gonzalez LC, Gaytan F, Zhang FP, Caminos JE, Pinilla L, Casanueva FF, Dieguez C, Aguilar E** 2002 Novel expression and functional role of ghrelin in rat testis. Endocrinology 143:717–725
29. **Caminos JE, Tena-Sempere M, Gaytan F, Sanchez-Criado JE, Barreiro ML, Nogueiras R, Casanueva FF, Aguilar E, Dieguez C** 2003 Expression of ghrelin in the cyclic and pregnant rat ovary. Endocrinology 144:1594–1602
30. **Dungan HM, Clifton DK, Steiner RA** 2006 Kisspeptin neurons as central processors in the regulation of GnRH secretion. Endocrinology 147:1154–1158
31. **Sekiguchi T, Mizutani T, Yamada K, Kajitani T, Yazawa T, Yoshino M, Miyamoto K** 2004 Expression of epiregulin and amphiregulin in the rat ovary. J Mol Endocrinol 33:281–291

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