Relationship and function of gonadotropin-releasing hormone and related bioactive peptides in rat granulosa cells

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ラットの顆粒層細胞におけるゴナドトロピン放出ホルモンと関連生理活性ペプチドの機能と相関

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Chapter 1 Introduction

Mammalian ovary contains follicles in various stages and corpus luteum. Primary follicles are already prepared in the ovary at birth after appropriate mitosis during embryonic stage [9, 11]. Follicles start to grow when animals reach puberty [14]. Rats show regular four or five day estrous cycle and ovulate 10 to 15 ova in the morning of estrous day [39]. Maturation of follicles is stimulated by pituitary follicle stimulating hormone (FSH) [70] and luteinizing hormone (LH) [13]. Almost double number of follicles grow in each cycle and half of them will not be ovulated. They are destined to atresia. Once ovulation occurs, theca cells and granulosa cells grow to fulfill the antrum of the ovulated follicle and they become corpus luteum [45]. Follicle and corpus luteum are both steroidogenic organ, but steroidogenic pathway is quite different. In a follicle, cholesterol to progesterone occurs solely in theca layer and progesterone is converted to estrogen in granulosa cells [69]. In contrast, only progesterone is produced in the corpus luteum. Cell differentiation from granulosa to luteal cells is called luteinization.

Luteinization accompanies tissue remodeling, the shift of steroidogenic system from estrogen to progesterone, exit cell cycle and changes in responsiveness to pituitary hormones [69]. LH surge induces ovulation and is thought to bring each changes for luteinization to granulosa cells. On the other hand, there are many local regulators reported in the ovary [69]. They include protein, peptide, steroid and lipids [35, 68]. They are involved in a vriety of changes in the ovary. Recently, gonadotropin releasing hormone (GnRH) and its related peptides (kisspeptin, neurokinin B (NKB) and dynorphin) were reported to express in the ovary and their expression changes in the afternoon of proestrus in rats by our laboratory [33].

GnRH is a hypothalamic neuropeptide hormone and stimulates pituitary gonadotropin secretion. GnRH is released into the hypophysial portal circulation and transported to the anterior pituitary in a pulsatile manner, where it binds to specific high-affinity receptors on the gonadotrope [18]. GnRH and its receptor are also expressed in the ovary [61]. Our laboratory

already has shown that GnRH augments annexin A5 (ANXA5) expression in the pituitary gonadotropes [28] and also in gonads [30, 82]. Ovarian GnRH is suggested to have a role in follicular atresia, luteolysis [1, 49] and luteal cell apoptosis [30]. GnRH agonist (GnRHa) was demonstrated to inhibit proliferation of ovarian cancer cell [21]. GnRHa suppresses proliferative activity of porcine granulosa cells in immature follicles and steroidogenic activity of granulosa cells in mature follicles[71]. Despite of these reports, the role of GnRH in the ovary is not well known yet.

ANXA5 is a member of the annexin family of calcium-dependent phospholipid-binding proteins [44, 77]. ANXA5 is expressed in a cell type-specific manner in various organs including reproductive organs (ovary and testis) [30, 82]. ANXA5 is used as a biomarker of GnRH action in various tissues [72]. It was demonstrated that GnRH stimulates ANXA5 expression and apoptosis in the corpus luteum of pseudopregnant rats [30]. ANXA5 is expressed only in the corpus luteum and not in granulosa cells [29]. Thus, ANXA5 synthesis may start during luteinization and GnRH is suggested to function during luteinization. Hence, it is of great interest to know the function of GnRH-ANXA5 in granulosa cells.

Kisspeptin is a hypothalamic peptide coded by KiSS-1 gene. KiSS-1 was first identified as metastasis-suppressor gene of human malignant melanoma [34]. Kisspeptin or metastin are the natural ligands of the orphan G protein-coupled receptor, GPR54 [32, 47]. Kisspeptin is a novel neuromodulator that acts upstream of GnRH in the hypothalamus [75]. Kisspeptin neurons co-expresses NKB and dynorphin at the arcuate nucleus (ARC) of the hypothalamus, called KNDy neurons [5]. KNDy neurons in the ARC are thought to be responsible for pulsatile GnRH and LH secretion [42]. NKB and neurokinin-3 receptor (NK3R) signaling stimulates kisspeptin release, whereas dynorphin and kappa opioid receptor (KOR) signaling suppresses the activation of KNDy neurons [76]. Interestingly, kisspeptin, dynorphin, NKB and their receptors (GPR54, NK3R, and KOR respectively) are also expressed in the ovary [7, 33, 38, 68]. Granulosa cells

were reported to have a characteristic of KNDy cells [33]. However, roles for KNDy in the ovary is not elucidated.

In this thesis, it was examined whether GnRH is involved in the process of luteinization and the relationship among GnRH, kisspeptin, dynorphin, and NKB in the granulosa-luteal cells. Finally, a function of ANXA5 in the granulosa cells during luteinization was also studied. Physiological roles for neuropeptides in the ovary will be discussed.

Chapter 2 Involvement of GnRH in luteinization process induced by hCG

Introduction

Gonadotropin-releasing hormone (GnRH) is a central hormone of mammalian reproduction [8]. It is well described as a hypothalamic neuropeptide that regulates gonadotropin (FSH and LH) secretion from pituitary gonadotropes and subsequently gonadotropin modifies the synthesis of steroid hormones of the gonad [25]. GnRH and its receptor are also expressed in various peripheral tissues including mammary gland, testis and ovary [54, 61, 82]. Ovarian GnRH has been reported to relate to follicular atresia and luteolysis [1, 49]. GnRHa has been shown to inhibit ovarian cancer cell growth [21]. GnRHa suppresses proliferative activity of porcine granulosa cells in immature follicles and steroidogenic activity of granulosa cells in mature follicles. GnRHa was also shown to stimulate apoptosis of granulosa cells regardless of follicular maturation stage [71]. Although it has been postulated that GnRH has important roles in the ovary, physiological function of GnRH in the ovary is still not clear.

ANXA5 is a member of the annexin family of calcium-dependent phospholipid-binding proteins, which comprises 12 members in mammals [44]. Studies from our group and others have reported that ANXA5 is expressed in a cell type-specific manner in the pituitary gland, ovary, testis, mammary gland, adrenal gland, thyroid gland, placenta, heart and muscle [17, 19, 29, 52, 54, 82]. Because ANXA5 expression is stimulated by GnRH not only in pituitary gonadotropes but also in peripheral tissues, it has been used as a biomarker of GnRH action in various tissues [55]. Our laboratory previously reported that GnRH stimulates ANXA5 expression and apoptosis in the corpus luteum of pseudopregnant rats [30]. Because ANXA5 is expressed in the corpus luteum and not in granulosa cells [29], its synthesis is hypothesized to begin during luteinization and hence GnRH is suggested to function during luteinization.

In the estrous cycle, ovulation is induced by LH surge. LH surge also causes transformation of granulosa cells to luteal cells and the process is called luteinization. Granulosa cells that synthesize estrogen differentiate into luteal cells that produce progesterone. Luteinization is characterized by abundant cell proliferation, exit from the cell cycle and angiogenesis [69]. The expression rate of FSH receptor (FSHR) mRNA decreases and LH receptor (LHR) mRNA increases in luteal cells [62]. Although all of these changes are triggered by LH surge, local regulators would be involved in the process. It is hypothesized that LH surge would induce GnRH synthesis in the ovary and GnRH would be involved in the process of luteinization.

To clarify this hypothesis, the expression and effects of ovarian GnRH on the differentiation of granulosa to luteal cells were examined in this chapter.

Materials and methods

1. Animals

Immature female Wistar-Imamichi rats bred in our laboratory were used in all experiments. The day of parturition was designated as day 0. Pups number was adjusted to 8 on day 1. The pups were weaned on day 21 after birth. Rats were housed at 23±3°C with a light-dark cycle of 14L:10D (light on 5:00–19:00 h). Food (laboratory chow, CE-2, Oriental Co., Tokyo, Japan) and tap water were supplied *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committee of Kitasato University (Approval No. 16-028).

2. Immature female rat model

a. In vivo experiment

Immature female 25-day old rats were given pregnant mare serum gonadotropin (PMSG, 15 IU/0.15 ml, IP, Zenoaq, Koriyama, Japan) for inducing follicular growth. After 48 h of PMSG administration, rats were given human chorionic gonadotropin (hCG, 20 IU/0.2 ml, IP, NOVARTIS, Tokyo, Japan) or saline (0.2 ml). Experimental protocol is summarized in Fig. 2-1.

b. In vitro experiment

PMSG (15 IU/0.15 ml, IP*)* was administered to immature female rats on day 25 to induce follicular growth. Rats were euthanized cervical dislocation under deep anesthesia with diethyl ether or isoflurane 48 h after PMSG administration. Ovaries were collected and placed in growth medium. Then, granulosa cells were dissociated (Fig. 2-1).

3. Sample preparation and collection

a. Ovary samples for RNA extraction

On day 25 after birth, rats were given PMSG and euthanized by cervical dislocation under deep anesthesia at 1, 3, 6, 12 and 24 h after the administration of hCG or saline (n=5) on day 27. RNA was extracted from whole ovary by Trizol Reagent. Ovaries were homogenized with a micro pestle in 1 ml Trizol Reagent and then snap frozen in liquid nitrogen. Samples were kept at -80°C until RNA extraction. The samples were homogenized again by Polytron homogenizer before RNA extraction.

b. RNA extraction from Granulosa cells

Granulosa cells were homogenized by pipetting up and drown 30 times in 0.5 ml Trizol reagent and then kept at -80°C until RNA extraction.

c. Blood collection for progesterone assay

To examine changes in plasma progesterone concentrations, blood samples were taken from the abdominal aorta (needle 23 gauge) under anesthesia with isoflurane at 1, 3, 6, 12 and 24 h after the administration of hCG or saline (n=5). Heparinized blood was centrifuged (700×g, 10 min, 4°C) and plasma was stored at -30°C until the assay of progesterone by time-resolved immunofluorometric assay (TR-IFMA). Plasma sample of 0.2 ml each was used for progesterone assay.

d. Conditioned medium for progesterone assay

At the end of culture, growth medium (1 ml) was collected and put into a glass tube with a cap. Samples were kept at -30°C until the extraction for progesterone assay by TR-IFMA.

e. Ovary for immunohistochemistry

Immunohistochemical analysis of 3β-Hydroxysteroid dehydrogenase (3β-HSD), ovaries were harvested from rats of 25 day old after birth and 48 h after PMSG administration on day 25. Immunohistochemical analysis of ANXA5, rats were treated as *in vivo* model described above. Ovaries were harvested 48 h after PMSG and 24 h after hCG administration. Rats were deeply anesthetized with diethyl ether and slowly infused 50 ml of PBS through left ventricle after cutting off the right atrium. Then solution was changed to 50 ml of 4% paraformaldehyde solution (4% PFA). Ovaries were removed and further fixed with 4% PFA overnight at 4˚C.

4. RNA extraction and reverse-transcription to cDNA

a. Reagents, solutions, and equipment

Trizol Reagent (Thermo Fisher Scientific, Tokyo)

UltraPure™ DNase/RNAse-Free Distilled Water (UltraPure water, Invitrogen Life Technologies, Grand Island, NY, USA)

Chloroform (Kanto Chemical Co., Tokyo, Japan)

2-propanol (Kanto Chemical Co.)

75% Ethanol (Kanto Chemical Co.)

High-Capacity cDNA Reverse Transcription kits (Applied Biosystems, Foster, CA, USA)

NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA)

Veriti™ 96 well Thermal cycler (Applied Biosystems)

b. Protocol

RNA was extracted from samples by Trizol reagent according to the protocol supplied by the manufacturer. Total RNA was measured with NanoDrop 2000 spectrophotometer. Ovary RNA concentration was adjusted to 500 ng/µl UltraPure water. Granulosa cell RNA was adjusted to 250 ng/µl. RNA samples were subjected to reverse transcription with High-Capacity cDNA Reverse Transcription kits using Veriti™ 96 well Thermal cycler. Briefly, 10 µl of RNA was mixed with 2 µl of 10× RT Random Primers, 2 µl of 10×RT buffer, 0.8 µl of 25× dNTP Mix (100 mM), 1 µl of MultiScribe™ Reverse Transcriptase and 4.2 µl of UltraPure water. Final volume was 20 µl. Reverse transcription was performed at 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min. Samples were used directly for real time PCR analysis or stored at -30 °C.

5. Quantitative Real-time PCR

a. Reagents, solutions, and equipment

THUNDERBIRD®SYBR® qPCR Mix (TOYOBO, Osaka, Japan)

Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK)

Sequences for forward and reverse primers were presented in Table 1.

StepOnePlus Real-time PCR system (Applied Biosystems)

96 well plate real time PCR (Thermo scientific)

UltraPure water

b. Protocol

The reaction mixture contained 1 µl of complementary DNA (cDNA) sample, 0.03 µl (100 µmol) of forward and reverse primers, 5 µl of THUNDERBIRD®SYBR® qPCR Mix, 0.2 µl of 50X ROX reference dye and 3.74 µl of UltraPure water. Final volume was 10 µl. Power SYBR Green PCR Master Mix, the reaction mixture of each sample contained 1 µl of cDNA

sample, 0.03 µl (100 µmol) of forward and reverse primers, 5 µl of Power SYBR Green PCR Master Mix and 3.94 µl of UltraPure water. Final volume was 10 µl. The quantitative real-time PCR was performed with 50 cycles of 95 °C for 15 sec, 60 °C for 1 min and one more cycle for melting curve result by StepOnePlus Real-time PCR system. The delta-delta Ct method was used for the normalization of gene expression. RPL19 was the reference gene.

6. Time-resolved immunofluorometric assay (TR-IFMA) of progesterone

Progesterone was measured by TR-IFMA**.**

a. Reagents, buffers, and equipment

Hexane (Kanto Chemical Co., Tokyo, Japan)

Assay buffer (pH 7.8)

- 50 mM tris-HCl buffered saline (Kanto Chemical Co.)
- 0.5% bovine serum albumin (BSA, Sigma-Aldrich, Inc., St. Louis, MO, USA)
- 0.05% Bovine gamma globulin (Millipore Corp., Billerica, MA, USA)
- 0.01% Tween 20 (Kanto Chemical Co.)
- 20 µM DTPA (Dojindo, Kumamoto, Japan)
- Phenol red 15 mg/l (WAKO PURE CHEMICAL INDUSTRIES. LTD., Osaka, Japan)
- 0.05% NaN_3 (Sodium azide, Kanto Chemical Co.)
- Milli Q water

Coating buffer

- 50 mM K_2HPO_4 buffered saline (Kanto Chemical Co.)
- 0.05% NaN_3 (Sodium azide, Kanto Chemical Co.)
- Milli Q water

Blocking buffer

- 50 mM Na_2HPO_4 (Kanto Chemical Co.)
- 0.1% BSA (BSA, Sigma-Aldrich, Inc., St. Louis, Mo)
- Milli Q water

Washing buffer

- 50 mM tris-HCl buffered saline (pH 7.8)
- 0.05% Tween 20 (Kanto Chemical Co.)

BSA conjugated progesterone (4-PREGNEN-3, 20-DIONE 3-O-CARBOXYMETHYLOXIME: BSA, Steraloids Inc., Newport, RI, USA)

Eu-labelling

- Eu-labelling reagent DELFIA® Eu-Labelling Kit (PerkinElmer, Turku, Finland)
- Anti-progesterone IgG solution (Homemade)
- Sodium carbonate buffer (pH 9.8)

Protein G HP Spin Trap (GE Healthcare life sciences, Uppsala, Sweden)

DELFIA® Enhancement Solution (PerkinElmer™, Wallac Oy)

96-well assay plate (Thermo Scientific, Roskilde, Denmark)

PerkinElmer's 2030 multi label reader

ImmunoWash™1575 microplate washer (Bio-Rad)

b. Protocol

Milli Q water (1 ml or 0.5 ml) was added to plasma and medium samples respectively for increasing sample volume. Progesterone fraction was extracted from plasma or medium with hexane. The hexane phase was decanted into a new glass tube and evaporated with nitrogen gas. The dried samples were dissolved in 2 ml assay buffer. BSA-conjugated progesterone was diluted with coating buffer to 3 µg/ml and added to each well of a 96-well assay plate at a volume of 100 µl/well, after which the plate was shaken at room temperature overnight. Then the wells were

washed three times with wash buffer, then 200 µl/well of blocking solution was added to the wells and the plate was shaken at 4°C overnight. Anti-progesterone IgG was prepared from homemade anti-progesterone rabbit serum using Protein G HP Spin Trap and IgG was labeled with Europium (Eu) using DELFIA® Eu-Labelling Kit. Eu-labeled IgG was diluted with assay buffer and added to the plate at a volume of 100 µl/well. Progesterone standards and samples (100 µl/well) were added to the plate. Fluorescence was measured by PerkinElmer's 2030 multi label reader after the addition of 100 µl enhancement solution and then shaken for 5 min.

7. Histology

Paraffin section preparation

a. Reagents, solutions and equipment

0.1 M Phosphate buffered saline (PBS, pH 7.4)

- 137 mM NaCl (Kanto Chemical Co.)
- 8.1 mM Na_2HPO_4 anhydrous or $Na_2HPO_4 \cdot 12H_2O$ (Kanto Chemical Co.)
- 2.68 mM KCl (Kanto Chemical Co.)
- 1.47 mM KH_2PO_4 (Kanto Chemical Co.)

4% paraformaldehyde solution (4% PFA)

- Paraformaldehyde (Merck KGaA, Darmstadt, Germany)
- 0.1 M PBS (pH 7.4)

Paraffin (Merck KGaA, Darmstadt, Germany)

Microtome (Leica RM2245)

Incubator

Micro slide glass (Matsunami Glass Ind., Ltd, Osaka, Japan)

b. Protocol

Ovaries were collected and fixed in 4% PFA in 0.1 M PBS at 4°C overnight. They were then incubated with 0.1 M PBS (pH 7.4) at 4°C overnight. Dehydration and paraffin embedding were performed as per standard procedures. Sections of 3 µm thickness were made and dried overnight in an incubator at 37°C.

8. Immunohistochemistry

a. Reagents, solutions and equipment

Antibody-binding buffer (ABB, pH 7.4)

- 50 mM tris-HCl buffered saline
- 5 mM EDTA (Kanto Chemical Co.)
- 0.25% gelatin (Kanto Chemical Co.)
- 0.05% Nonidet P-40 (Sigma-aldrich®)

1% hydrogen peroxide (H_2O_2)

- Hydrogen peroxide (Kanto Chemical Co.)
- Methanol (Kanto Chemical Co.)

Blocking solution

- 2.5% normal horse serum (ImmPRESS anti-rabbit IgG, Vector Laboratories, Burlingame, CA, USA)

Primary antibody

- Rabbit polyclonal antiserum against ANXA5 developed in our laboratory (1:10,000 dilution)

- Rabbit polyclonal antiserum against 3β-HSD developed in our laboratory (1:5,000 dilution)
- Normal rabbit serum (1: 10,000 or 1: 5,000 dilution)
- 3% Fetal bovine serum (Gibco® Life Technologies, Grand Island, NY, USA)

Second antibody

- Anti-rabbit IgG (ImmPRESSTM horseradish peroxidase reagent kit, Vector Laboratories, Burlingame, CA, USA)

Diaminobenzidine (Roche, Mannheim, Germany)

Hematoxylin (MUTO PURE CHEMICALS CO., LTD, Tokyo, Japan)

Mounting medium MX (Matsunami, Kishiwada, Japan)

Coverslip (Matsunami Glass Ind., Ltd, Osaka, Japan)

Light microscope

b. Protocol

After dewaxing, sections were soaked in 1% hydrogen peroxide in methanol for 20 min to suppress internal peroxidase, and washed with ABB for 5 min for 3 times. Then the sections were incubated with 2.5% normal horse serum for 1 h at room temperature in dark and moist box. Sections were incubated overnight at 4°C with a 1:10,000 dilution of rabbit polyclonal antiserum against ANXA5 or a 1: 5,000 dilution of rabbit polyclonal antiserum against 3β-HSD. Normal rabbit serum used for negative control. Then the sections were washed with ABB 5 min for 3 times and incubated with second antibody for 2 h. The specimens were washed again with ABB 5 min for 3 times. Visualization was performed with diaminobenzidine and counter-stain was done with hematoxylin. Finally, the samples were dehydrated and mounted on coverslips.

9. Primary culture of granulosa cells

a. Reagents, solutions and equipment

Growth medium

- DMEM F-12 (Gibco® Life Technologies, Grand Island, NY, USA)
- 10 mM HEPES (Gibco® Life Technologies)
- 1% antibiotic-antimycotic (Gibco® Life Technologies)
- 10% fetal bovine serum (Gibco® Life Technologies)

24-well plates (IWAKI brand, ASAHI Glass CO., LTD., Tokyo, Japan)

26-gauge needle

Pasteur Pipette

Falcon® 70 μm pore Cell strainer (Corning Incorporated, corning, NY, USA)

Stereo microscope (Leica)

Counting chamber

b. Protocol

Rats were treated with PMSG as *in vitro* model (Fig. 2-1B). Ovaries were excised under a stereo microscope. Granulosa cells were liberated from large follicles to the medium by puncturing the antral follicle with a 26-gauge needle. Cell dissociation was facilitated by efflux and influx of cell suspension through a Pasteur pipette. Cells were filtered through a cell strainer and centrifuged at 133 x g for 10 min, after which the supernatant was discarded. Cells were re-suspended with growth medium and counted. The cells were adjusted to a density of 500,000 cells/ml and seeded onto a 24-well culture plate with a volume of 1 ml/well. Cells were pre-incubated for 24 h before the experiments. Then the medium was replaced with fresh growth medium containing reagents according to each experimental design.

10. Experimental design for primary culture of granulosa cell

To examine the effects of hCG on progesterone production of granulosa cells, the cells were treated with different concentration of hCG (0.001, 0.01, 0.1 IU/ml) for 3 h.

To examine the effects of hCG on GnRH mRNA expression and effects of GnRHa on ANXA5 mRNA expression of granulosa cell, cells were incubated with or without hCG 0.01 IU/ml and with or without GnRH agonist (GnRHa, Fertirelin acetate, Des-Gly10 [Pro9]-GnRH ethylamide, Takeda Pharmaceuticals Co., Osaka, Japan) for 3 h.

To examine the effects of GnRH (Sigma), GnRHa and Cetrorelix (GnRH antagonist) on progesterone production stimulated by hCG, granulosa cells were incubated with 0.01 IU/ml hCG, 10^{-7}M GnRH, 10^{-8}M GnRHa and 10^{-8}M GnRH antagonist for 3 and 6 h.

To examine the effects of GnRHa on the mRNA expression of various genes, the cells were treated with 10^{-8}M GnRHa with or without 0.01 IU/ml hCG for 3 h.

11. Immunocytochemistry

a. Reagents, solutions and equipment

Coating of glass coverslips with poly-L-lysine hydrobromide

- 0.5 mg/ml Poly L lysine hydrobromide (Sigma-Aldrich, Inc., St. Louis, Mo, USA)
- Coverslip (Matsunami Glass Ind., Ltd, Osaka, Japan)

Micro slide glass (Matsunami Glass Ind., Ltd, Osaka, Japan)

4% PFA

Cold 99.5% Acetone (Kanto Chemical Co., Tokyo, Japan)

0.1 M PBS (pH 7.4)

Antibody-binding buffer (ABB, pH 7.4)

- 50 mM tris-HCl buffered saline
- 5 mM EDTA
- 0.25% gelatin
- 0.05% Nonidet P-40

Blocking solution

- 3% Fetal bovine serum (Gibco® Life Technologies, Grand Island, NY, USA)

Primary antibody

- Anti 3β-HSD (1:5,000 dilution) rabbit serum
- Normal rabbit serum (1: 5,000 dilution)
- 3% Fetal bovine serum (Gibco® Life Technologies, Grand Island, NY, USA)

Second antibody

- Alexa 488 Goat anti rabbit IgG (1:1,000 dilution) (Life Technologies Corporation, Oregon, USA)

Mounting medium

- VECTASHIELD® mounting medium for fluorescence with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA)

Confocal laser scanning microscopy

b. Protocol

Ovaries and granulosa cells were prepared as same *in vitro* protocol (2-1B) and primary culture of granulosa cell. Granulosa cells were adjusted to 100,000 cells/ml for each sample and incubated for 24 h. Growth medium was replaced with 4% PFA for 10 to 15 min at room

temperature. Cold acetone (30°C) was added to samples for 2 to 3 sec. The samples were rinsed with PBS for 5 min for 3 times and then with ABB for 5 min. Fetal bovine serum was added to samples for 1h at room temperature to prevent non-specific staining. Primary antibody was anti-rabbit serum 3β-HSD. Normal rabbit serum was used for negative control. Samples were incubated with primary antibody for overnight at 4°C and washed with ABB for 5 min 5 times. Then, samples were incubated with Alexa 488 Goat anti rabbit IgG for 2 h at room temperature and rinsed with ABB for 5 min 3 times. Specimens were mounted with VECTASHIELD® mounting medium for fluorescence with DAPI.

12. Statistical analysis

Statistical significance was assessed by analysis of variance followed by Tukey's test, with p-values less than 0.05 considered statistically significant. Single comparisons were analyzed by Student's *t*-test with p-values less than 0.01 considered statistically significant.

Results

1. Changes in plasma progesterone levels in response to hCG administration

Plasma progesterone was dramatically increased after hCG administration to PMSG pre-treated immature female rats (Fig. 2-2). A peak was observed at 6 h (P<0.05) then the levels gradually decreased (Fig. 2-2). The peak at 6 h was five times more than at 1 h after hCG administration. There was no obvious change in saline treated group.

2. ANXA5 expression in granulosa cells

ANXA5 expression in the ovary of gonadotropin treated immature rat was examined by immunohistochemistry. Rats of 25-day old were given PMSG and then hCG two days later (Fig. 2-1A). ANXA5 was observed mainly in interstitial tissues with slightly positive theca layer. Granulosa cells were negative for ANXA5 (Fig. 2-3a). ANXA5 appeared on luteal cells after 24 h of hCG treatment (Fig. 2-3b, d).

3. Changes in ovarian GnRH mRNA expression rates after hCG administration

PMSG was administered to immature rats on day 25 and ovarian GnRH mRNA expression was examined after the administration of hCG or saline on day 27 (Fig. 2-4). GnRH mRNA expression was significantly higher in hCG-treated group at 3 h (P<0.05, Fig. 2-4). There was no significant difference between hCG and saline groups except for the time point of 3 h.

4. Confirmation of 3β-HSD in the ovary and granulosa cells after dissociation

For, immunohistochemical analysis of 3β-HSD in the ovary, ovaries were collected from rats on day 25 after birth (Fig. 2-5A). While granulosa cells were negative for 3β-HSD, after 48 h of PMSG treatment, granulosa cells became positive for 3β-HSD (Fig. 2-5B). Granulosa cells

were harvested on day 27 from PMSG treated rats (Fig. 2-1B). Then granulosa cells were incubated for 24 h and subjected to immunocytochemistry for 3β-HSD. It was demonstrated that majority of dissociated cells were 3β-HSD positive while negative cells were also included (Fig. 2-6).

5. Changes in progesterone levels in response to hCG

Primary culture of granulosa cells was established 48 h after PMSG administration (Fig. 2-1B). After 24 h of pre-incubation, cells were challenged with hCG and the dose-dependent response in progesterone production was confirmed by 3 h incubation (Fig. 2-7). Granulosa cells responded to 0.001 IU/ml hCG to produce a significant amount of progesterone ($P<0.05$). Hereafter, 0.01 IU/ml hCG was used for each experiment.

6. The effects of hCG on GnRH and GnRHa on ANXA5 expression in granulosa cells

In the primary culture of granulosa cells, GnRH mRNA expression was significantly increased by hCG during 3 h incubation. In turn, ANXA5 mRNA expression was increased by the treatment with GnRHa (Fig. 2-8).

7. The effects of GnRH, GnRHa, and GnRH antagonist on the progesterone production

Granulosa cells were treated with a combination of GnRH, Cetrorelix (GnRH antagonist) or GnRHa with hCG to examine their effects on the hCG-stimulation of progesterone production in the primary culture of granulosa cells. The results showed that GnRH, GnRHa and GnRH antagonist all suppressed progesterone production stimulated by hCG at 3 and 6 h (Fig. 2-9). The effect of GnRH on progesterone at 6 h was not significant even though there was still a suppressive tendency on progesterone production. Incubation only with GnRH, GnRH antagonist or GnRHa showed no effect on basal progesterone production.

8. The effects of GnRHa on gene expression in granulosa cells

The effects of GnRHa on the expression of genes related to luteinization were examined. To this end, the mRNA expression of LHR, FSHR, p21, p27, FOXO1 and PRLR were measured. GnRHa affected the expression of all genes examined. While hCG showed a tendency to increase the expression of LHR mRNA expression, GnRHa itself and addition of GnRHa to hCG significantly augmented LHR expression (Fig. 2-10A). GnRHa and hCG showed additive effects on the mRNA expression of LHR mRNA. On the contrary, FSHR mRNA expression was suppressed by GnRHa and GnRHa enhanced the suppressive effect of hCG (Fig. 2-10B). The effects of GnRHa on the endogenous Cdk inhibitors, p21 and p27, were stimulatory and inhibitory, respectively (Fig. 2-10C, D). GnRHa significantly increased p21 expression, but hCG did not show any effect (Fig. 2-10C). GnRHa and hCG synergistically reduced the mRNA expression of p27, FOXO1 and PRLR (Fig. 2-10D, E and F).

Discussion

The results of this study clearly demonstrate that GnRH synthesized in the ovary is involved in the process of hCG stimulation, the biological equivalent of LH. It was confirmed that PMSG treatment facilitated follicular growth and that hCG stimulated progesterone production. It was observed that hCG administration made granulosa cells ANXA5-positive after 24 h. As ANXA5 would be stimulated by local GnRH [30], this result suggests a GnRH action on granulosa cells after hCG administration. Then it was shown that ovarian GnRH mRNA expression was significantly increased until 3 h after hCG administration.

Primary culture of granulosa cells was established from PMSG stimulated follicles for further analysis. It was first confirmed that the liberated follicular cells consist mainly of 3β-HSD positive cells. Although it is not known what 3β-HSD negative cells are, liberated cells were used for the primary culture of granulosa cells. It would be possible that 3β-HSD negative cells are premature luteal cells, germ cells or contaminated interstitial cells. Only theca cells but not granulosa cells of smaller follicle were reported to express 3β-HSD [10]. FSH (PMSG) induces LHR and 3β-HSD. In the present study, majority of granulosa cells became 3β-HSD positive 48h after PMSG treatment. So, cells negative for 3β-HSD would be not well differentiated granulosa cells. Even though the primary culture of granulosa cells of this study are not homogeneous cell population, they would represent a characteristic of preovulatory granulosa cells and luteal cells after hCG stimulation [6]. Using these cells, GnRH mRNA expression was confirmed to increase by hCG treatment *in vitro*, and that ANXA5 mRNA expression was stimulated by GnRHa during 3 h incubation of primary granulosa cells. GnRH is suggested to be an autocrine of granulosa cells. It may be necessary to confirm which cells are the source of GnRH. Present data suggest that GnRH produced in the follicle may play a role in the differentiation of granulosa cells to luteal cells under the effect of LH.

The relationship between hCG and GnRH was examined during luteinization. Progesterone production of primary culture of granulosa cells was stimulated by hCG. GnRH, GnRHa and GnRH antagonist all suppressed hCG enhanced progesterone production. The effect of GnRHa on granulosa steroid production have been studied and results are varied among reports [62, 71]. It seems that the response of granulosa cells to GnRH would relate to a stage of a follicle [71]. In other words, complete differentiation of granulosa cells to luteal cells would require *in vivo* environment. It was interesting that GnRH antagonist also showed a similar negative effect on progesterone production in this study. GnRH antagonist shows antagonist activity at the gonadotrope on gonadotropin secretion [57] and also in the ovary [50]. In latter case, folicular atresia is inhibited by GnRH antagonist. So, it is suggested that there is different signal transduction mechanism for GnRH stimulation in the gonadotrope and at differentiation to luteal cells.

ANXA5 is well known for its ability to detect early apoptotic cells [31]. Its physiological function remains unknown. It was previously demonstrated that GnRH stimulates ANXA5 synthesis and ANXA5 increases gonadotropin secretion from the pituitary gonadotrope [28]. It was also examined the relationship between GnRH and ANXA5 in peripheral tissues and found that GnRH stimulates the expression of ANXA5 in the corpus luteum, mammary epithelial cells and Leydig cells [30, 54, 82]. In the corpus luteum of pseudopregnant rats, suppression of prolactin secretion caused an increase in GnRH-mediated ANXA5 expression and the apoptosis of luteal cells [30]. In addition, GnRH increased ANXA5 expression and the apoptosis of mammary epithelial cells after weaning [54]. It was demonstrated that the expression of the GnRH receptor increases in mammary tissues after weaning [72]. Together, these results suggest close functional relationship between GnRH and ANXA5. By observing the expression of ANXA5, it was found that the action of GnRH in Leydig cells are influenced by LH [82].

In this study, ANXA5 was demonstrated to increase in granulosa-luteal cells 24 h after hCG administration. As GnRHa stimulated ANXA5 mRNA expression in granulosa cell culture, it is suggested that GnRH mediates hCG actions in granulosa cell. We previously observed that LH stimulation of ANXA5 expression in Leydig cell is mediated by testicular GnRH [82]. A similar relationship was thought to present in granulosa cells. In this study, GnRH and GnRHa suppressed progesterone production that was augmented by hCG. These results indicate that the GnRH action on granulosa cell is not a simple enhancement of hCG action. Thus, we decided to examine the effects of GnRH on the expression of various genes.

Luteinizaton is the process of cell proliferation, exit from the cell cycle, and changes in steroid synthesis [69]. In granulosa cells, the FSHR is decreased and the LHR is increased after LH surge [62, 69]. The steroid synthesis system shifts from for estradiol to for progesterone [69]. The present data demonstrated an increase in the mRNA expression of LHR and a decrease in FSHR upon treatment with hCG and GnRHa. Interestingly, hCG itself did not show a significant effect on LHR; however, it augmented the GnRH stimulation of LHR mRNA. FSHR mRNA was suppressed slightly but significantly by hCG. GnRH significantly reduced the expression of FSHR mRNA and augmented the suppressive effect of hCG. Present results on LHR and FSHR indicate that GnRH and hCG collaborate to proceed the differentiation of granulosa to luteal cells. If the augmentation of GnRH by enough hCG stimulation was attained, hCG would show more apparent effects on LHR gene expression. Changes in FSHR and LHR are the most apparent feature of the differentiation of granulosa cells. GnRH expression in the follicle was stimulated relatively early phase of hCG stimulation in the present study. It is hypothesized the physiological sequence of events in the follicle that LH surge would induce follicular expression of GnRH to facilitate luteinization.

The cyclin-dependent kinase inhibitors, p21 and p27, were examined in the early phase of hCG action. It has been reported that the expression of p21 and p27 change along with

luteinization [22, 56]. GnRHa augmented p21 and suppressed p27 mRNA expression. The effects of GnRHa on p27 was additive to hCG action although the difference between GnRHa group and hCG+GnRHa group was not significant. These results are again consistent with the idea that GnRH is involved in the transition of granulosa cells to luteal cells. FOXO1 is a transcription factor that is decreased by FSH action in granulosa cells [64, 65]; its expression is further suppressed by LH. Present data demonstrated that the synergistic actions of hCG and GnRH on FOXO1 expression. It was shown that PRLR was decreased by hCG and GnRH in this study. Another study showed that granulosa cells obtained from hypophysectomized rats treated with 17β-estradiol, FSH and hCG showed the angmentation of PRLR. So, PRLR is thought to increase after full-luteinization [59].

Summary of interaction in this chapter was shown in Fig. 2-11. GnRH mRNA expression in the granulosa cell is stimulated by hCG/LH (Fig. 2-11a). LH surge of proestrous day would trigger the sequence of events those would start from the augmentation of local GnRH synthesis in a provulatory antral follicle. GnRH mediates at least partly the action of hCG/LH. GnRH stimulates ANXA5, p21, LHR mRNA (Fig. 2-11b), while GnRH is suppressive on FSHR, p27, FOXO1 and PRLR mRNA expression (Fig. 2-11c). Most of these effects are augmentation of hCG action. As hCG stimulates GnRH expression in the granulosa cells, GnRH expression would be a part of luteinization process.

The present study showed that mature granulosa cells synthesize GnRH under the effects of hCG (probably LH surge), and that GnRH at least partly mediates the effects of hCG/LH on the transition of granulosa cells to luteal cells. Thus, GnRH may be a local regulator of cell differentiation and ANXA5 may be involved in the process of GnRH action.

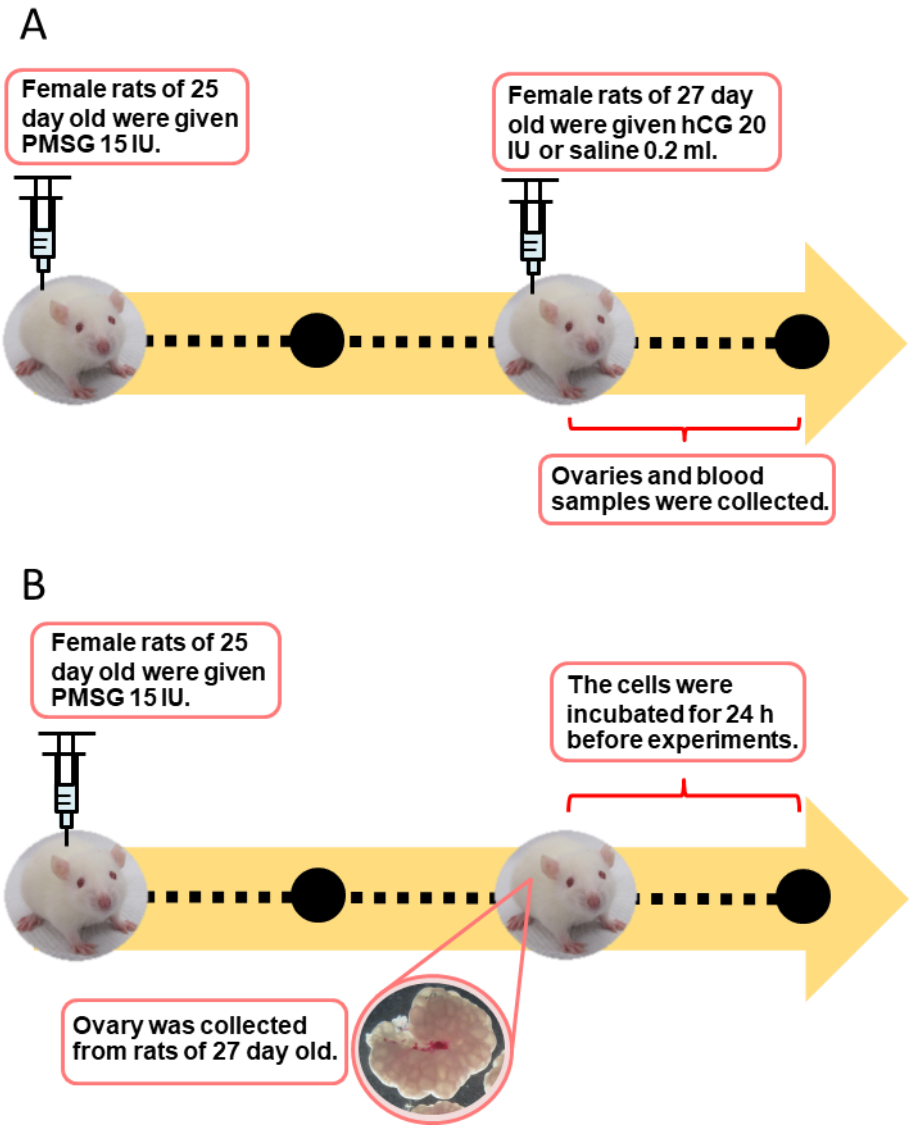

Fig. 2-1 Protocols of *in vivo* and *in vitro* experiments

In vivo experiment, rats were administered hCG 20 IU/0.2 ml after 48 h of PMSG 15 IU/0.15 ml treatment and then ovaries and blood samples were collected (A). *In vitro* experiment, rats were given PMSG 15 IU/0.15 ml and ovaries were harvested for granulosa cell dissociation after that 48 h (B).

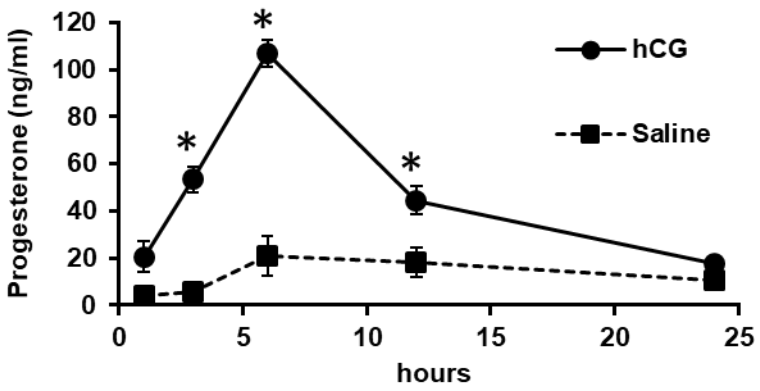

Fig. 2-2 Changes in progesterone levels of immature PMSG treated rats in response to hCG
PMSG-treated rats were administered hCG or saline after 48 h of PMSG treatment and plasma samples were collected after hCG or saline administration for 1, 3, 6, 12 and 24 h. Values are mean±SEM of five rats. Asterisk indicates significant difference from the saline-treated control of the same sampling point ($P<0.05$).

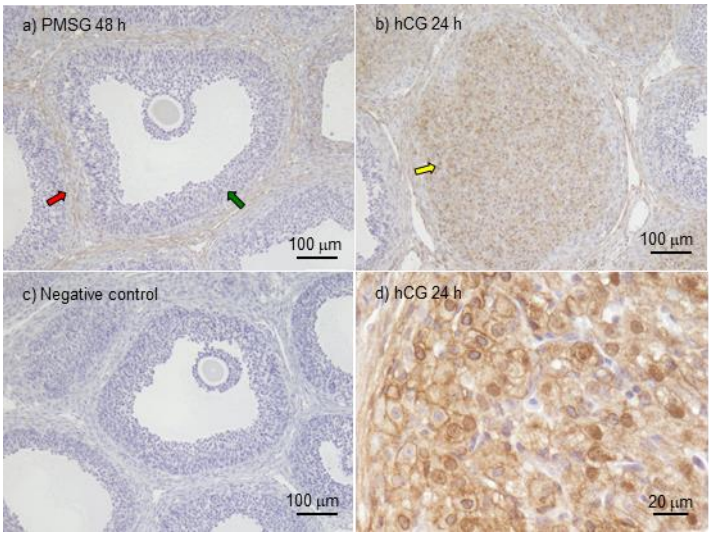

Fig. 2-3 Expression of ANXA5 in the corpus luteum after hCG treatment

Immunohistochemical analysis of ANXA5 in the ovary. a) PMSG (15 IU) was administered to 25-day-old rats. Red arrow shows the interstitial cell layer and green arrow indicates granulosa layer. b) Ovaries were collected 48 h after PMSG treatment and 24 h after hCG administration. Yellow arrow is corpus luteum. c) Negative control of immunohistochemistry. Normal rabbit serum was used instead of antiserum. d) Corpus luteum of hCG treated rat as b) in higher magnification.

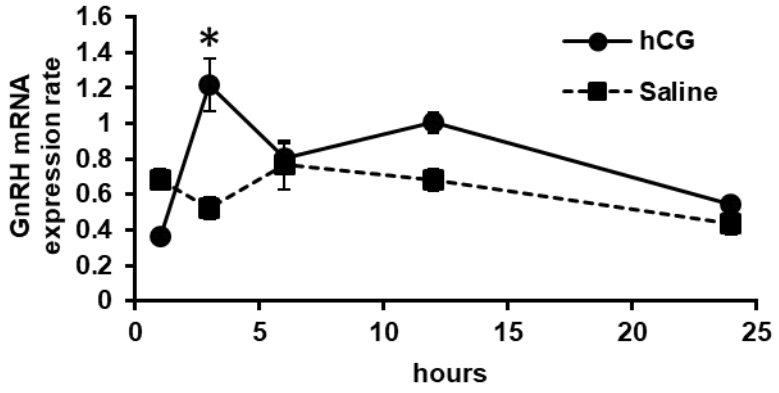

Fig. 2-4 Changes in ovarian GnRH mRNA expression rates in response to hCG

PMSG-treated rats were administered hCG or saline after 48 h of PMSG treatment and ovary samples were collected after hCG or saline administration at 1, 3, 6, 12 and 24 h. Values are mean±SEM of five rats. Asterisk indicates significant difference from the saline-treated control of the same sampling point (P<0.05).

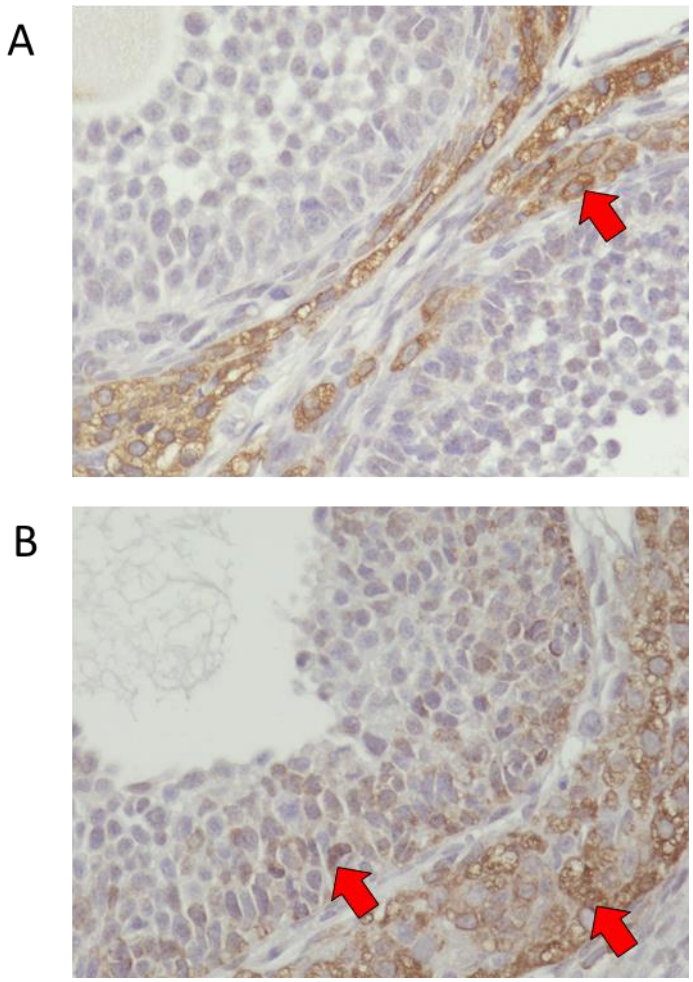

Fig. 2-5 Expression of 3β-HSD in the granulosa cells after PMSG treatment

Immunohistochemical analysis of 3β-HSD in the ovary. A) 25 day old of rat. B) Ovaries were collected 48 h after PMSG treatment to 25 day old rats. Red arrows indicate immunoreaction for 3β-HSD.

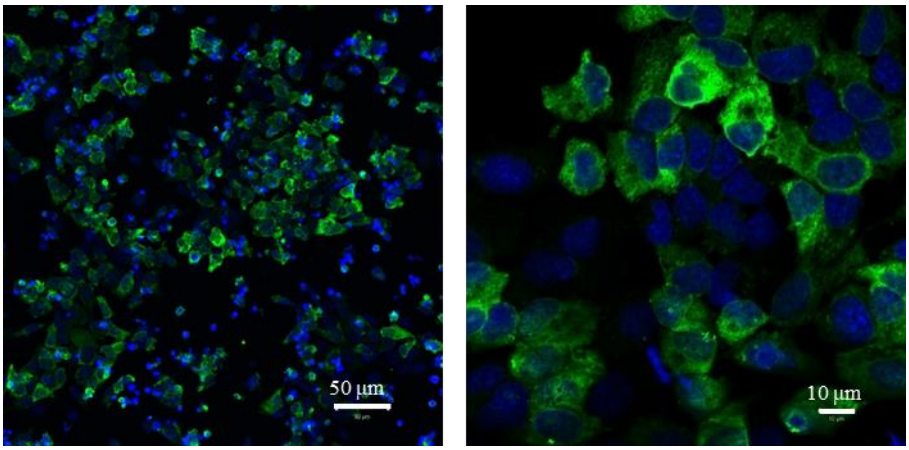

Fig. 2-6 Immunopositive cells for 3β-HSD in the primary culture of granulosa cell

Granulosa cells were incubated for 24 h after dissociation and subjected to the immunocytochemistry for 3β-HSD. Green color is 3β-HSD and blue color is DAPI.

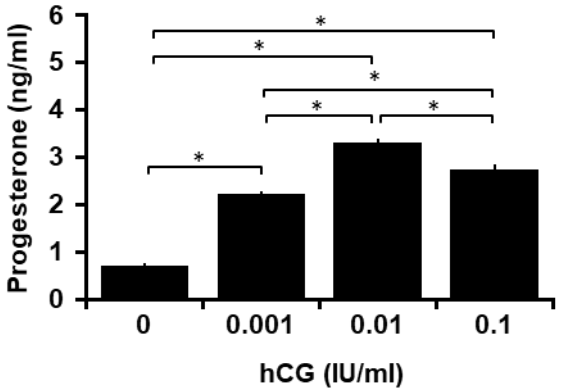

Fig. 2-7 Changes in progesterone levels in response to hCG

Granulosa cells were treated with various concentrations of hCG (0.001, 0.01, 0.1 IU/ml) for 3 h. Values are mean±SEM (n=6). Asterisks indicate values that are significantly different between groups (P<0.05).

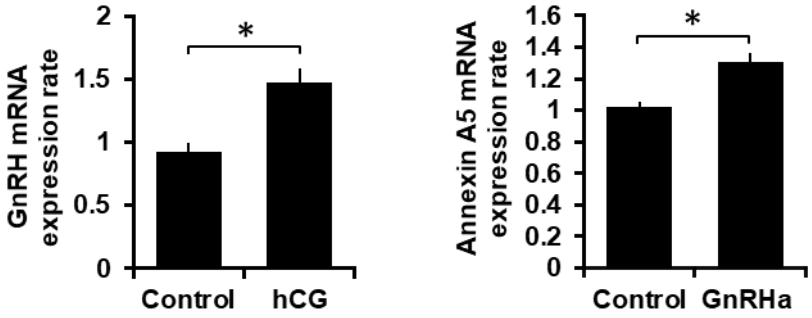

Fig. 2-8 The effects of hCG on GnRH and GnRHa on ANXA5 expression in the primary culture of granulosa cell

Effects of hCG (0.01 IU/ml) on GnRH mRNA expression and effects of GnRHa (10^{-8} M) on ANXA5 mRNA expression in the primary culture of granulosa cells were examined respectively. Values are mean±SEM (n=6). Asterisk shows significant difference (P<0.01).

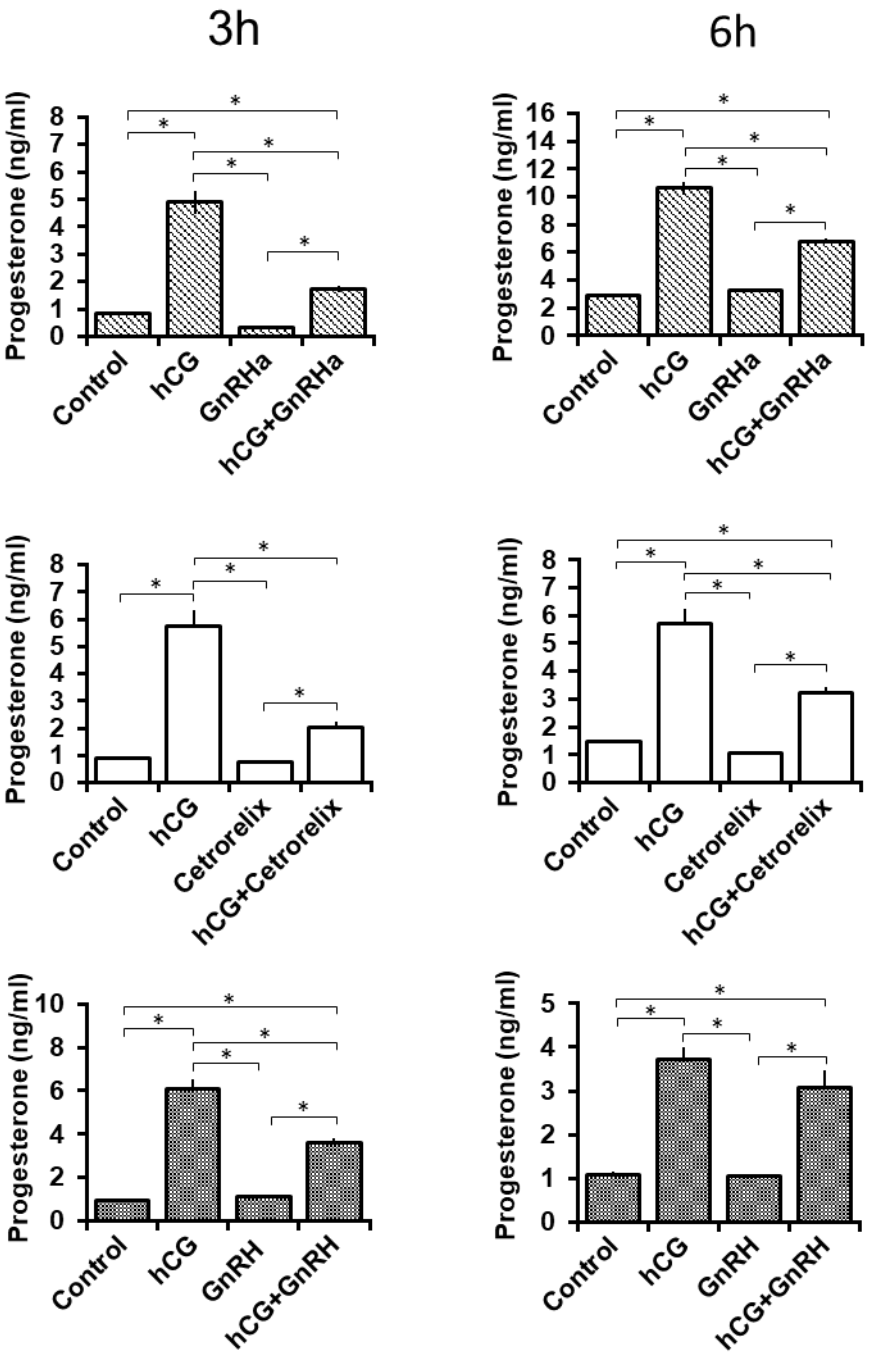

Fig. 2-9 Effects of GnRH, GnRHa, and GnRH antagonist (Cetrorelix) on progesterone production

Granulosa cells were treated with 0.01 IU hCG and 10^{-7} M GnRH or 10^{-8} M GnRHa or 10^{-8} M cetrorelix (GnRH antagonist) for 3 and 6 h. Values are mean±SEM (n=6). Asterisks reveal statistical significance (P<0.05).

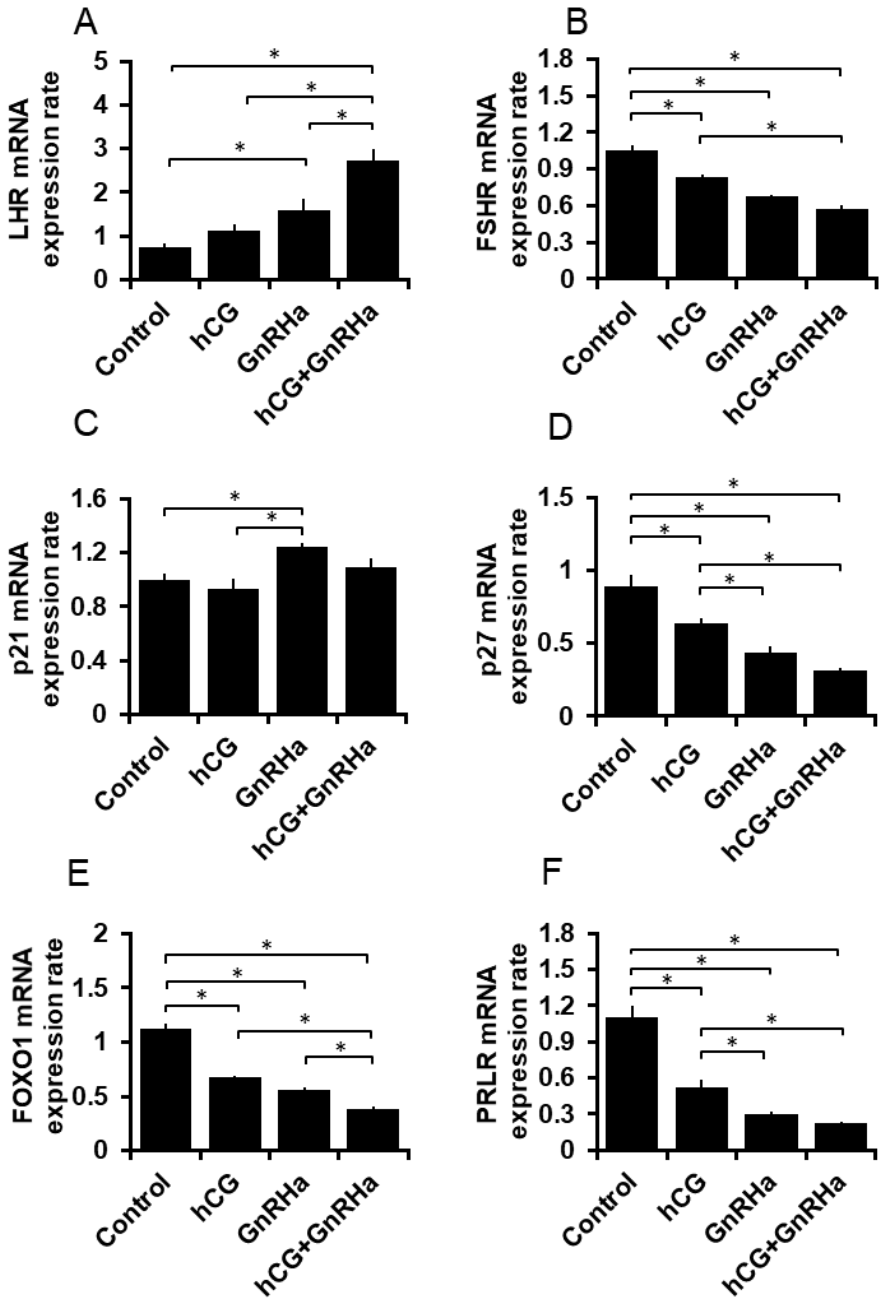

Fig. 2-10 Effects of GnRHa on the mRNA expression of genes related to granulosa cell differentiation

Granulosa cells were incubated with 0.01 IU hCG and 10^{-8} M GnRHa for 3 h. RNA samples were subjected to RT-qPCR to examine mRNA expression of LHR (A), FSHR (B), p21(C), p27 (D), FOXO1(E), and PRLR (F). Values are mean±SEM (n=6). Asterisks indicate values that are significantly different ($p<0.05$).

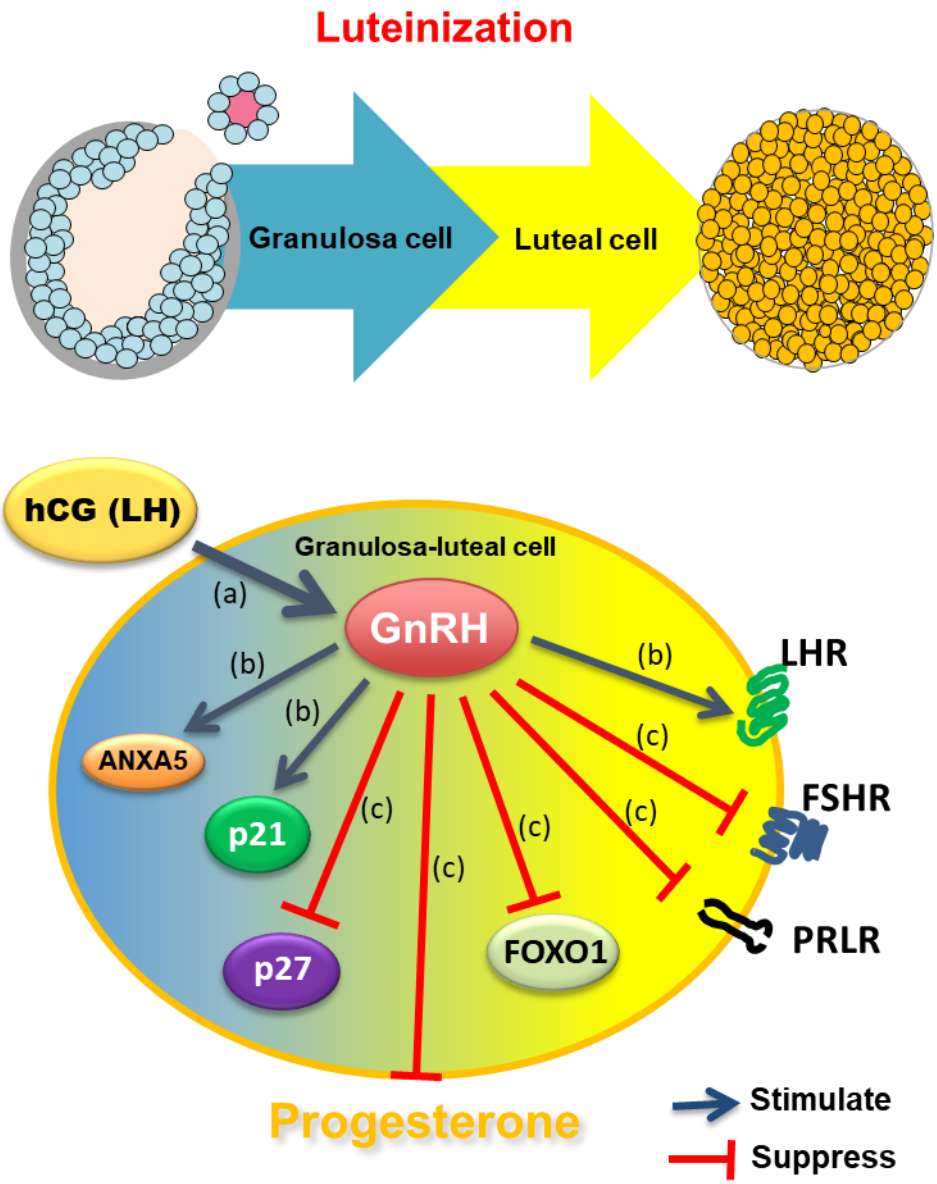

Fig. 2-11 Summary of the chapter

(a) hCG stimulates GnRH mRNA expression in granulosa cell. (b) GnRH increases ANXA5, p21 and LH receptor mRNA expression. (c) GnRH decreases p27, FSH receptor, FOXO1 and prolactin receptor mRNA expression, and also suppresses progesterone production.

Table 1. Primer sequences for RT-qPCR and PCR

Target	Forward primer	Reverse primer
GnRH	GGCAAGGAGGAGGATCAAA	CCAGTGCATTACATCTTCTTCTG
ANXA5	GGAAACCATTGACCGAGAGA	TCTCTGCAAGGTAGGCAGGT
p21	CACGAAACAGGCTCAGGAGT	GCATCGTCAACACCCTGTCT
p27	CCAGACGTAAACAGCTCCGAA	CTCAGTGCTTATACAGGATGTCC
LHR	GGCACACCATCACCTATGCT	AAAAGAGCCATCCTCCGAGC
FSHR	TCTCTGCATGGCCCCAATTT	GGCCTTGGACACAGTGATGA
FOXO1	AGGAGTTAGTGAGCAGGCAAC	GGGTGAAGGGCATCTTTGGA
PRLR	TGTTGTGATGTTCGGCCGTC	TCAGGGCTCATGTGCAAAAC
RPL19	CAGGAGATACCGGGAATCTAAG	TGCCTTCAGTTTGTGGATGTG
Prodynorphin	ACAGGCTTTGAGATCTGCGT	GGCAGTCTGCTGTAACCTCA
NKB	GTGAGGAACCTCAGGAGCAG	GCTAGCCTTGCTCAGCACTT
Kisspeptin	ATGATCTCGCTGGCTTCTTG	AGGCTTGCTCTCTGCATACC
KOR	CCTGGAAAACTGACGCTGAT	TGTAGATGTTGGTTGCGGTCT
NK3R	TCGCTGGTGTCCTTTCATCC	GTGGTGGAGGCAGATTTGGA
GPR54	TCCCCTGTTTTTCGCTACAC	GTAGAGGAGTGCGGTGAAGG

Chapter 3 Relationship between GnRH, kisspeptin, dynorphin and NKB in the granulosa cells

Introduction

KiSS-1 was first identified as metastasis-suppressor gene of human skin cancer cell (malignant melanoma) in 1996 [34]. KiSS-1 inhibits cancer cells metastasis to other tissues [34]. The KiSS-1 gene codes metastin also called kisspeptin which bind to the G-protein-coupled receptor (GPR54) [32, 47]. New aspect for the function of this molecule was introduced by two independent groups in 2003 and 2004 [58, 63]. They demonstrated that kisspeptin-GPR54 system in the hypothalamus plays a critical role in reproduction by reporting that loss of function mutations of the GPR54 gene in humans and mice led to a hypogonadotropic phenotype characterized by low circulating levels of gonadotropins and sex steroids with immature gonads [58, 63]. Kisspeptin acts as a strong activator of GnRH neuron [43, 75]. Several studies have shown that some kisspeptin neurons co-expressed neurokinin B (NKB) and dynorphin, and they were called KNDy neurons at the arcuate nucleus (ARC) of the hypothalamus [5]. Kisspeptin neurons in the ARC are thought to be responsible for pulsatile GnRH and LH secretion [42]. NKB and dynorphin expressed in those neurons would be involved in kisspeptin regulation [20]. It has been postulated that NKB and neurokinin-3 receptor (NK3R) signaling plays an accelerating role for kisspeptin release, whereas dynorphin and kappa opioid receptor (KOR) signaling would suppress the activation of KNDy neurons [76]. Granulosa cells were reported to have a characteristic of KNDy cells, but the relationship among these neuropeptides is remained unknown [33].

It has been demonstrated that expression of kisspeptin, dynorphin, NKB, and their receptors (GPR54, NK3R, and KOR respectively) in the ovary [7, 33, 38, 67]. Furthermore,

granulosa cells are KNDy cells that express kisspeptin, dynorphin and NKB. Expression pattern of these neuropeptides and GPR54 mRNA in the ovary of cycling rat have been revealed [33]. The data indicated that the neuropeptides expressions were stimulated by proestrous LH surge and administration of hCG to the rats [33].

Effects of these neuropeptides on the granulosa cell have reported in human [15] and various animals [27, 37]. When granulosa cells of swine were treated with kappa receptor agonists, it suppressed basal androstenedione, testosterone and estrogen release [27]. Kiss-10 inhibited proliferation and promoted apoptosis of bovine granulosa cells [37]. Kisspeptin caused an increase in intracellular free Ca^{2+} levels in human granulosa cells. Kisspeptin antagonist (p234) suppressed progesterone production stimulated by hCG in primary culture of granulosa cell of immature rat [33]. These reports indicate physiological significance of kisspeptin in the ovary, but it is not known its primary function on GnRH expression in the ovary. Further, KNDy and GnRH may have unknown relationship.

In this chapter, to clarify a functional network of these neuro-peptides in the ovary, especially in the granulosa cells after luteinization, the mutual effect of these peptides on respective mRNA expression was investigated and their effect on progesterone production was examined.

Materials and methods

1. Animals

Immature female Wistar-Imamichi rats were used as chapter 2. The day of delivery was designated as day 0. Number of pups per litter was adjusted to 8 on day 1. The pups were weaned on day 21 after birth. Rats were housed under controlled light-dark cycle of 14L:10D (light on 5:00–19:00 h) at 23±3°C. Food and tap water were supplied *ad libitum*. All animal experiments were approved by the institutional Animal Care and Use Committee of Kitasato University.

2. Immature female rat model

Follicular growth was induced by PMSG in immature female rats and they were utilized for *in vivo* experiments. Primary culture of granulosa cells was established from the ovary of PMSG treated immature female rats also. Both protocols were described in chapter 2 (Fig.2-1).

3. Sample preparation and collection

a. Ovary sample for RNA extraction

Immature rats were treated with PMSG on day 25 after birth and hCG two days later as described in chapter 2 (Fig. 2A). Rats were euthanized by cervical dislocation under deep anesthesia at each sampling time after hCG administration. Ovaries were quickly harvested into Trizol reagent (Thermo Fisher Scientific, Tokyo) and snap frozen in liquid nitrogen. They were kept at -80 °C until RNA extraction.

b. Granulosa cells for RNA extraction

Primary culture of granulosa cell was used for determining mRNA expression *in vitro*. RNA sample was collected from cells and subjected to reverse transcription as described in chapter 2.

c. Conditioned medium for progesterone assay

Medium samples of the primary culture were collected for progesterone assay as demonstrated in chapter 2.

d. Ovary collection for immunohistochemistry

Ovary was also subjected to histological analysis. They were harvested at 1, 3, 6, 12 and 24 h after hCG or saline administration (n=5) as demonstrated in Fig. 2-1A. Ovaries were fixed in 4%PFA and processed for histology.

4. Experimental design for primary culture of granulosa cell

Interrelations among neuropeptides on mRNA expression and progesterone secretion were examined with the primary culture of granulosa cells as explained below.

The effects of hCG on KNDy (kisspeptin, NKB and prodynorphin) mRNA expression was examined. Granulosa cells were incubated with 0.01 IU/ml hCG for 3 h and RNA was harvested.

To examine the relationship between kisspeptin and GnRH in granulosa cells, granulosa cells were treated with 10^{-11} M kiss10 (Peptide institute. Inc., Osaka, Japan) under the effect of 0.01 IU/ml hCG. Then GnRH mRNA was evaluated. GnRHa (10^{-8} M) was administered to cells

with or without 0.01 IU/ml hCG for 3 h and the mRNA expression rate of kisspeptin was determined.

The effects of GnRH on NKB and prodynorphin mRNA expression were examined after incubation of granulosa cells with 10^{-8} M GnRHa and 0.01 IU/ml hCG for 3 h.

The effects of kiss 10 on NKB mRNA expression and progesterone production which was stimulated by hCG were examined by incubation with 10^{-11} M kiss 10 and 0.01 IU/ml hCG for 3 h.

To examine the effects of dynorphin A on GnRH, kisspeptin, and NKB mRNA expression and progesterone production stimulated by hCG, granulosa cells were incubated with 10^{-6} M dynorphin A (Peptide institute. Inc., Osaka, Japan) with or without 0.01 IU/ml hCG for 3 h.

To see the effects of NKB on GnRH, kisspeptin, and prodynorphin mRNA expression and progesterone production stimulated by hCG, granulosa cells were administered with 10^{-6} M NKB (Peptide institute. Inc., Osaka, Japan) with or without 0.01 IU/ml hCG for 3 h.

To examine the effects of dynorphin antagonist and NKB antagonist on progesterone production stimulated by hCG, granulosa cells were incubated with 10^{-6} M dynorphin antagonist (nBNI, Sigma) and NKB antagonist (SB 222200, Sigma-Aldrich, Inc., St. Louis, MO, USA) on progesterone production.

5. RNA extraction and reverse-transcription to cDNA

RNA was extracted from ovaries and granulosa cells. The protocol of RNA extraction and reverse-transcription to cDNA were same as already explained in chapter 2.

6. Quantitative Real time PCR

Expression rate of kisspeptin, dynorphin, and NKB in the ovary and GnRH were measured by quantitative real time PCR. The protocol of quantitative Real time PCR was the same as in chapter 2. Sequences for forward and reverse primers were shown in Table 1.

7. Time-resolved immunofluorometric assay (TR-IFMA) of progesterone

Granulosa cells were incubated with a combination of hCG and other reagents (kiss 10, dynorphin A, NKB, nBNI, and 222200). Progesterone level in culture medium was analyzed by TR-IFMA. The protocol of TR-IFMA was same as in chapter 2.

8. Histological analysis

Paraffin sections of ovary samples were subjected to immunohistochemistry.

9. Immunohistochemistry

The protocol of immunohistochemistry was already described in chapter 2. The distribution of kisspeptin in the ovary was proved by using rabbit anti-kisspeptin 10 polyclonal antibody (Millipore, Temecula, CA, USA) as a primary antibody. Normal rabbit serum was used

as a negative control. The primary antibody was diluted with ABB to 1:1,000. Process of immunohistochemistry was described in chapter 2.

10. Polymerase chain reaction (PCR) and gel electrophoresis

a. Reagents, solutions and equipments

Premix Taq™ (Ex Taq version 2.0, TAKARA BIO INC, Otsu, Shiga, Japan)

Sequences for forward and reverse primers were presented in Table 1.

10×TBE buffer sterile

- 890 mM Tris (Kanto Chemical Co., Tokyo, Japan)
- 890 mM Boric acid (Kanto Chemical Co.)
- 20 mM EDTA (Kanto Chemical Co.)
- Milli Q water

Agarose gel

- 1.8% Certified™ Molecular Biology Agarose (Bio-Rad Laboratories, Inc., Hercules, CA, USA)
- 1×TBE buffer

100 bp DNA ladder (TAKARA BIO INC, Otsu, Shiga, Japan)

6×loading buffer (TaKaRa)

Ethidium bromide solution (1:1,000 dilution)

VeritiTM 96 well Thermal cycler (Applied Biosystems)

Microwave

Electrophoresis tank

Power supply

Ultraviolet imaging system

b. Protocol

Mixture of PCR reaction consists of 1 µl cDNA sample, 0.02 µl (100 µM) forward and reverse primers, 5 µl Premix Taq™, and 3.96 µl Milli Q water. The final volume was 10 µl per each sample. Complementary DNA samples were amplified by PCR using VeritiTM 96 well thermal cycler. Each sample was added 2 µl of 6×loading buffer. The PCR products were separated by gel electrophoresis in 1.8% agarose gel, stained with ethidium bromide (EtBr) and gently shaken for 10 min. EtBr was washed out with distilled water for 10 min shaking. The PCR products were visualized and taken the photo under ultraviolet illumination. Amplicon sizes were verified by comparison with 100 bp DNA Ladder. PCR condition was adjusted according to each specific primer empirically as shown below.

Kappa opioid receptor: 33 cycles of amplification are denature template 94°C 30 sec, annealing primers 58°C 30 sec, and extension 72°C 1min.

Tachykinin receptor 3: 36 cycles of amplification are denature template 94°C 30 sec, annealing primers 62°C 30 sec, and extension 72°C 1min.

Kiss-1 receptor (GPR54): 40 cycles of amplification are denature template 94°C 30 sec, annealing primers 70°C 30 sec, and extension 70°C 1min.

To know whether there are GPR54, NK3R and KOR in the primary culture of granulosa cells after dissociation from follicles and after hCG treatment. Granulosa cells were harvested after dissociation and centrifugation (25°C, 800 rpm, 10 min). Granulosa cells were harvested after incubation with 0.01 IU/ml hCG for 1, 3, 6, 12 and 24 h. The control sample was hypothalamus of mature female rat. All samples were stored in Trizol reagent at -80 °C until RNA extraction.

11. Statistical analysis

Statistical significance was assessed by analysis of variance being followed by Tukey's test and p-values less than 0.05 was considered statistically significant. Single comparisons were analyzed by Student's *t*-test with p-values less than 0.01 considered statistically significant.

Results

1. Changes in ovarian kisspeptin, prodynorphin and NKB mRNA expression after hCG administration

PMSG was administered to immature female rats on day 25 after birth and ovarian kisspeptin, prodynorphin and NKB mRNA expression rates were examined after hCG or saline treatment on day 27 (Fig. 2-1A). The expression rate of kisspeptin mRNA dramatically increased after hCG treatment until 3 h ($P<0.05$), then the level was gradually decreased for 24 h during the experimental period. Saline administered control group also showed an increase of kisspeptin mRNA expression but delayed. Prodynorphin mRNA expression rates significantly increased at 3, 6 and 12 h. NKB mRNA was gradually increased but not significant (Fig. 3-1).

2. Immunohistochemical analysis of kisspeptin in the ovary after hCG administration

Immature female rats were given PMSG on day 25 after birth. Then hCG or saline was administered to the rats on day 27 (protocol: Fig. 2-1A). Ovaries were obtained at 1, 3, 6, 12 and 24h after hCG or saline administration. The highest expression of kisspeptin was observed at 6h after hCG treatment (Fig. 3-2). Kisspeptin was increased also in control ovary again delayed to hCG treated ovary.

3. Expression of GPR54, NK3R, and KOR in granulosa cells

GPR54, NK3R and KOR expression were examined by PCR. GPR54 was shown in granulosa cells throughout the experiment before and after hCG treatment (Fig. 3-3A). It gradually increased after hCG treatment. While there was no NK3R expression in the granulosa cell after dissociation, hCG stimulated NK3R expression in the granulosa cells (Fig. 3-3B). KOR expression was only seen 6 h after hCG stimulation (Fig. 3-3C).

4. The effect of hCG on mRNA expression of kisspeptin, prodynorphin, and NKB

Granulosa cells were treated with or without 0.01 IU/ml hCG. hCG significantly increased all kisspeptin, prodynorphin and NKB mRNA expression in primary culture of granulosa cells during 3 h incubation (P<0.01) (Fig. 3-4).

5. The effects of kiss 10 on GnRH and GnRHa on kisspeptin mRNA expression

To examine whether kisspeptin stimulates GnRH expression in granulosa cells as seen in the hypothalamus. Granulosa cells were incubated with hCG, kiss 10 and a combination of kiss 10 and hCG. The data showed kiss 10 did not stimulate GnRH mRNA expression. Contrary, GnRHa augmented hCG action on the expression of kisspeptin at 3 h incubation (P<0.05) (Fig. 3-5).

6. The effects of GnRHa on NKB and prodynorphin mRNA expression

Effects of GnRHa on NKB and prodynorphin mRNA expression was examined. The data showed that GnRHa augmented mRNA expression of NKB and prodynorphin synergistically with hCG during 3h incubation (P<0.05) (Fig. 3-6).

7. The effect of kiss 10 on NKB mRNA expression

Granulosa cells were challenged with a combination of hCG and kiss 10. The data showed kiss 10 increased NKB mRNA expression synergistically with hCG (P<0.05) (Fig. 3-7).

8. The effects of dynorphin A on GnRH, kisspeptin, and NKB mRNA expression

It was examined in the primary culture of granulosa cells whether there is negative effect of dynorphin on kisspeptin and NKB as seen in the hypothalamus. The results showed dynorphin

A itself increased NKB mRNA. It also augmented hCG action on kisspeptin and NKB mRNA expression rates at 3h incubation (P<0.05) but dynorphin A did not show any effect on GnRH mRNA expression (Fig. 3-8).

9. The effects of NKB on GnRH, kisspeptin, and prodynorphin mRNA expression

NKB itself suppressed GnRH mRNA expression rates. It also suppressed GnRH and kisspeptin mRNA expression stimulated by hCG at 3 h incubation (P<0.05). NKB showed tendency to suppress hCG effect on prodynorphin (Fig. 3-9).

10. Effects of kiss-10, dynorphin A, NKB, dynorphin antagonist (nBNI, Norbinaltorphimine) and NKB antagonist (SB 222200) on progesterone production

The data showed kiss 10, dynorphin A and NKB suppressed progesterone production stimulated by hCG. Interestingly, nBNI and SB 222200 also showed negative effect as agonist at 3 h incubation (P<0.05) (Fig. 3-10).

Discussion

GnRH was shown to be involved in a luteinization process under the effect of hCG/LH in chapter 2. It has been well documented that relationship among GnRH, kisspeptin, dynorphin and NKB in the hypothalamus [42]. These neuropeptides are also expressed in the ovary [7, 33]. In chapter 3, GnRH and its related neuropeptides were studied on functional relations and intimate functional links were demonstrated.

It was clearly shown in the present study that immature female rats treated with hCG showed increased ovarian kisspeptin mRNA expression until 3 h then it was gradually decreased for 24 h during the experimental period. Saline-treated control group also showed an augmentation of kisspeptin mRNA expression but it was delayed. Administration of hCG to PMSG treated immature female rats induces ovulation and luteinization [41, 51]. Even in saline control rats, spontaneous ovulation, or gonadotropin surge, would occur by intrinsic estradiol from grown follicels [40]. So, delayed increase of kisspeptin expression in the control rats are supposed to be derived by intrinsic LH secretion induced by the increase of estradiol from preovulatory follicles. It has been clearly demonstrated already that kisspeptin, dynorphin and NKB mRNA increase in granulosa cells in the afternoon of proestrus by LH surge [33]. So, it is suggested that the observed increase in the expression of kisspeptin and prodynorphin after hCG treatment is physiological changes in preovulatory follicles.

Prodynorphin mRNA expression rates also significantly increased at 3, 6 and 12 h after hCG administration. Unlike kisspeptin, its level did not change significantly in the control group. As the response of prodynorphin expression to hCG was very big when comparing to that of kisspeptin and GnRH *in vitro*, it is suggested that spontaneous changes in gonadotropin secretion of the control rats would not be sufficient to increase prodynorphin expression. In other words,

the effect by gonadotropin secretion induced by intrinsic estradiol would be much lower than hCG stimulation [40].

Ovarian NKB mRNA was gradually increased after hCG treatment but not significant when compared with saline control group. The data would be consistent with the recent report in which ovarian NKB mRNA increased after LH surge, but the peak was delayed by 6 h to kisspeptin and dynorphin [33].

Distribution of kisspeptin in the ovary in adult rat has been shown by others that kisspeptin is in the theca layer of growing follicles, corpora lutea and interstitial tissues with a stage-specific pattern along with the estrous cycle [7]. In the present study, kisspeptin was shown in interstitial tissues after 1 h of hCG treatment and then granulosa cells became kisspeptin positive after 6 h of hCG administration. This is consistent with the data about mRNA expression in which hCG stimulated kisspeptin mRNA expression solely in granulosa cells [33]. After 24 h of hCG administration, newly formed corpus luteum was negative for kisspeptin. As in adult rats corpus luteum at early estrus also showed the low intensity of kisspeptin [7], kisspeptin is hypothesized to have a specific role rather during luteinization.

GPR54, NK3R and KOR are the receptors for kisspeptin, NKB and dynorphin, respectively. These were already demonstrated in granulosa cells by others and the expression of all genes were confirmed also in this study [15, 16, 67]. GPR54 was shown in granulosa cells throughout the experiment and gradually increased after hCG treatment. The expression of KOR and NK3R were also stimulated by hCG treatment. Interestingly, KOR expression was seen only 6 h after hCG stimulation. These results suggest that granulosa cells would be the targets of kisspeptin, dynorphin and NKB those are increased by hCG/LH in granulosa cells.

Expression of kisspeptin, prodynorphin and NKB mRNAs were stimulated by hCG in primary culture of granulosa cells prepared from PMSG pretreated immature rats. It is reported that co-expression of kisspeptin, NKB and dynorphin in kisspeptin neurons at ARC of

hypothalamus [20] and they are called KNDy neurons being responsible for synchronizing GnRH pulse generation [46]. Kisspeptin stimulates GnRH secretion and it is followed by paracrine stimulatory and inhibitory inputs by neurokinin B and dynorphin, respectively [76]. Thus, it was very interesting whether these relations existed also in granulosa cells under the effect hCG/LH. So, primary culture of granulosa cell was examined with neuropeptides for their relationships.

Functional relationship among GnRH, kisspeptin, dynorphin and NKB was observed in granulosa cells in this study. This study is the first to discover the relationship of these neuropeptides in the granulosa cells. The relationship among the neuropeptides in granulosa cell is different from those in KNDy neurons, where NKB augments kisspeptin, kisspeptin stimulates GnRH release and dynorphin suppresses kisspeptin and NKB [76]. Interestingly, in the granulosa cells, kiss-10 (a bioactive kisspeptin peptide) did not stimulate GnRH expression but GnRHa augmented the expression of kisspeptin mRNA. This result suggests granulosa cell specific relationship among peptides and response to kiss-10 stimulation. Another example has been shown for a different relationship between kisspeptin and GnRH from that seen in the hypothalamus. In the primary culture of placental tissue of rat, kisspeptin failed to stimulate GnRH mRNA expression [48]. While GnRH increases kisspeptin mRNA expression [48]. Inverse relationship between kisspeptin and GnRH in the granulosa cells is interesting. As GPR54 is expressed in granulosa cells, kisspeptin is hypothesized to drive a different signal transduction in these cells from GnRH neuron.

In the present study, a sequence of events was shown among neuropeptides in the ovary (Fig.3-11). Ovulation inducible level of hCG/LH would induce ovarian GnRH expression, and then GnRH would augment kisspeptin, dynorphin and NKB. Kisspeptin and dynorphin may contribute to increase each other and NKB. Finally, NKB would suppress all GnRH, kisspeptin and dynorphin terminating the sequence of function started by hCG. The effect of kiss-10 on prodynorphin may be necessary to examine also, but it was not done in this occasion. These data

demonstrate that the relationship of these neuropeptides obviously different from that of the hypothalamus. This sequence of events would occur in preovulatory matured follicles by LH surge and would be probably necessary changes for luteinization of granulosa cells.

Finally, to know the function of neuropeptides on progesterone production of granulosa-luteal cells, effects of kiss-10, dynorphin and NKB on hCG stimulated progesterone production was examined. Simultaneously, inhibitors of dynorphin and NKB were also tested. Results of present study showed kisspeptin, NKB and dynorphin A all suppressed progesterone production stimulated by hCG, but none of the peptides by themselves affected progesterone production. On the other hand, when granulosa cell was treated with antagonist of dynorphin and NKB, it was shown also negative effect on hCG-enhanced progesterone production. GPR54 antagonist (p234) was already reported to suppress progesterone production stimulated by hCG [33]. It was expected that kiss-10, dynorphin and NKB would stimulate progesterone production, since there were synergistic effects with hCG was observed for some gene expression. It is difficult to interpret this, but augmentation of progesterone production by granulosa cells would need more factors supplied from outside granulosa cells. For example, prodynorphin is synthesized in granulosa cells, but quantity of its expression is extremely high in interstitial tissues [33]. It is necessary to study what is necessary for full differentiation of granulosa cells to luteal cells.

Present results clearly demonstrate the existence of granulosa cell specific relationship between GnRH, kisspeptin, NKB and dynorphin. These neuropeptides are hypothesized to be involved in granulosa cell differentiation.

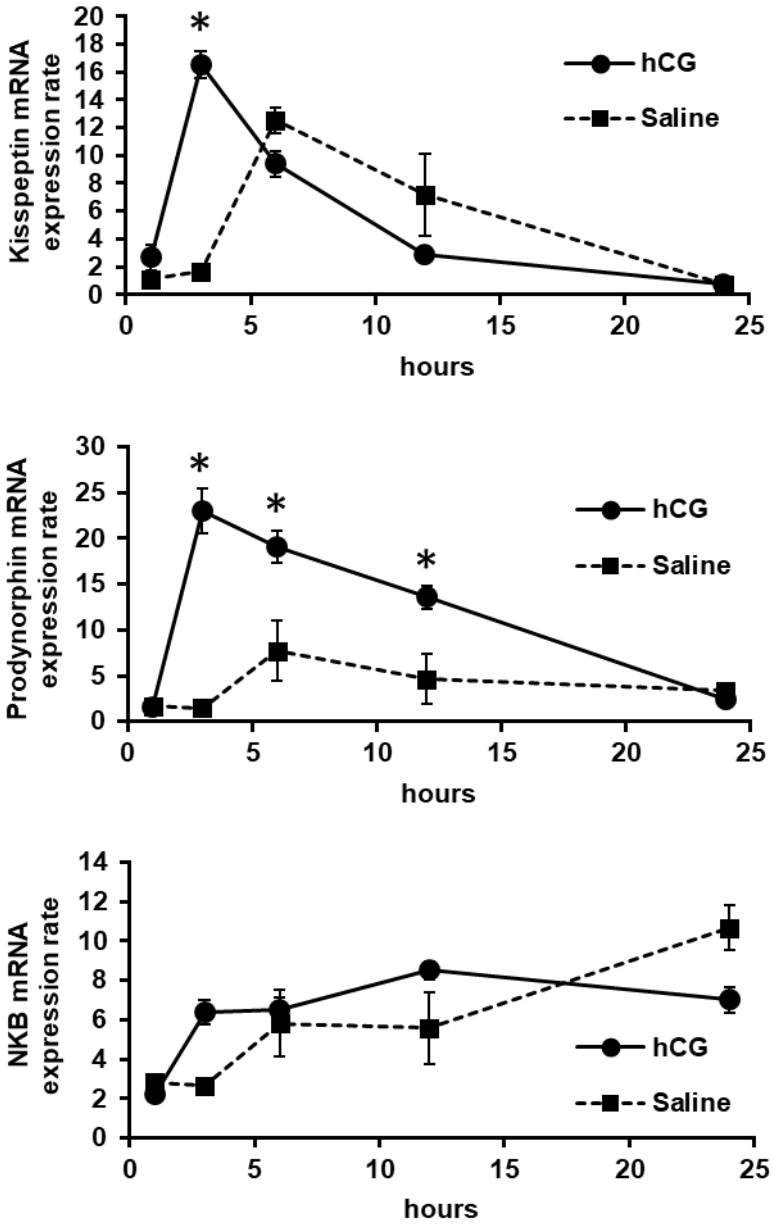

Fig. 3-1 Changes of kisspeptin, prodynorphin and NKB mRNA expression in the ovary after hCG administration.

PMSG-treated rats were administered with hCG after 48 h of PMSG treatment and ovary samples were collected after hCG administration at 1, 3, 6, 12 and 24 h. Values are mean±SEM of five rats. Asterisk indicates significant difference from the saline-treated control of the same sampling point (P<0.05).

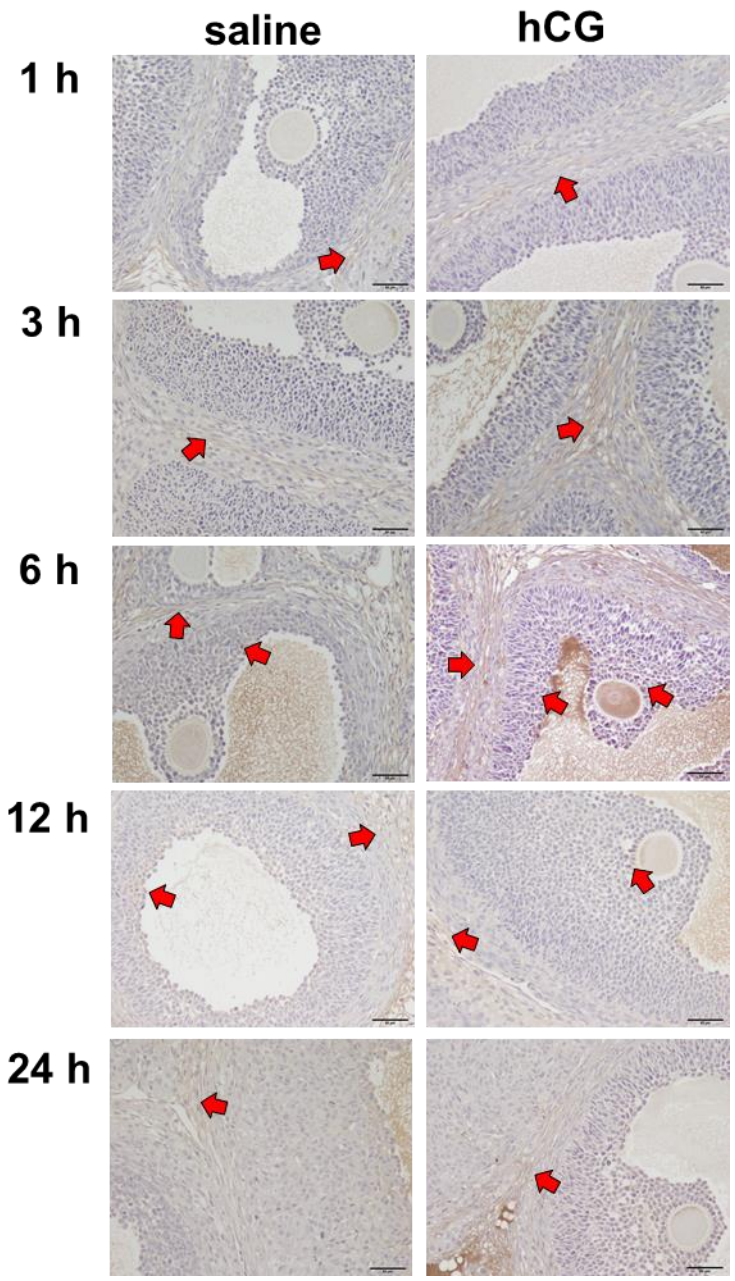

Fig. 3-2 Immunohistochemical analysis of kisspeptin in the ovary

Immunohistochemistry for kisspeptin in the ovary. PMSG (15 IU) was administered to 25-day-old rats. Ovaries were collected 1, 3, 6, 12 and 24 h after hCG or saline treatment on day 27 of birth. Red arrows indicate immunoreaction for kisspeptin.

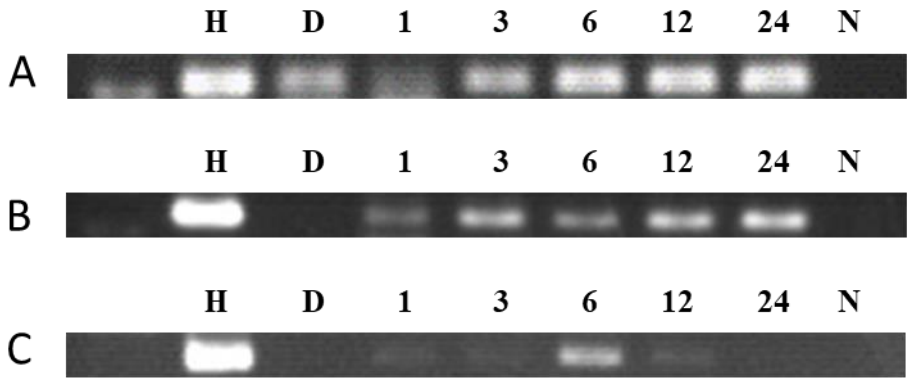

Fig. 3-3 Expression of GPR54, NK3R and KOR in granulosa cells.

Granulosa cells were treated with 0.01 IU/ml hCG then samples were harvested different h. (A) GPR54, (B) NK3R, (C) KOR, (H) hypothalamus, (D) granulosa cell after dissociation, (Number) incubation time, and (N) negative control.

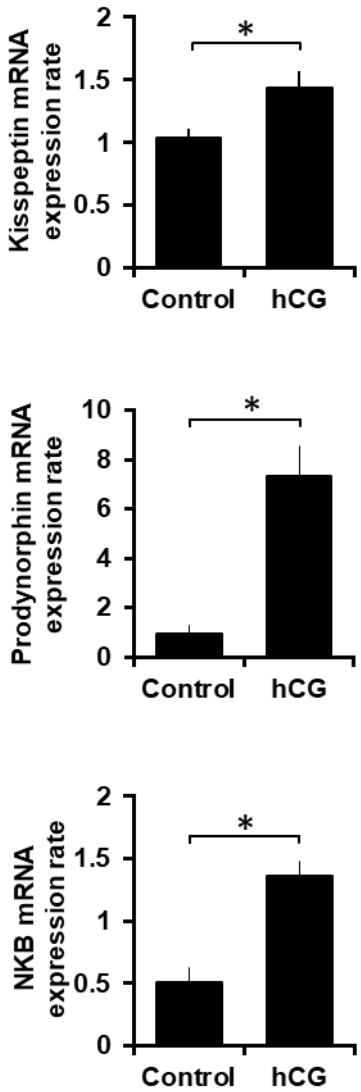

Fig. 3-4 The effect of hCG on kisspeptin, prodynorphin and NKB mRNA expression

Granulosa cells were incubated with or without hCG 0.01 IU for 3 h. Values are mean±SEM (n=6). Asterisk reveals significant difference $P<0.01$.

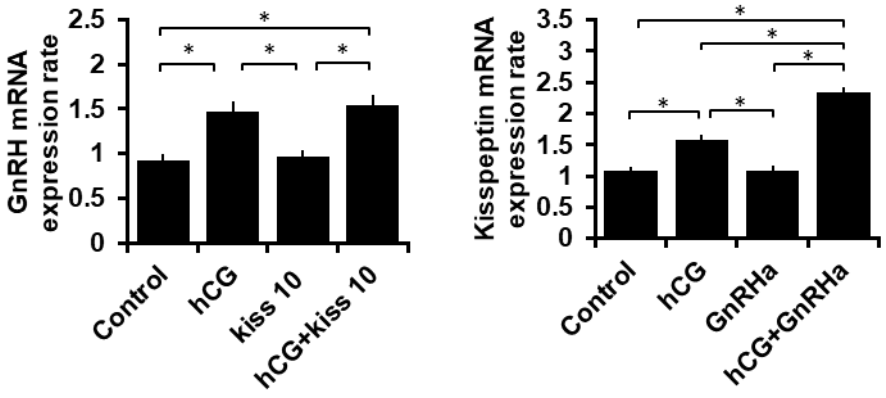

Fig. 3-5 The effects of kiss 10 on GnRH and GnRHa on kisspeptin mRNA expression

GnRHa (10^{-8} M) or kiss 10 (10^{-11} M) was administered with hCG 0.01 IU for 3 h. Values are mean±SEM (n=6). Asterisks reveal significant difference (P<0.05).

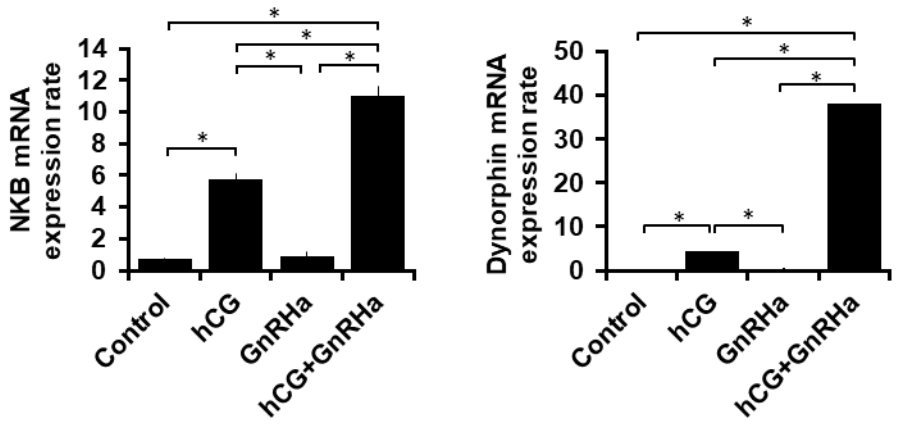

Fig. 3-6 The effects of GnRHa on NKB and dynorphin mRNA expression

GnRH agonist (10^{-8} M) was administered with hCG 0.01 IU for 3 h. Values are mean±SEM (n=6). Asterisks reveal significant difference (P<0.05).

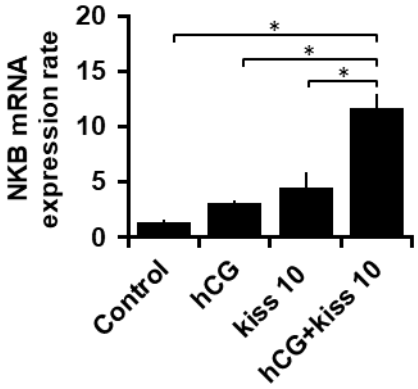

Fig. 3-7 The effects of kiss 10 on NKB mRNA expression

Kiss 10 (10^{-11} M) was administered to the primary granulosa cells with hCG 0.01 IU for 3h. Values are mean±SEM (n=6). Asterisks reveal significant difference (P<0.05).

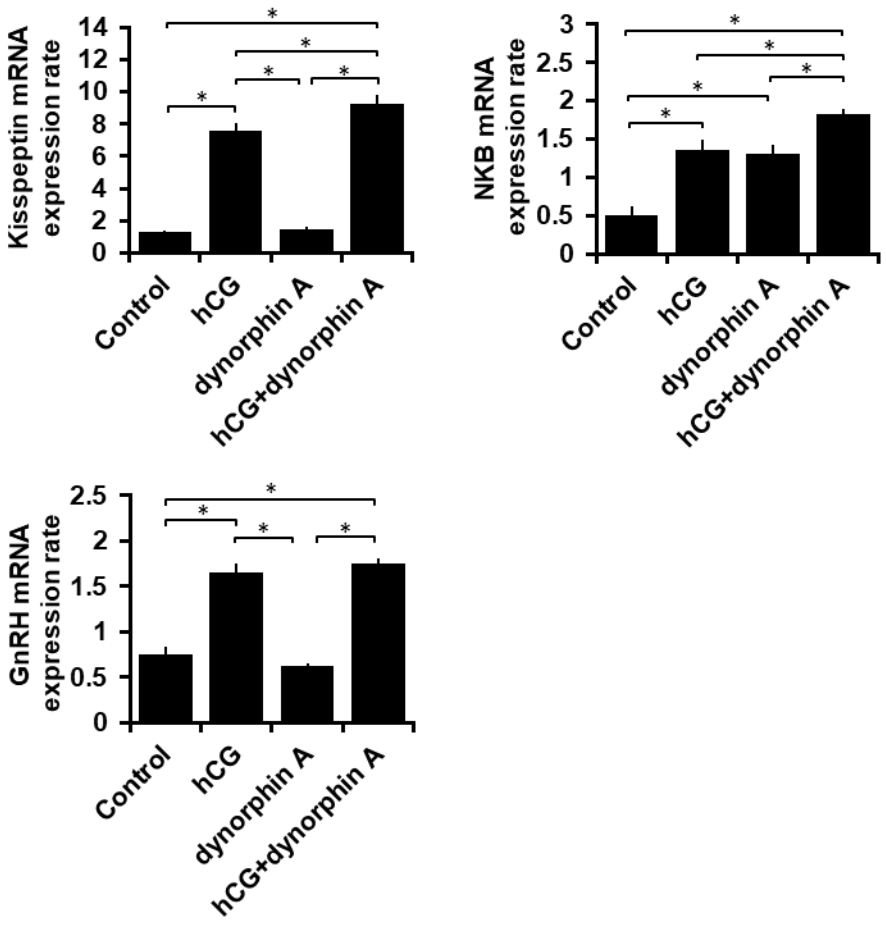

Fig. 3-8 The effects of dynorphin A on kisspeptin, GnRH and NKB mRNA expression Dynorphin (10^{-6} M) was administered to primary granulosa cells with hCG (0.01 IU) for 3 h. Values are mean±SEM (n=6). Asterisks reveal significant difference (P<0.05).

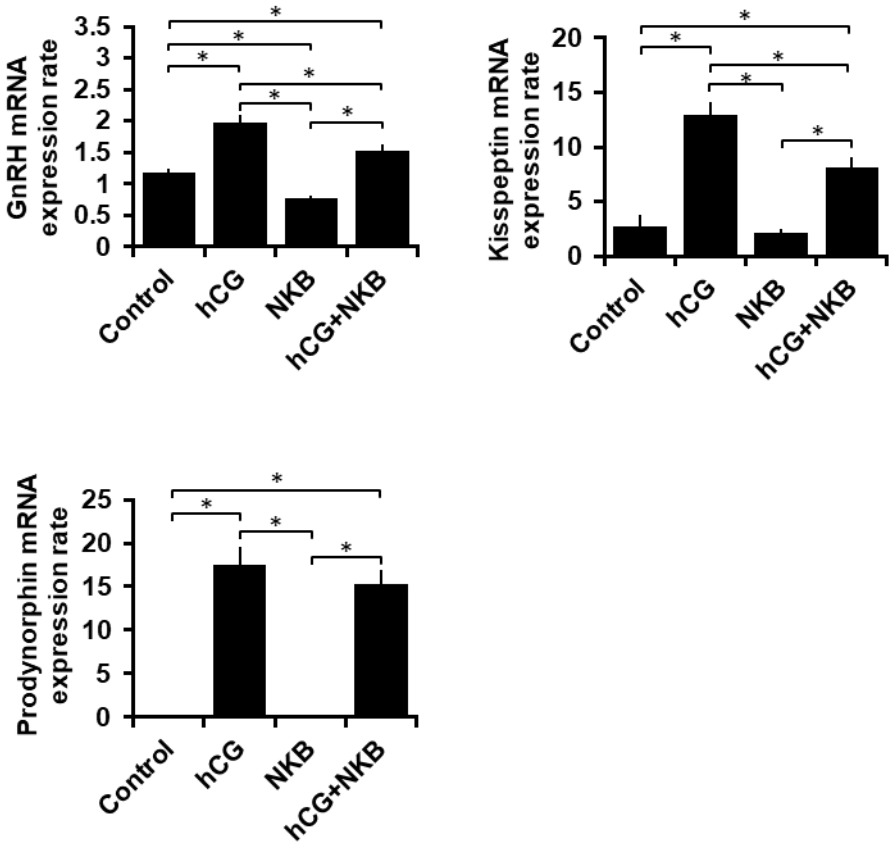

Fig. 3-9 The effects of NKB on kisspeptin, GnRH and prodynorphin mRNA expression

NKB (10^{-6} M) was administered to primary granulosa cells with hCG (0.01 IU) for 3 h. Values are mean±SEM (n=6). Asterisks reveal significant difference (P<0.05).

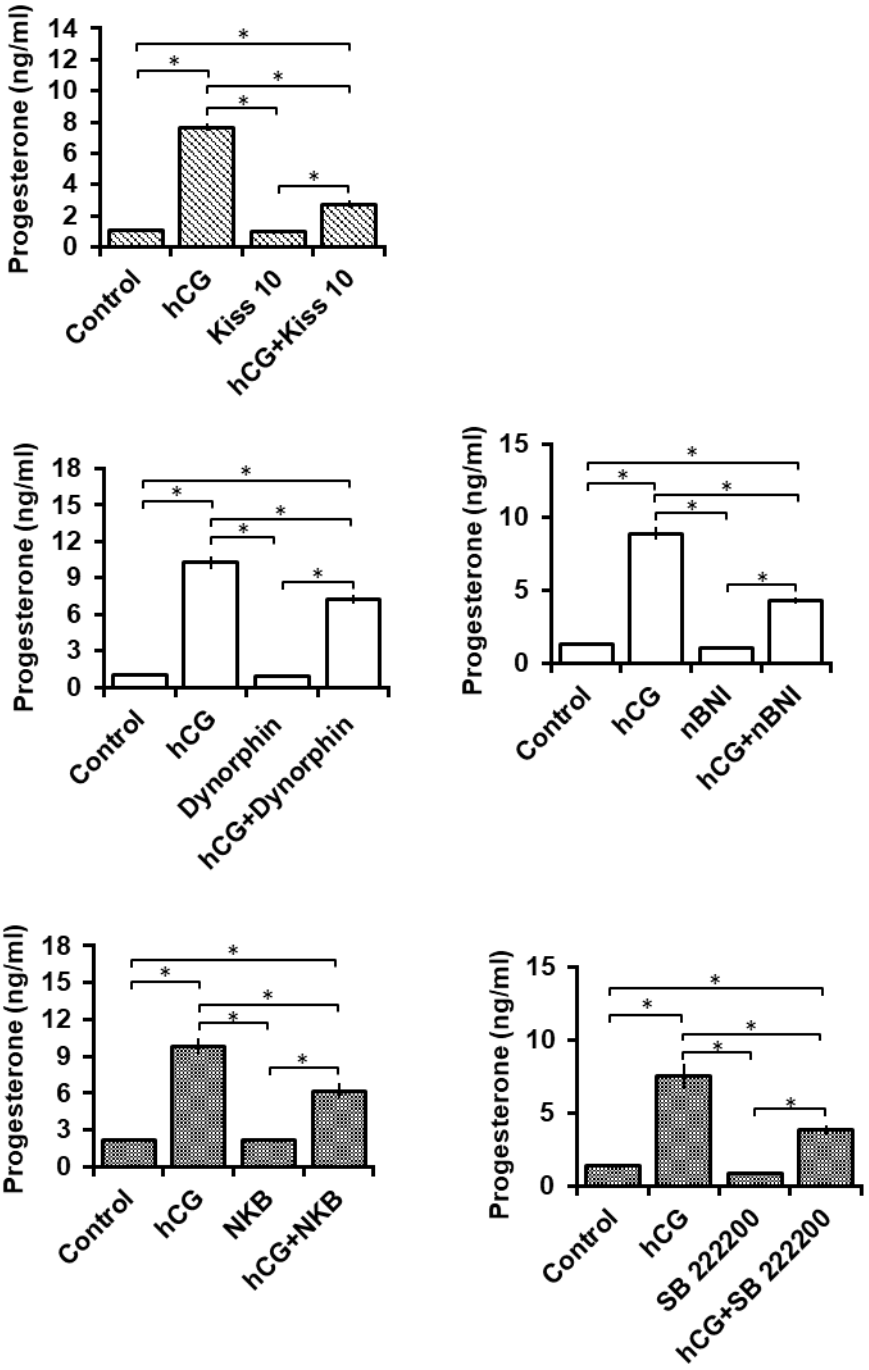

Fig. 3-10 Effects of kiss 10, dynorphin A, NKB, Norbinaltorphimine (nBNI) and SB 222200 on progesterone production

Granulosa cells were treated with hCG (0.01 IU) and kiss 10 (10^{-11} M), dynorphin A (10^{-6} M), NKB (10^{-6} M), nBNI (10^{-6} M) and NKB antagonist (SB 222200, 10^{-6} M) for 3 h. Values are mean±SEM (n=6). Asterisks reveal statistical significance (P<0.05).

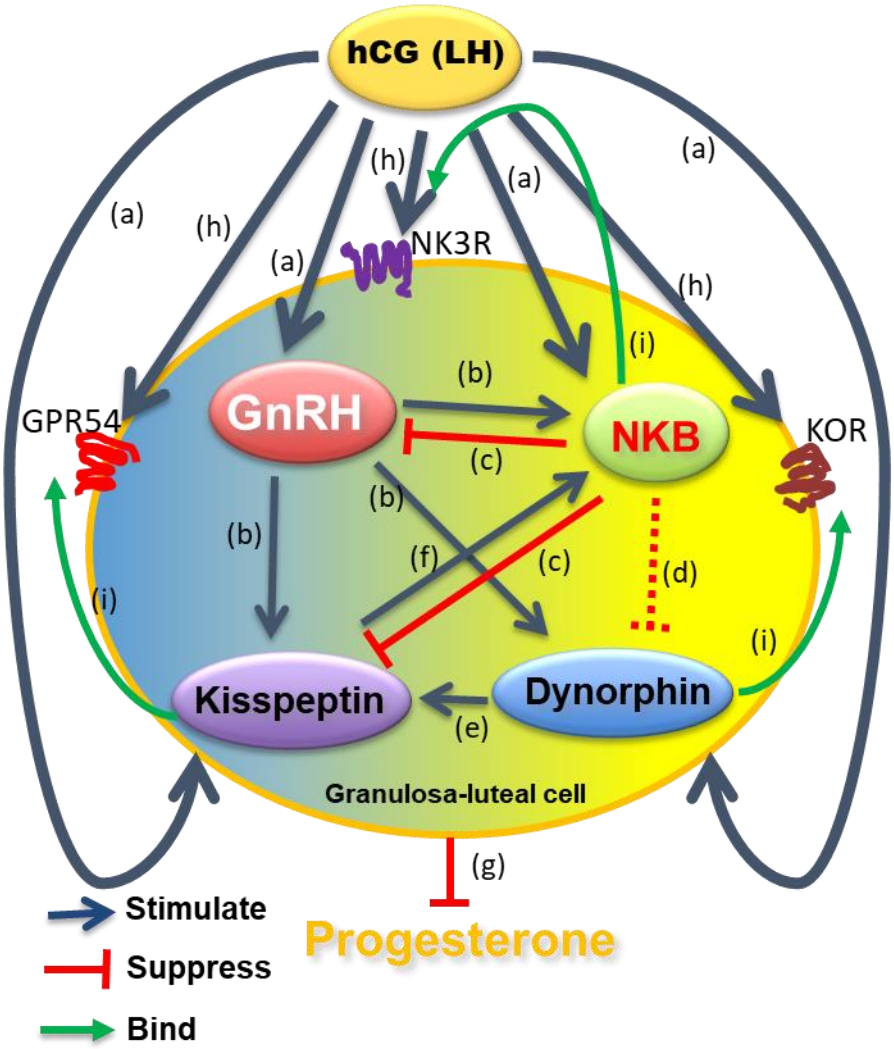

Fig. 3-11 Summary of interaction

(a) hCG stimulates GnRH, kisspeptin, dynorphin and NKB mRNA expression in granulosa cell. (b) GnRH increases kisspeptin, dynorphin and NKB mRNA expression. (c) NKB decreases GnRH and kisspeptin mRNA expression. (d) NKB may also suppress dynorphin mRNA expression. (e) Dynorphin increases kisspeptin mRNA expression. (f) Kisspeptin increases NKB mRNA expression. (g) All of peptides suppress progesterone production stimulated by hCG. (h) hCG increases GPR54, NK3R and KOR. (i) kisspeptin, dynorphin and NKB will bind their receptors.

Chapter 4 GnRH-Annexin A5 function in granulosa cells

Introduction

Annexin A5 (ANXA5) was first isolated from human placenta as PP10 in 1979 [2]. ANXA5 belongs to a large family of calcium and phospholipid-binding proteins [17]. It has been reported that ANXA5 is involved in cell membrane repair [4], a marker of apoptotic cells [31] and inhibitor of blood coagulation [53]. ANXA5 is utilized as a biomarker of GnRH action in cells expressing GnRH receptor [55, 72].

ANXA5 was also shown in this study to be stimulated its expression by GnRH in granulosa cells in immature rat model. Granulosa cells were collected from PMSG stimulated immature rats. It has been already demonstrated in adult female rats that ANXA5 is expressed in regressing corpus luteum but not in granulosa cells [30]. In the present study, ANXA5 was increased after hCG stimulation of granulosa cells. Based on this phenomenon, the involvement of GnRH in luteinization was discovered in chapter 2. GnRH in the hypothalamus is neuroendocrine peptide hormone and the master molecule of reproduction. It plays critical role to control reproductive function. In mammals, the pattern of gonadotropin secretion consists of pulsatile and surge phases, that have been thought to depend on different kisspeptin neurons [26]. GnRH and its receptor also distribute in peripheral tissues such as gastrointestinal tract [60], pancreas [78], testis [3] and ovary [73]. Interestingly, GnRH mRNA was enhanced by hCG in the primary culture of testicular interstitial cells [18]. GnRH induced apoptosis of luteal cells in pseudopregnant rats [30]. Peripheral GnRH and GnRH receptor systems also play crucial roles in antiproliferation and apoptosis [83]. It was found that GnRHa induced apoptosis in the cultured leiomyoma cells [79]. GnRH has been postulated to mediate the anti-proliferative effect of GnRH agonist on the endometrial cancer cell and leiomyoma [66, 80].

Our laboratory previously showed that ANXA5 is involved in gonadotropin secretion and its expression is under the control of GnRH at pituitary gonadotrope [28]. GnRH also promotes ANXA5 synthesis in Leydig cell and mammary epithelial cell [54, 82]. The expression of ANXA5 in the rat corpus luteum is stimulated by GnRH and decreases during pseudopregnancy by prolactin [30]. It is interesting to clarify the direct effect of ANXA5 on granulosa cells. In this chapter, a series of experiments were performed to clarify a nature of GnRH-ANXA5 system and its function in granulosa-luteal cells with immature female rat model.

Materials and methods

1. Animals

The animals were prepared as chapter 2. Shortly, immature female Wistar Imamichi rats were kept in controlled light-dark cycle of 14L:10D (light on 5:00–19:00 h) at 23±3°C. Food and tap water were supplied *ad libitum*. The day of delivery was designated as day 0. Number of pups per litter was adjusted to 8 on day 1. The pups were weaned on day 21 after birth. All animal experiments were approved by the Institutional Animal Care and Use Committee of Kitasato University.

2. Immature female rat model

The immature female rat was treated with PMSG according to *in vitro* model as shown in the chapter 2 (Fig.2-1) and establishing primary culture of granulosa cells.

3. Sample preparation and collection

a. Granulosa cell for RNA extraction

Primary culture of granulosa cell was used for various *in vitro* experiments. RNA sample was obtained from cells and subjected to reverse transcription as described in chapter 2.

b. Conditioned medium for progesterone assay

Medium samples of the primary culture were used for progesterone assay. Collection of medium was documented in chapter 2.

4. Primary culture of granulosa cells

Primary culture of granulosa cell was also established in this chapter for *in vitro* experiments. Protocol was same as already described in chapter 2.

5. Experimental design for primary culture of granulosa cells

Changes in mRNA expression, progesterone production and cell growth were examined by means of the primary culture of granulosa cells.

Granulosa cells were treated with 10^{-8}M GnRHa and 0.01 IU hCG for 3, 6 and 24 h and then ANXA5 mRNA was measured. The effect of hCG on GnRH mRNA expression was also examined by granulosa cells being incubated with or without 0.01 IU hCG for 3 and 6 h.

The effects of hCG pre-treatment on the effect of ANXA5 on progesterone production were studied. Granulosa cells were administered with recombinant ANXA5 (10^{-12}, 10^{-11}, 10^{-10}, 10^{-9} and 10^{-8} M) for 24h. Pre-incubation with 0.01 IU hCG for 1 h was applied for a group. Culture medium was collected and subjected to progesterone assay.

The effects of ANXA5 on progesterone production stimulated by hCG were tested. Granulosa cells were treated with 10^{-9} M recombinant ANXA5 with or without 0.01 IU hCG for 3, 6 and 24 h. Medium was subjected to progesterone assay.

6. RNA extraction and reverse-transcription to cDNA

RNA was extracted from granulosa cells by means of Trizol. The protocol of RNA extraction and reverse-transcription to cDNA were already explained in chapter 2.

7. Quantitative Real time PCR

Expression rate of GnRH and ANXA5 in the primary culture of granulosa cell were measured by quantitative Real time PCR. The protocol of quantitative Real time PCR was the same as in chapter 2. Sequences for forward and reverse primers were shown in Table 1.

8. Time-resolved immunofluorometric assay (TR-IFMA) of progesterone

Granulosa cells were incubated with a combination of hCG and ANXA5. Progesterone level in culture medium was analyzed by TR-IFMA. The protocol of TR-IFMA was same as in chapter 2.

9. Immunocytochemistry

Changes in the distribution of ANXA5 in granulosa cells after hCG or GnRHa treatment were observed by immunocytochemistry. Granulosa cells were incubated with 0.01 IU hCG or 10^{-9}M GnRHa for 6 h. The protocol of immunocytochemistry was described in chapter 2. Granulosa cells were cultured in Glass Bottom dish coated with Poly-Lysine (Matsunami GLASS IND., LTD, Osaka, Japan). Primary antibody was anti-ANXA5, that was diluted with ABB and 3% fetal bovine serum (1:5,000). Normal rabbit serum was used for negative control (1:5,000).

10. Counting granulosa cell

The effects of ANXA5 on cell number were examined**.**

a. Reagents, solutions and equipment

TC20 Automated Cell Counter (BioRad)

Counting slide (BioRad)

Trypan blue solution

- 0.4% Trypan blue (WAKO PURE CHEMICAL INDUSTRIES. LTD., Osaka, Japan)
- 0.81% NaCl
- 0.06% K_2HPO_4

Trypsin solution

- 0.25% Trypsin inhibitor, soybean (GIBCO^{TM}, Scotland, UK)
- 10mM EDTA

Growth medium

b. Protocol

Granulosa cells were prepared as explained in chapter 2. Cells were adjusted to 250,000 cells/ml per each well of a 24-well culture plate. Cells were pre-incubated for 24 h before experiments. Growth medium was removed and replaced with fresh medium containing reagents. Cells were treated according to experimental designs that are described below.

- To examine the effects of GnRHa on granulosa cell growth, granulosa cells were treated with GnRHa (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} and 10^{-7}M) for 12h.
- To examine the effects of ANXA5 on granulosa cell growth, cells were treated with recombinant ANXA5 (10^{-12}, 10^{-11}, 10^{-10}, 10^{-9} and 10^{-8} M) for 24h.
- To examine the effects of anti-ANXA5 on granulosa cell growth, cells were treated with anti-ANXA5 (1:1,000, 1:500 and 1:250) for 24h.

- To examine the effects of hCG on granulosa cell growth suppressive effect of ANXA5, granulosa cells were incubated with 0.01 IU/ml hCG for 3 h and then challenged with recombinant ANXA5 (10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, and 10^{-8} M) for 24 h.

At the end of experiments, medium was discarded and the cells were taken photos. Each sample was added 200 µl trypsin solution and incubated for 3 min. Then 200 µl of growth medium was added into every well. To determine cell viability, mix 1-part trypan blue dye and 1-part cell suspension. Cells were counted by TC20 Automated Cell Counter. Cell size 7-11µm was measured.

11. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Apototic cells were detected by TUNEL method**.**

a. Reagents, solutions and equipments

In Situ Cell Death Detection Kit, POD (Roche Diagnostics GmbH Mannheim, Germany)

Washing buffer

- Phosphate buffered saline (PBS)

Blocking solution

- 3% H_2O_2 (Kanto Chemical Co., Tokyo, Japan)
- Methanol

Fixation solution

- 4% PFA

Permeabilisation solution

- 0.1% Triton[1)] X-100 (Wako Pure Chemical Industries, Ltd., Osaka, Japan)
- 0.1% sodium citrate (Kanto Chemical Co., Tokyo, Japan)

Mounting medium

- VECTASHIELD® mounting medium for fluorescence with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA)

Confocal laser scanning microscopy

Glass Bottom dish coated Poly-Lysine (Matsunami GLASS IND., LTD, Osaka, Japan)

Incubator

b. Protocol

Granulosa cells were prepared as explained in chapter 2. Number of granulosa cell was adjusted to 100,000 cells/ml. Cells were seeded in glass bottom dishes as 2 ml/sample. Experiments were performed after 24 hrs pre-incubation.

Cells were treated with or without 10^{-9}M ANXA5 for 24 h. Apoptotic cells were detected by TUNEL reaction using In Situ Cell Death Detection Kit POD according to the recommended protocol of manufacturer. Concisely, samples were dried by air and then added 4%PFA for 1h at room temperature. PBS was used for rinse. Samples were incubated with blocking solution for 10 min at room temperature, rinsed and incubated in permeabilisation solution for 2 min on ice. Samples were rinsed for 2 times and incubated in TUNEL reaction mixture for 60 min in an incubator. Samples were rinsed for 3 times and analyzed after mounting with a drop of VECTASHIELD® mounting medium for fluorescence with DAPI by Confocal laser scanning microscope.

12. Statistical analysis

Statistical significance was assessed by analysis of variance followed by Tukey's test, with p values less than 0.05 considered statistically significant. Single comparisons were analyzed by Student's *t*-test with *p* value less than 0.01 was considered statistically significant.

Results

1. Effects of GnRHa on ANXA5 mRNA expression and effects of hCG on GnRH mRNA expression

Granulosa cell was treated with hCG and GnRH agonist for 3, 6 and 24 h. GnRHa significantly stimulated ANXA5 mRNA expression in early times of incubation at 3 and 6h (P<0.05). while, hCG did not show any evident effects on ANXA5 mRNA (Fig. 4-1).

GnRH mRNA expression was significantly augmented by hCG at 3 h as shown in chapter 2 but it significantly suppressed at 6 h incubation (P<0.05) (Fig. 4-2).

2. Effects of recombinant ANXA5 on progesterone production

Granulosa cell was pre-treated with 0.01 IU/ml hCG for 1 h. Then various concentration of recombinant ANXA5 was administered for 24 h. Without pre-incubation of hCG, ANXA5 did not show any effect (Fig. 4-3A). While ANXA5 significantly suppressed progesterone production after hCG pre-treatment (P<0.05) (Fig. 4-3B). This data reveals that hCG would necessary for sensitize granulosa cells to ANXA5 on progesterone production.

Granulosa cell was treated with a combination of hCG and ANXA5. ANXA5 significantly inhibited progesterone production stimulated by hCG at 3, 6 and 24h of incubation. However, suppressive effect on basal progesterone production was only seen at 3 h (P<0.05) (Fig. 4-4).

3. Effects of GnRHa, recombinant ANXA5 and anti-ANXA5 on granulosa cell growth

Granulosa cells were treated with various concentrations of GnRHa for 12 h. GnRHa (10^{-9}, 10^{-8} and 10^{-7} M) significantly inhibited cell growth (P<0.05) (Fig. 4-5A). The effect of ANXA5 on granulosa cell growth was also confirmed. The cells were treated with different concentrations

of recombinant ANXA5 for 24h. Recombinant ANXA5 (10^{-10}, 10^{-9} and 10^{-8} M) significantly inhibited cell growth (P<0.05) (Fig. 4-5B). The effect of Anti-ANXA5 on granulosa cell growth was examined. The cells were treated with various dilutions of Anti-ANXA5 for 24h. Anti-ANXA5 (1:500 and 1:250) significantly increased cell growth (P<0.05) (Fig. 4-5C).

4. Effects of recombinant ANXA5 on granulosa cells

Granulosa cells were treated with 10^{-8} M recombinant ANXA5 (Fig. 4-6A) for 24h. Cell morphology was observed with a light microscope. We found fragmented cells in granulosa cells treated with ANXA5 (Fig. 4-6B).

5. Inhibition of suppressive effect of ANXA5 on cell growth

To examine whether hCG would affect ANXA5 action. Granulosa cells were pre-incubated with 0.01 IU hCG for 3h and then treated with various concentrations of ANXA5. The suppressive effect of ANXA5 was only seen in hCG pre-treated cells (Fig. 4-7).

6. Effects of ANXA5 on granulosa cell apoptosis

To examine whether ANXA5 is involved in granulosa cell apoptosis. Granulosa cells were challenged with recombinant ANXA5 (10^{-9} M) for 24 h. The results showed ANXA5 increased TUNEL positive cells (Fig. 4-8).

7. Effects of hCG and GnRHa on ANXA5 distribution

Granulosa cells were incubated with 0.01 IU/ml hCG or 10^{-9} M GnRHa for 6h. The results showed an increase in immunofluorescence of ANXA5 and membrane blebs in granulosa cells treated with hCG (Fig. 4-9B).

ANXA5 distributed close to cell membrane and there were membrane blebs on granulosa cells treated with GnRHa (Fig. 4-9C).

Discussion

In this chapter, close relationship between local GnRH and ANXA5 was again demonstrated in the granulosa cells. It has been reported that GnRH augments ANXA5 expression in luteal cells of pseudopregnant rats when luteotropic hormone is inhibited by dopamine agonist [30]. Present study showed that GnRHa stimulated ANXA5 mRNA expression in granulosa cells relatively early phase of hCG stimulation (3 and 6 h incubation). It is suggested that ANXA5 synthesis would be directly stimulated by hCG or by GnRH at granulosa cells .

It has been already reported by this author that hCG enhanced GnRH mRNA in the primary culture of granulosa cells [74]. While the results of this study demonstrated hCG raised GnRH mRNA in granulosa cells at 3 h incubation but it was suppressed when the incubation was prolonged to 6 h. GnRH synthesis seems to be augmented by hCG but the effect is phasic and does not last long. This is consistent with the result that ANXA5 expression was stimulated in relatively early phase of hCG stimulation. In other words, it is suggested that hCG/LH would stimulate ANXA5 expression through GnRH synthesis. ANXA5 was significantly decreased by hCG at 3 and 6 h incubation and hCG also suppressed the effect of GnRHa on ANXA5 mRNA expression at 3, 6 and 24 h incubation. As hCG stimulates GnRH expression, GnRH is expected to augment ANXA5 expression. As hCG actually suppressed ANXA5 and later GnRH expression, hCG would affect other mechanisms than stimulating GnRH expression also and it may be suppressive to ANXA5 expression. So far, it is not known the exact effect of hCG on ANXA5 expression, but it would be shown following to the stimulating effect on GnRH expression and be related to cell differentiation process, namely luteinization.

Pretreatment with hCG was shown to change the response of granulosa cells to ANXA5 on progesterone production. ANXA5 did not show any effects on progesterone production before hCG treatment. However, hCG pretreatment made cells responsive to ANXA5. As ANXA5 was

shown to be related to apoptosis of luteal cells during pseudopregnancy, the responsiveness to ANXA5 would indicate at least partly a characteristic of differentiated luteal cells. Newly formed luteal cells would become to be cells with a life span. When luteotropic hormone will not be supplied, GnRH-ANXA5 would drive cells to regression.

Former studies indicated that peripheral GnRH and GnRH receptor systems play a crucial role in antiproliferation and apoptosis [83]. GnRH receptors in peripheral tissues differ from those in pituitary gonadotrophs being coupled with Gi protein in uterine leiomyosarcoma, ovarian carcinoma and endometrial carcinoma [23, 24]. Activation of these peripheral GnRH receptor leads to down-regulation of gene transcription in the cell nucleus to mediate antiproliferative activity of GnRH and synthesis of GnRH receptors in tumor cells [36]. GnRHa in vitro suppresses the growth of endometrial cancer and ovarian cancer [12, 81]. It was found that GnRHa induced apoptosis in the cultured leiomyoma cells associated with increased expression of Fas and induction of Fas ligand [79]. GnRH antagonist inhibits apoptosis in preovulatory follicles [50]. This study demonstrated that the negative effect of GnRHa on granulosa cell growth. So, it was thought that GnRH would suppress granulosa cell growth by means of ANXA5 expression. Thus, the effect of recombinant ANXA5 on granulosa cell growth was examined. As expected, the result showed ANXA5 significantly inhibited cell growth. Furthermore, anti-ANXA5 significantly increased cell growth. These data indicate that intrinsic ANXA5 is working during primary culture of granulosa cells.

ANXA5 increased number of TUNEL positive cells. It is suggested that the suppression of cell growth by ANXA5 is due to apoptosis. GnRH and ANXA5 would induce apoptosis in granulosa cells. On the other hand, the suppressive effect of ANXA5 on granulosa cell growth was disappeared when granulosa cells were pre-incubated with hCG. It seems that cell growth suppressive effect of ANXA5 would be exerted only on granulosa cells while luteal cells were not sensitive to pro-apoptotic action of ANXA5. This is very interesting since 1) GnRH was

reported to induce apoptosis in granulosa cells of atretic follicles and 2) effects on progesterone production and cell growth can be distinguished. Biphasic action of GnRH-ANXA5 action is proposed, for granulosa cells and for differentiated luteal cells.

GnRHa stimulated blebbing of cell membrane of granulosa cells. Blebs could be formed as apoptotic bodies since increase in TUNEL positive cells was observed after GnRH stimulation. However, we need to remind ectosome formation as a function of cells. It is observed in the pituitary gonadotropes in this laboratory.

Summary of interaction shows ANXA5 involves in granulosa cell apoptosis (Fig. 4-10A). ANXA5 inhibits progesterone production facilitated by hCG treatment of luteal cells (Fig. 4-10B)

Present study clearly shows that GnRH-ANXA5 acts on granulosa cells in biphasic as proapototic for granulosa cells and suppressive on progesterone synthesis in differentiated luteal cells. The latter effect may be a part of differentiation to luteal cells. GnRH-ANXA5 would be a novel local mechanism for regulation of granulosa and luteal cells.

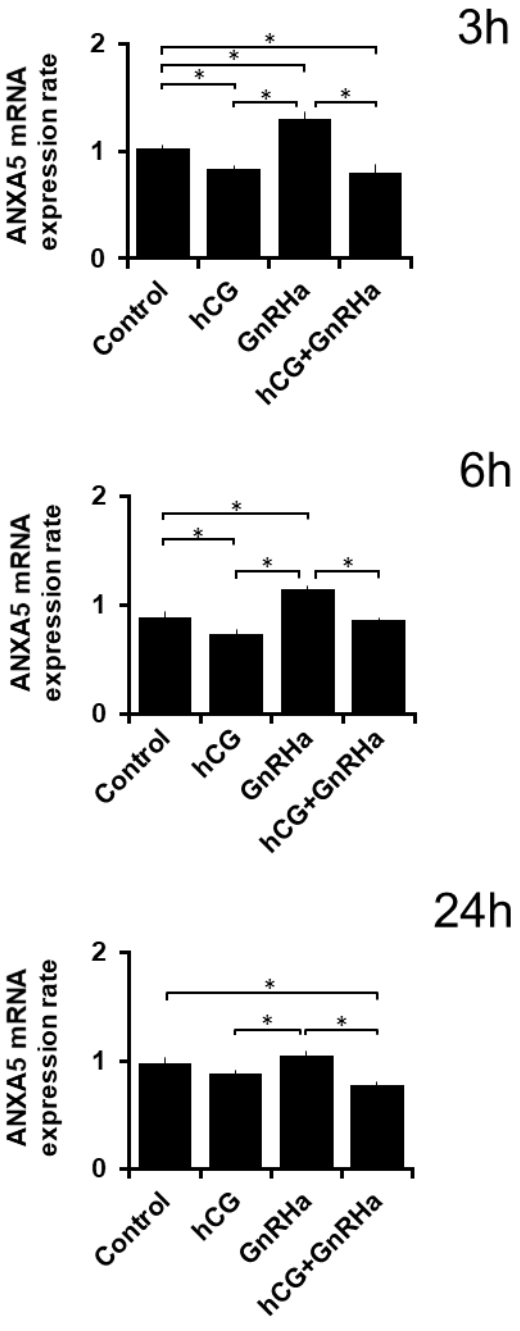

Fig. 4-1 The effect of GnRHa and hCG on ANXA5 mRNA expression in granulosa cells

GnRHa (Fertirelin, 10^{-8} M) and hCG (0.01 IU) was administered to primary granulosa cells for 3, 6 and 24 h. Values are mean±SEM (n=6). Asterisks indicate significant difference (P<0.05).

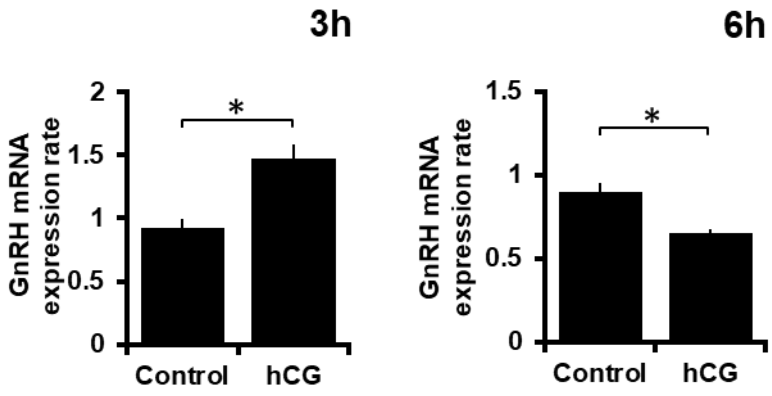

Fig. 4-2 The effect of hCG (0.01IU) on GnRH mRNA expression in the granulosa cells

Primary granulosa cells were incubated with hCG (0.01IU/ml) for 3 and 6h. Values are mean±SEM (n=6). Asterisk indicates values significantly different (P<0.01).

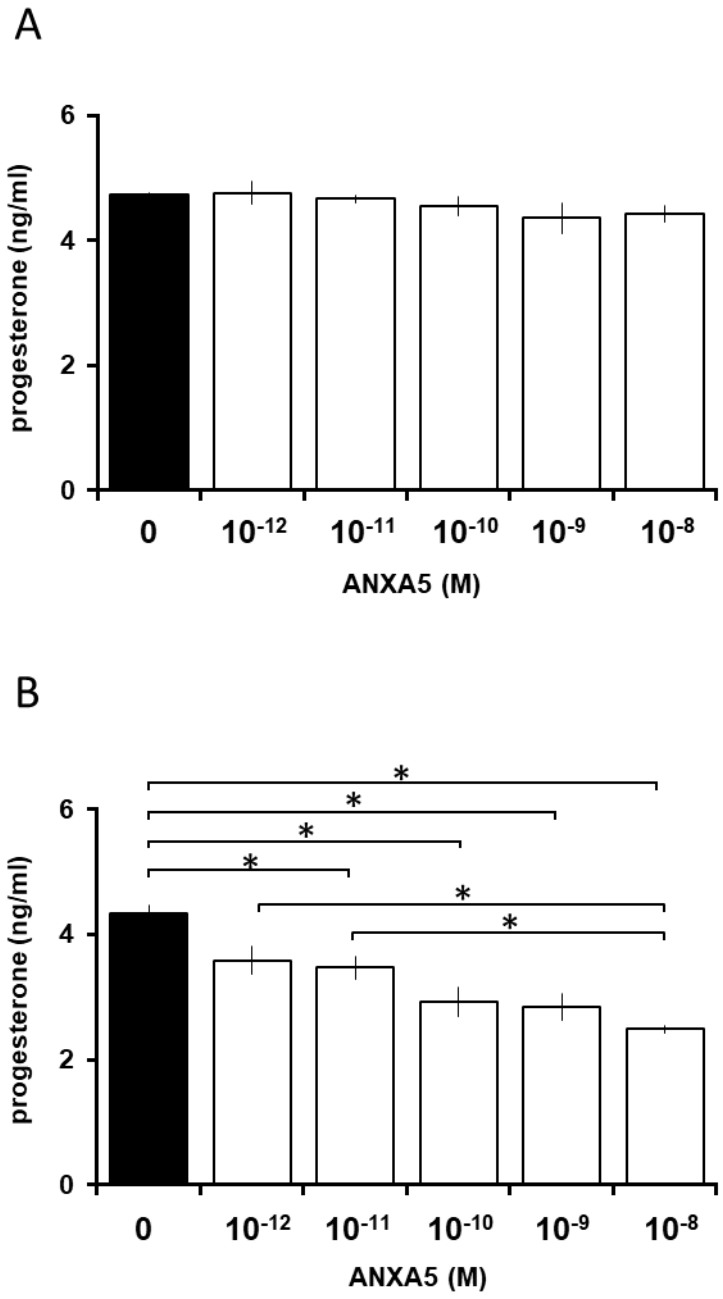

Fig. 4-3 The effect of recombinant ANXA5 on progesterone production

Granulosa cells were treated with ANXA5 for 24h without (A) or with (B) pre-incubation with hCG (0.01 IU) for 1 h. Values are mean±SEM (n=4). Asterisks indicate values significantly different (P<0.05).

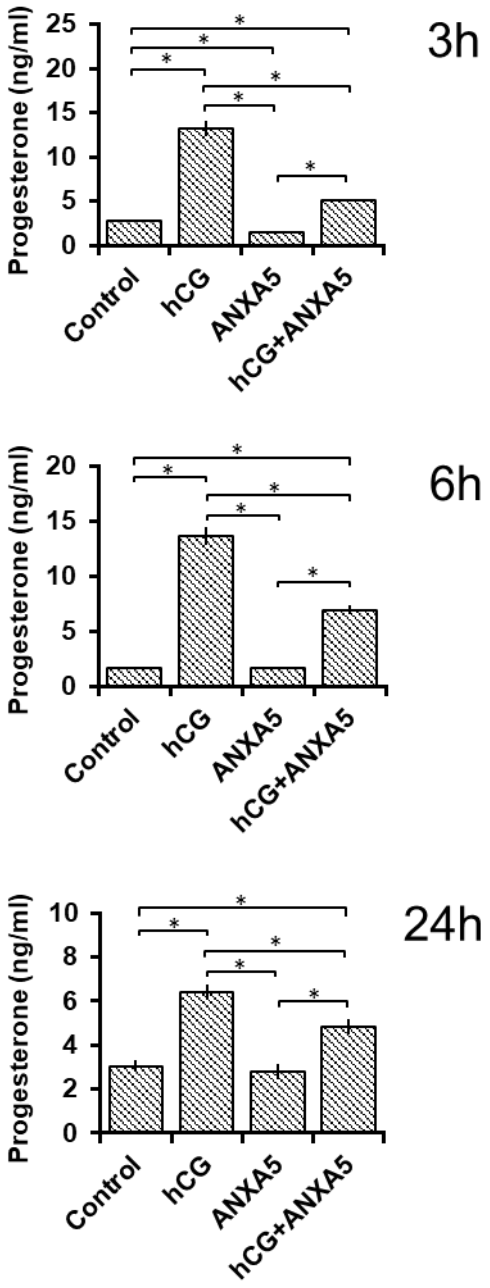

Fig. 4-4 The effect of recombinant ANXA5 on progesterone production stimulated by hCG

Recombinant ANXA5 (10^{-9} M) was administered with hCG (0.01 IU) for 3, 6 and 24 h. Values are mean±SEM (n=6). Asterisks indicate values significantly different (P<0.05).

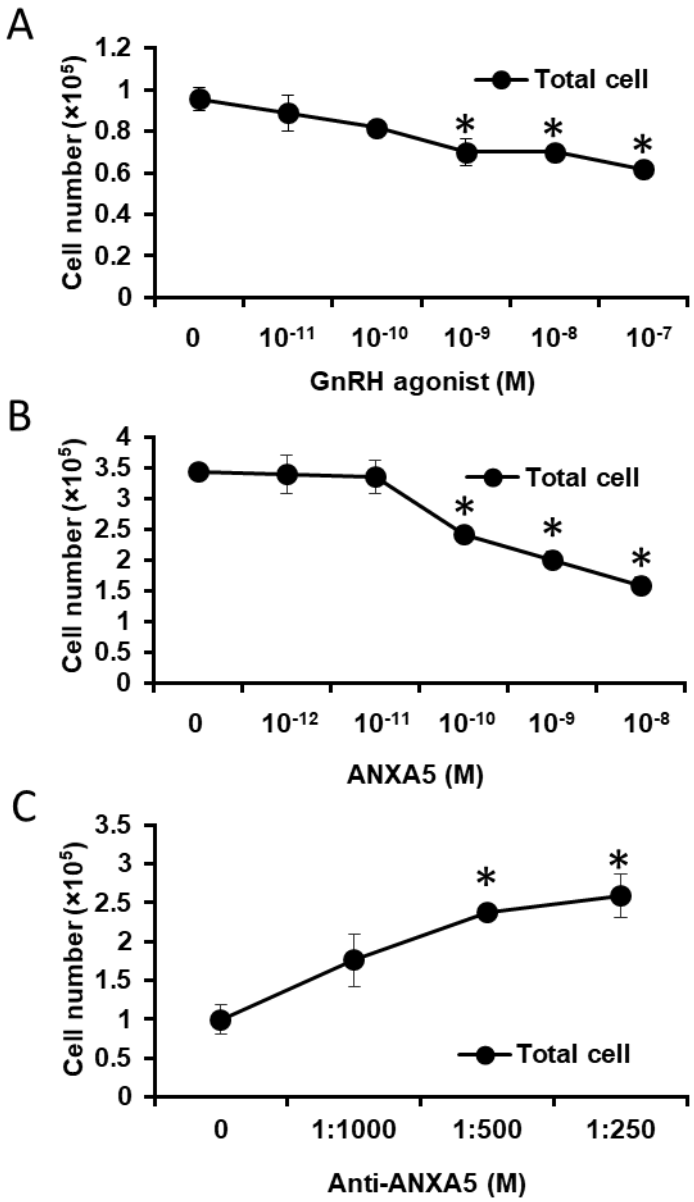

Fig. 4-5 Effects of GnRHa, recombinant ANXA5 and anti-ANXA5 on cell growth of primary granulosa cells

Granulosa cells were treated with GnRHa for 12h. The cells were treated with recombinant ANXA5 or anti-ANXA5 for 24h. Values are mean±SEM (n=4). Asterisks indicate values that are significantly different $P<0.05$.

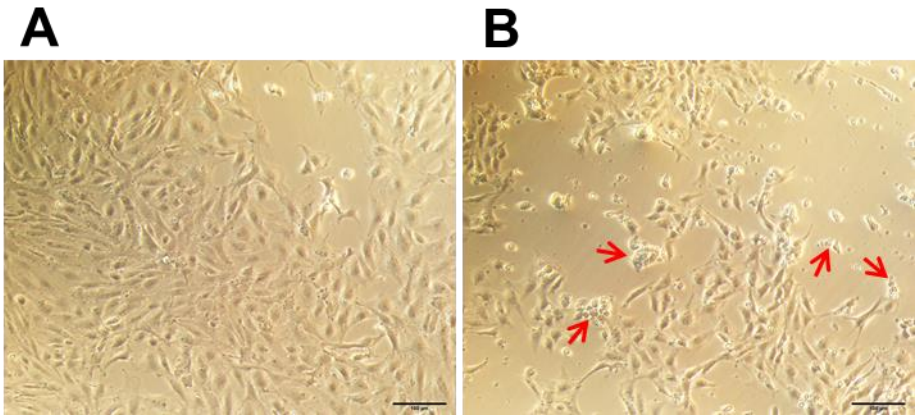

Fig. 4-6 The effect of recombinant ANXA5 on granulosa cells

Primary granulosa cells were incubated with recombinant ANXA5 (10^{-8} M) for 24 h. Control incubation (A) and recombinant ANXA5 treated cells (B). Red arrows indicate fragmented cells.

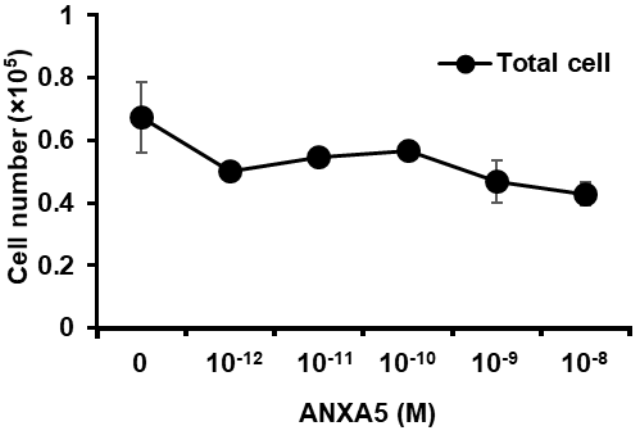

Fig. 4-7 The effect of hCG pre-treatment on the suppressive effect of ANXA5 on cell growth
Granulosa cells were treated with hCG 0.01 IU for 3 h and then the effect of various concentration of ANXA5 was observed for 24h.

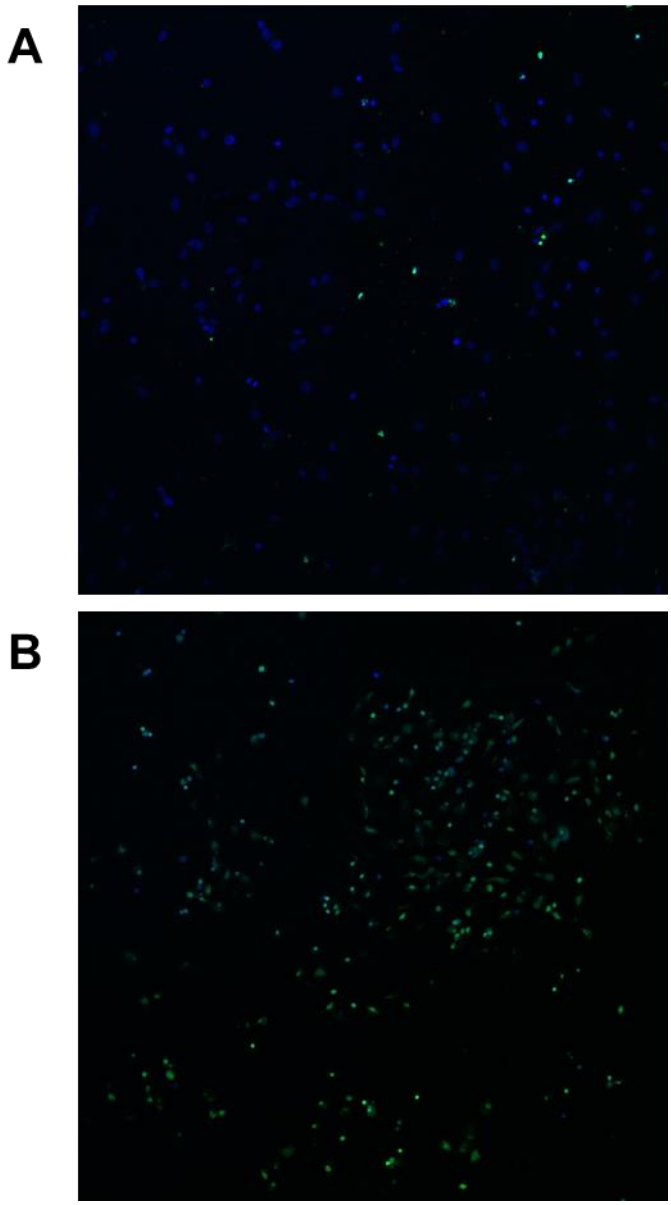

Fig. 4-8 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis of primary granulosa cells

The effect of ANXA5 (10^{-9} M) on granulosa cells was examined by TUNEL method. Control incubation is A and cell incubated with ANXA5 is B. Green signal is TUNEL positive cell. Blue is DAPI stained nucleus

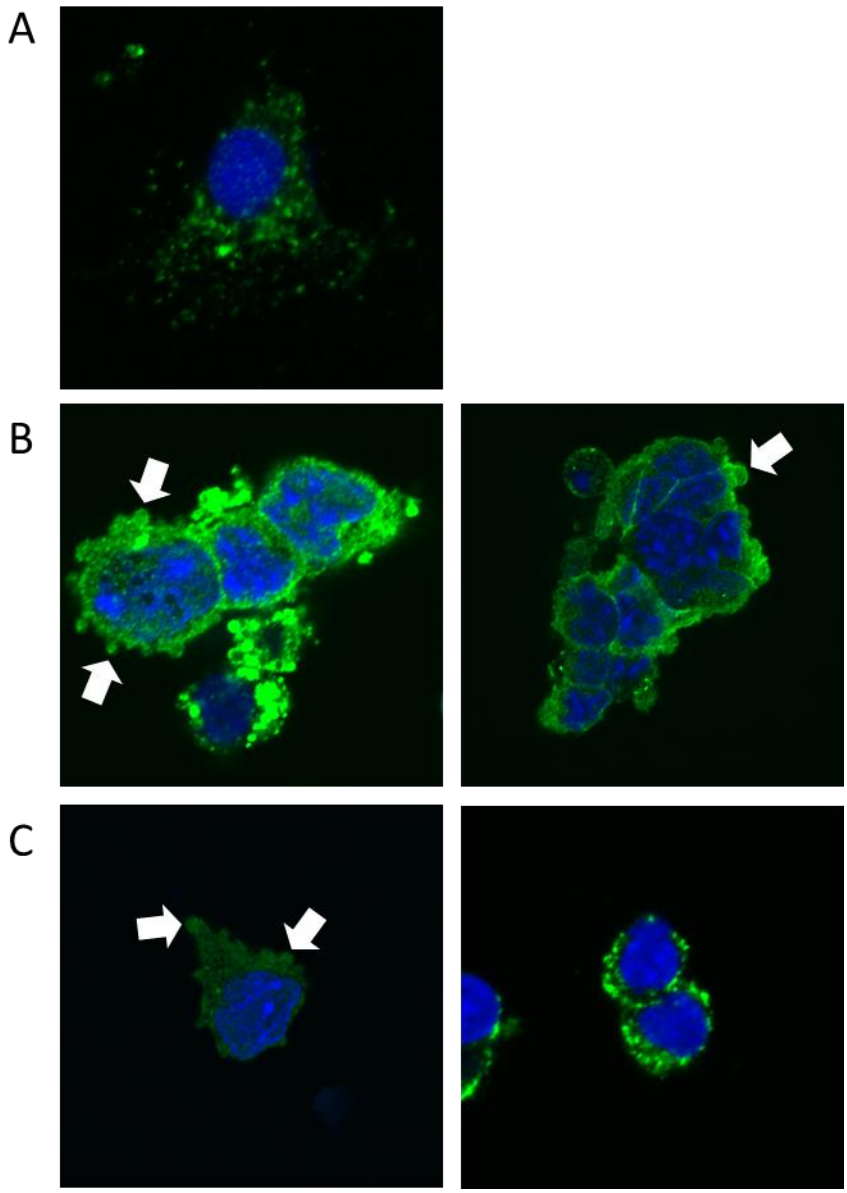

Fig. 4-9 The effect of hCG and GnRHa on ANXA5 distribution

Granulosa cells were incubated with fresh medium (A) or hCG 0.01 IU (B) or GnRHa, 10^{-9} M (C) for 6 h. The green and blue color indicated ANXA5 and DAPI respectively. White arrows indicate membrane blebs.

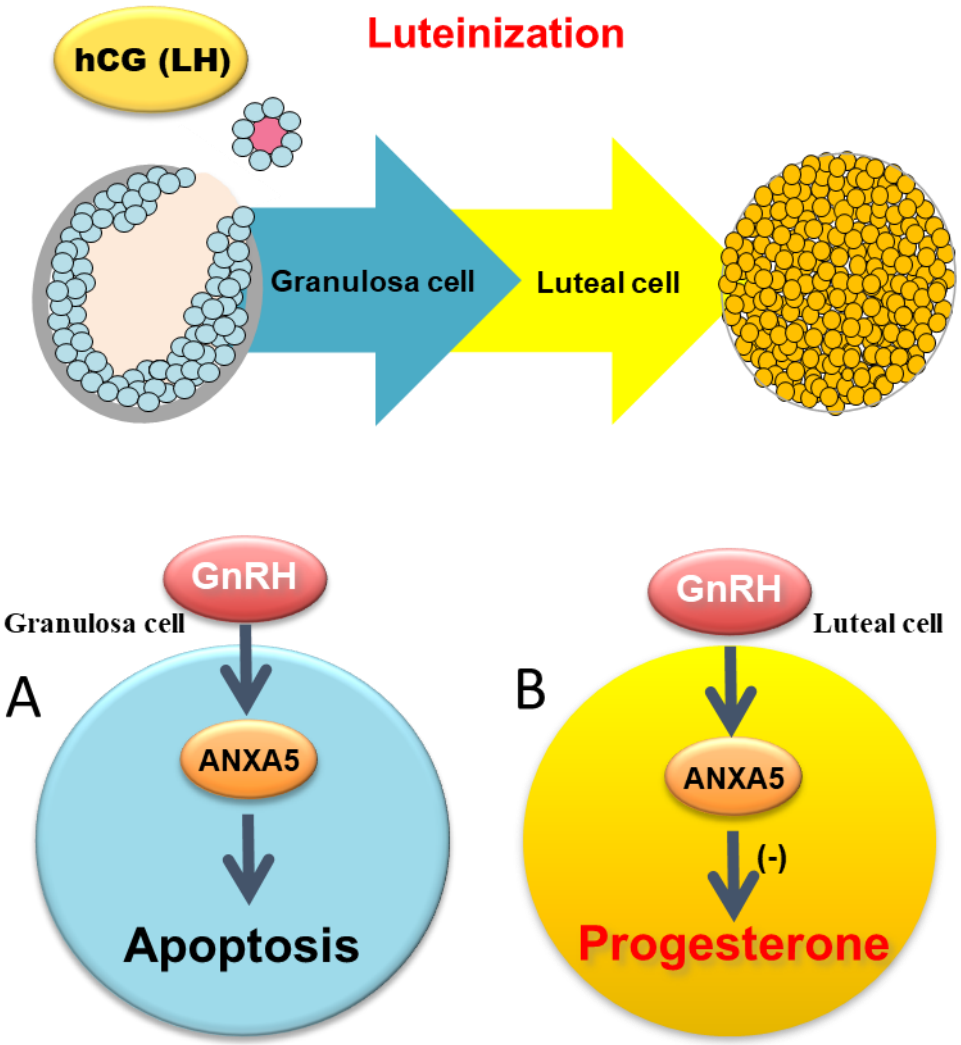

Fig. 4-10 Summary of interaction

(A) GnRH and ANXA5 stimulates granulosa cell apoptosis. (B) GnRH and ANXA5 has suppressive effects on progesterone production in early phase of luteinization.

Chapter 5 Summary

Introduction: Mammalian ovary contains follicles in various stages and corpus luteum. In the ovary of rats, a set of follicles starts to grow in each estrous cycle and ovulation recurs with a short interval. After ovulation, theca and granulosa cells of the follicle proliferate and become corpus luteum. This process is called luteinization and it accompanies tissue remodeling, the shift of steroidogenic pathway, exit cell cycle and changes in responsiveness to pituitary hormones. While ovulation and luteinization is induced by LH surge in the afternoon of proestrus, it is becoming clearer that various local hormones in the ovary also contribute to the periodical changes in the ovary. Interestingly hypothalamic neuro-peptides are also expressed in the ovary. GnRH augments ANXA5 expression in the pituitary gonadotropes and also in other peripheral tissues including the ovary. GnRH is expressed in the ovary, but its physiological function in the ovary is still obscure. GnRH related neuro-peptides seen in the hypothalamus, kisspeptin, dynorphin and NKB are also expressed in granulosa cells. Hence, it is great interest to know the relationship and function of GnRH and related neuro-peptides in granulosa cells, especially during luteinization. In the present study, it was examined whether GnRH is involved in the process of luteinization and the relationship between GnRH, kisspeptin, dynorphin, and NKB in the granulosa-luteal cells. Finally, a function of ANXA5 in the granulosa cells during luteinization was also studied.

Involvement of GnRH in luteinization process induced by hCG: In the present study, ovarian GnRH was examined whether it is involved in the process of luteinization using immature rat model. Follicular growth was induced by pregnant mare serum gonadotropin (PMSG, 15 IU/0.15 ml) given to 25-day old female rats. Luteinization was induced by human chorionic gonadotropin (hCG, 20 IU/0.2 ml) administration on day 27 after PMSG administration. Plasma level of progesterone was increased by hCG with a peak at 6 hr. ANXA5, a biomarker of GnRH action,

expression in the granulosa cells was increased after hCG administration. GnRH mRNA was increased in the ovary 3 h after hCG administration. Primary culture of granulosa cells was established by liberating cells from large follicles obtained 2 day after the PMSG treatment. The majority of cells was proved to express 3β-HSD by immunocytochemistry. Progesterone synthesis was augmented by hCG in a dose-dependent manner. GnRH mRNA was also increased by 0.01 IU hCG in the primary culture of granulosa cells and GnRH agonist (GnRHa, des-Gly10 [Pro9]-GnRH ethylamide) increased ANXA5 mRNA expression. GnRH (10^{-7} M) or GnRHa (10^{-8} M) suppressed hCG stimulated progesterone synthesis during 3 h incubation, revealing GnRH stimulation is rather suppressive to progesterone synthesis. Interestingly, concomitant administration of GnRHa and hCG clearly increased LH receptor mRNA expression and decreased follicle-stimulating hormone receptor. As these changes are the characteristics of luteinization, GnRH is suggested to have a cooperative role with LH in the differentiation of granulosa cells to luteal cells. GnRHa also affected the expression of genes related to differentiation (p21, p27, FOXO1 and prolactin receptor). GnRH and hCG affected synergistically on these genes (p27, FOXO1 and prolactin receptor). Present data clearly show that GnRH is involved in the effect of hCG on the transformation of granulosa cells to luteal cells. ANXA5 is suggested to have a role under GnRH receptor.

Relationship between GnRH, kisspeptin, dynorphin and NKB in the granulosa cells: Kisspeptin, dynorphin and NKB mRNA are all augmented by LH surge in the ovary during the estrous cycle of rats. The relationship between these peptides in the granulosa cells and the effect on progesterone production were examined. Kisspeptin and dynorphin mRNA expression in the ovary were augmented by 3 hr after hCG administration on day 27. NKB mRNA was gradually increased but not significant. GnRH, kisspeptin, dynorphin and NKB mRNA expression were all stimulated by hCG also in the primary culture of granulosa cells until 3 hr. Progesterone production stimulated by hCG was suppressed by concomitant incubation with kiss-10 (bioactive

peptide of kisspeptin), dynorphin A and NKB. Inter-relationship between neuro-peptides was examined on mRNA expression rate and results suggest a sequence of events after LH surge. GnRH would stimulate the expression of kisspeptin, dynorphin and later NKB mRNA. Then NKB would suppress at least GnRH and kisspeptin mRNA expression. Immunohistochemistry showed kisspeptin in granulosa cells after 6 h of hCG administration. Kisspeptin receptor (GPR54), NKB receptor (Tachykinin receptor 3) and dynorphin receptor (kappa opioid receptor) mRNA were confirmed in granulosa cells. Functional relationship among neuro-peptides in granulosa cells and suggest changes in the expression of the neuro-peptides are related to the differentiation of granulosa cells, luteinization.

GnRH-ANXA5 function in granulosa cells: It is known that the expression of ANXA5 in the rat corpus luteum is suppressed by prolactin during pseudopregnancy and increased by GnRH when luteolysis occurs. ANXA5 is not seen in the granulosa cells and it appears in luteal cells. Granulosa cells were incubated with GnRHa with or without 0.01 IU hCG. GnRHa increased ANXA5 mRNA expression at 3 and 6 hr but the stimulating effect gradually decreased and disappeared until 24 h. Although hCG stimulated GnRH expression in early phase of incubation, ANXA5 mRNA was suppressed by concomitant administration of hCG in granulosa cell culture. Even though ANXA5 is induced by hCG treatment as shown by immunohistochemistry, changes induced by hCG in granulosa cells seem to become suppressive for ANXA5 expression. Recombinant ANXA5 (10^{-9}M) did not show any effect on progesterone production of granulosa cells. However, when hCG was given first, the suppressive effect of ANXA5 appeared. These data suggest that changes induced by hCG would sensitize granulosa cells to inhibitory action of ANXA5 on progesterone production. On the other hand, GnRHa and ANXA5 reduced cell number of granulosa cells not treated with hCG. Recombinant ANXA5 decreased while anti-ANXA5 increased cell growth during 24 h incubation of granulosa cells. The reduction of cells by ANXA5 was shown to accompany the increase of terminal deoxynucleotidyl transferase nick

end labeling (TUNEL) positive cells. Pro-apoptotic function of ANXA5 on granulosa cells disappeared after hCG treatment. It was clearly demonstrated that granulosa cells would change by hCG that is evaluated by responses to GnRH and ANXA5.

Bold: GnRH action was assumed by the expression of ANXA5 in granulosa cells after hCG stimulation**.** Actually, GnRH expression was augmented by hCG in early phase of hCG action**.** GnRH was demonstrated to be involved in the luteinization process by hCG**.** GnRH related neuro-peptides, kisspeptin, prodynorphin and NKB were all stimulated by hCG**.** NKB was suggested to cease these reactions by suppressing each expression**.** ANXA5 was shown to be pro-apoptotic on granulosa cells and to be suppressive on progesterone production of luteal cells**.** The former response is suggested to relate to follicular atresia and the latter to luteinization**.** Present study shows a network of ovarian neuro-peptides in different way from that seen in the hypothalamus**.** They are suggested to have a role to initiate luteinization**.** GnRH-ANXA5 would be a novel mechanism for regulation of granulosa and luteal cells in the ovary**.**

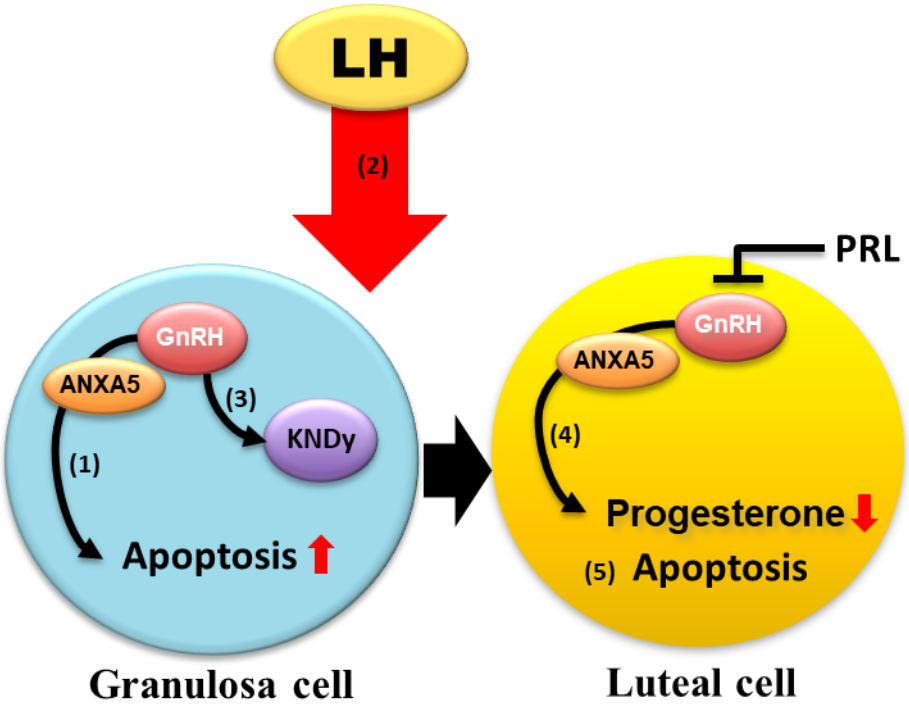

Fig. 5-1 Possible sequence of events

(1) GnRH-ANXA5 could induce apoptosis of granulosa cells before LH surge. (2) LH stimulates granulosa cells to transform to luteal cells. (3) There is interaction among GnRH-ANXA5 and KNDy, facilitating luteinization. (4) In the luteal cells, GnRH-ANXA5 would suppress progesterone synthesis and PRL inhibits GnRH action during luteal phase. (5) At the end of luteal phase, PRL secretion ceases and GnRH-ANXA5 induces apoptosis of luteal cells.

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