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Establishment of Primary Culture System of Porcine Granulosa Cells and Construction of Normalized cDNA library

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Gonadotropins like follicle stimulating hormone (FSH) and luteinizing hormone (LH) are known to be essential for the development and maturation of ovarian follicles and to exert their effects by inducing a group of genes in granulosa cells. In this study, we established a serum-free defined culture system for porcine granulosa cells and, under the conditions, porcine cells responded to FSH stimulation. Furthermore, making use of the culture system, we successfully constructed a normalized cDNA library to isolate and characterize FSH inducible genes.

KEY WORDS: Ovarian follicle / Chemical defined medium / FSH / LH receptor / Differentiation

INTRODUCTION

Mammalian ovarian follicle is the functional and structural unit of oogenesis where granulosa cells play important roles under the influence of follicle stimulating hormone (FSH)[1-4]. Oocytes remain arrested at the dictyate state for a prolonged period until leuteinizing hormone (LH) triggers the resumption of meiosis of a selected follicle and ultimately leads to ovulation[5]. After ovulation, granulosa cells differentiate into luteal cells and form the corpus luteum[6]. Gonadotropins such as FSH and LH thus function cooperatively in follicles and exert their effects by inducing various enzyme activities or substances within granulosa cells through membrane bound receptors[7-11]. However, the details of their signaling pathways and inducible genes are totally unclear.

We are interested in identifying gonadotropin-regulated genes which play key roles in the follicular development and maturation. Porcine system is suitable for this purpose because a large quantity of cells can be obtained easily which are fairly synchronized in the stage before luteinization due to its prolonged ovulatory cycle compared to other species[12]. Several trials have been reported to establish primary culture systems of granulosa cells so far, but there have been difficulties to maintain porcine primary cells as gonadotropin responsive cells and hence sufficiently reproducible conditions have not been established yet[13-17]. In this study, we first tried to set up optimal conditions for maintaining FSH responsive porcine cells *in vitro* and then constructed a normalized cDNA library successfully to isolate FSH-inducible or-repressible genes.

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MATERIALS AND METHODS

Media and sera

McCoy's 5A medium, Dulbecco's modified eagle medium (DEM) and nutrient mixture Ham F-12 (F-12) were obtained from GIBCO. 4F medium consists of DEM/F-12 (1:1) supplemented with insulin (2 μg/ml; Takara Shuzo, Japan), transferrin (5 μg/ml, Takara Shuzo, Japan), hydrocortisone (40 ng/ml; Sigma), fibronectin (8 μg/ml; BTI) and 5% fetal bovine serum (FBS; Hyclone, USA). All media were supplemented with gentamycin (40 μg/ml; Wako Chemicals, Japan) and $NaHCO_3$ (1.2 g/liter), and were manitained at 37℃ in a humidified atmosphere of 95% air-5% CO_2.

Porcine ovaries and granulosa cells

Ovaries (Fig.1) were obtained from immature prepubuvertal gilts at a local slaughterhouse and were transported in Hank's balanced salt solution (Flow laboratories) containing penicillin (50 units/ml; Meiji Seika, Japan), streptomycin (50 μg/ml; Meiji Seika, Japan), fungizone (500 ng/ml; GIBCO, USA), and $NaHCO_3$ (0.7 g/liter) on ice. Granulosa cells were isolated from small (1-2 mm) or medium (3- to 5- mm) follicles by aspirating with a needle and syringe as described previously[12]. The harvested cells were then suspended in cold Hank's solution and centrifuged at 300xg for 10 min. The process was repeated and the resulting pellet was resuspended in cold Hank's solution. Viable cells were counted by staining the cell suspension with trypan blue.

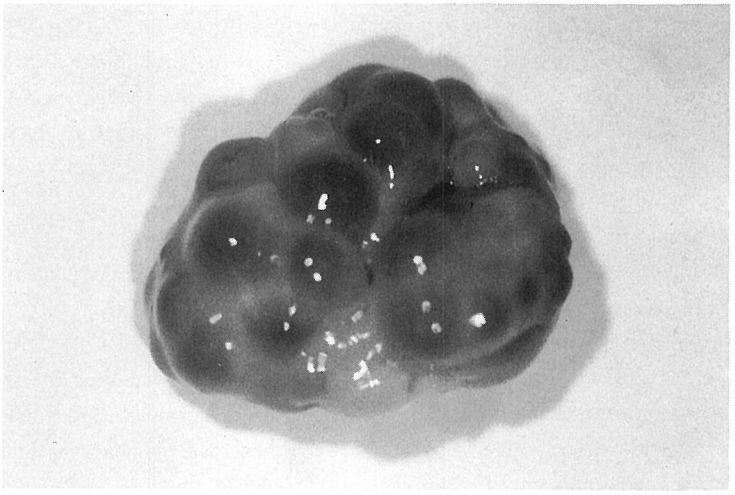

Fig.1 A porcine ovary containing small (1-2 mm) and medium (3-5 mm) follicles from which granulosa cells were obtained.

RNA isolation

For the isolation of mRNA, cells were inoculated in 4F medium at a desity of $1x10^7$ cells per 100-mm dish and incubated for the indicated period in the absence or presence of FSH (2.5 μg/ml, 1 NIH-FSH-SI U/mg; Sigma). Total RNA was extracted from cultured granulo-

sa cells as described[18]. Briefly, cells were lysed with 4M guanidinium isothiocyanate, 20 mM sodium acetate (pH5.2), 0.1 mM dithiothreitol and 0.5% N-lauryl sarcosine. The viscous solution was drawn up and down four times through a 20 G needle, layered onto a 5.7 M CsCl solution and centrifuged at 150,000xg (Beckman SW-50 Ti rotor) for 20 h at 18℃ or at 150,000xg (Beckman SW-41 Ti rotor) for 15 h at 18℃. The pellet was dissolved in TES [10 mM Tris·HCl (pH7.5), 5 mM EDTA, and 1% sodium dodecyl sulfate (SDS)] and extracted with chloroform/1-butanol (4:1, vol/vol). The RNA was precipitated with sodium acetate and ethanol and dissolved in diethylpyrocarbonate-treated distilled water. After rep-recipitation, the RNA was stored at -70℃. The concentration of nucleic acid was determined by the absorbance at 260 nm.

RNA blots

For slot blot analysis, 130 ng of total RNA was blotted on a nylon membrane as described[19]. For plaque hybridization, 500 plaques from a cDNA library were immobilized on nitrocellulose filters. Plasmid DNA containing the porcine inhibin α-subunit gene [20], the rat β-actin gene (M. Matsui, Nihon Medical School), or the human ribosomal RNA gene (JCRB) was labeled by using a random primer labeling kit (Takara Shuzo, Japan) and ^{32}P-dCTP. Hybridization was performed at 42℃ overnight and membranes were washed three times with 2xSSC (1xSSC = 150 mM NaCl and 15 mM trisodium citrate, pH 7.0) - 0.1% SDS, followed by washing three times with 0.2xSSC-0.1% SDS at room temperature.

Human chorionic gonadotropin (hCG) binding assay

For LH/hCG binding assay, $1x10^6$ viable cells were plated per well (25mm) of collagen-coated 12-multiwell culture dishes (Corning) and were cultured for 24h in DEM containing serum or in 4F medium. The dose-dependent effects of FSH on the induction of LH receptors was examined after 24 h by the extent of [^{125}I]-hCG (New England Nuclear, USA) binding as previously described [21]. Radioactivities of the bound hCG were counted by Beckman LS-230 liquid scintillation counter.

Construction of normalized cDNA library

Construction of a cDNA library of porcine granulosa cells and its normalization were carried out as described [22] with some modifications. Briefly, total RNA (102 μg) was extracted from granulosa cells cultured for various times with or without FSH (2.5 μg/ml) and they were finally combined. Poly(A)$^+$ RNA was prepared by passing oligo(dT)-cellulose column and was primed with a mixture of high concentration of random hexanucleotides (7.4 μg). cDNA was synthesized by Time-Saver cDNA Synthesis Kit (Pharmacia) and cloned into λgt10 vector. Packaging and amplification were performed by using GIGAPACK II PACKAGING EXTRACT (Stratagene). Inserts of whole clones were amplified by the polymerase chain reaction (PCR) and denatured; after reassociation at 65℃ for 72 h, the mixture was applied to a hydroxyapatite column (Bio-Rad, Bio-Gel HT). An unabsorbed fraction consisting of single stranded DNA was subjected to another PCR and finally cloned into λgt10 vector.

RESULTS

Primary culture of porcine granulosa cells

Granulosa cells were prepared from small (1-3 mm) and medium (3-5mm) follicles and plated in DEM without FBS. Cells were observed to attach on the dish substratum within 4 h. However, they were released easily into the medium and were completely lost after 4 days. On the other hand, when incubated with FBS, many flat cells could be observed shortly after plating and became confluent after 10 days (Fig. 2a). This confluency seemed, not due to proliferation, but mainly due to enlargement of an individual cell as was reported by Orly *et al.* [23] on rat cells.

Although FBS seemed to be essential for the maintenance of granulosa cells in its primary culture, the addition of serum in culture medium has been reported to abrogate the cellular response to FSH [17,23,24]. Therefore, we tried 4F medium which had been successfully used for the maintenance of rat granulosa cells without serum supplement [23]. Porcine cells were also found to be able to be maintained in 4F medium for 2 days without an apparent reduction of attached cells, although cells did not proliferate significantly either.

Effect of FSH on the expression of the inhibin and actin genes

It is well established that FSH induces the inhibin gene in granulosa cells *in vivo*. The induction of the inhibin mRNA in a cultured system also has been reported in rat granulosa cells [25,26]. Together, the inducibility of the inhibin mRNA can be an indicator of FSH stimulation in granulosa cells, and this has been examined in porcine granulosa cells under the culture condition described above.

The administration of FSH to cells in 4F medium caused the suppression of cell viability and, at the same time, increased the population of cells with round-shaped morphology (Fig. 2b,2c). The inhibin mRNA level in FSH-stimulated cells was 1.8-fold higher than that of unstimulated cells after 12 h and 3.7-fold higher after 24 h (Fig. 3), indicating that FSH actually exerted its effects in the cultured porcine cells and that primary cells still retained, at least, some of their characteristics under the conditions used. In addition, FSH-stimulated cells maintained the inhibin mRNA level up to 24h, whereas the level decreased to half by 24h without FSH treatment. On the other hand, the expression of the actin gene was found to be unaffected by the FSH stimulation.

Effect of serum and FSH on the expression of LH receptor

Primary cells were further characterized by the inducibility of LH/hCG receptors after FSH stimulation in DEM containing serum or in 4F medium (Fig.4). The basal level of hCG binding activity was much higher in 4F-medium than in DEM, indicating that the addition of serum strongly reduced the responsiveness of porcine cells to FSH as had been described in rat granulosa cells [24]. Moreover, the binding activity of LH/hCG to granulosa cells did not change with increased concentrations of FSH in either medium, and this could be explained by the reduction of viable cells (Fig. 2b,2c) as described above. The possibility, however, that cells had already been committed in luteinization cannot to excluded (see below).

Construction of normalized cDNA library of granulosa cells

Several genes including the inhibin gene have been known to be expressed abundantly in granulosa cells and those might hamper the identification and isolation of rare mRNAs. To overcome this problem, it was obviously preferable to construct a normalized cDNA library

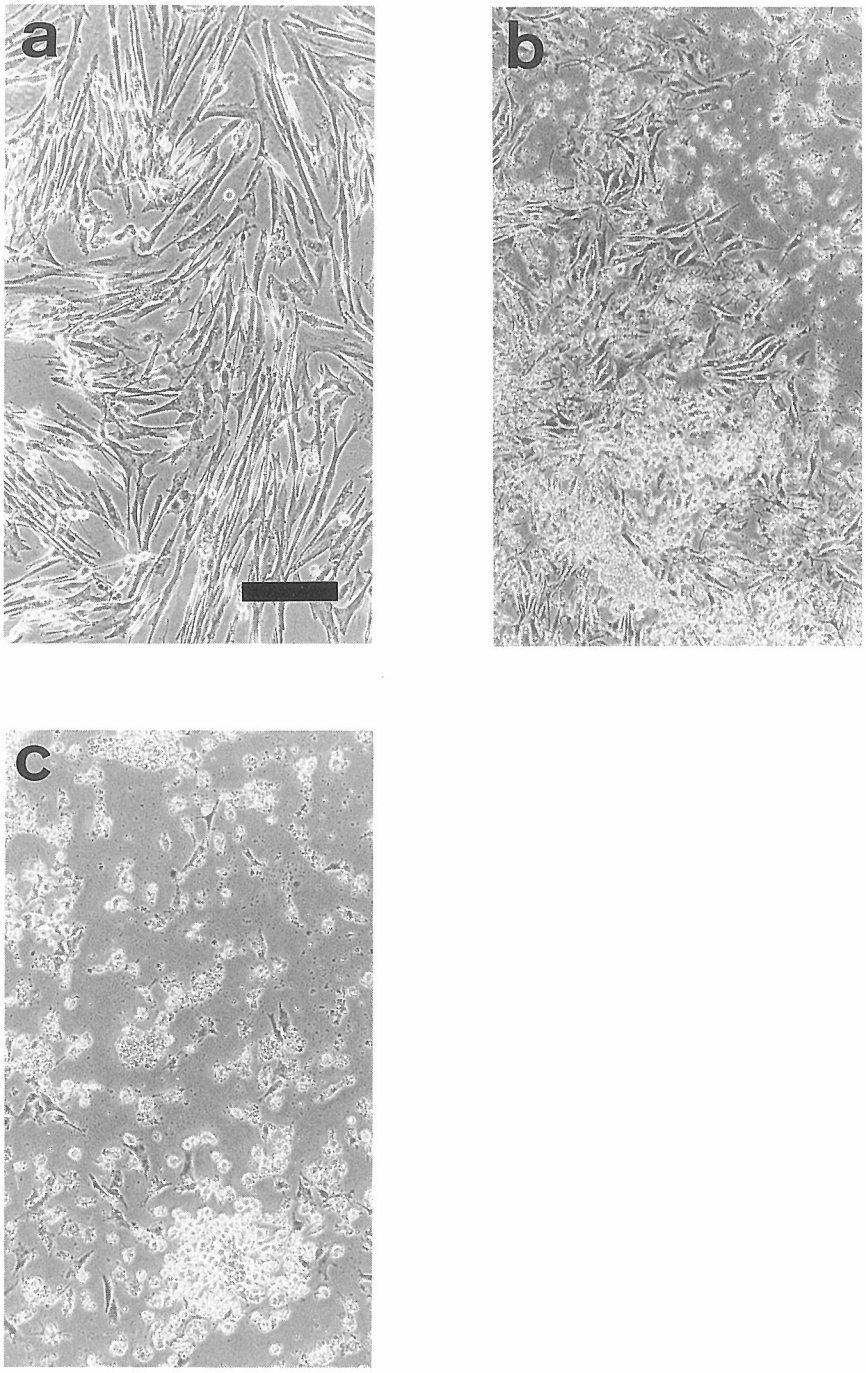

Fig.2 Morphologies of porcine granulosa cells in DEM containing serum or in 4F medium. Cells were plated on a 100-mm plate and cultured in DEM with 8% FBS for 10 days (a). Cells were plated on a collagen-coated 12-multiwell culture dish (22 mm) and cultured for 24 h in 4F medium in the absence (b) or in the presence (c) of FSH (2.5 μg/ml). The bar corresponds to 200 μm.

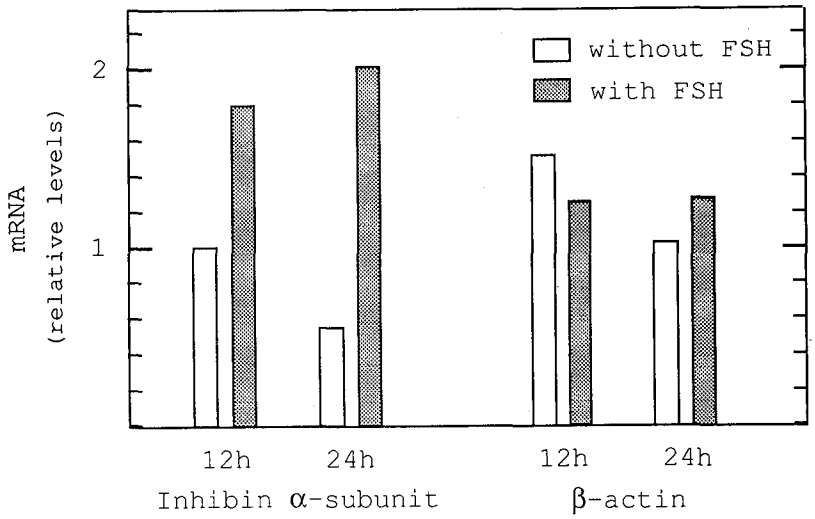

Fig.3 Effects of FSH stimulation on the expression of the inhibin and actin mRNA. Granulosa cells were cultured for 12 h or 24h in 4F medium with or without FSH (2.5 μg/ml). Total RNA (130 ng/slot) was spotted to a nylon membrane using a slot blot manifold and hybridized with ^{32}P-labeled α-inhibin and β-actin cDNA probes, respectively. Values are expressed relative to inhibin mRNA level at 12 h without FSH.

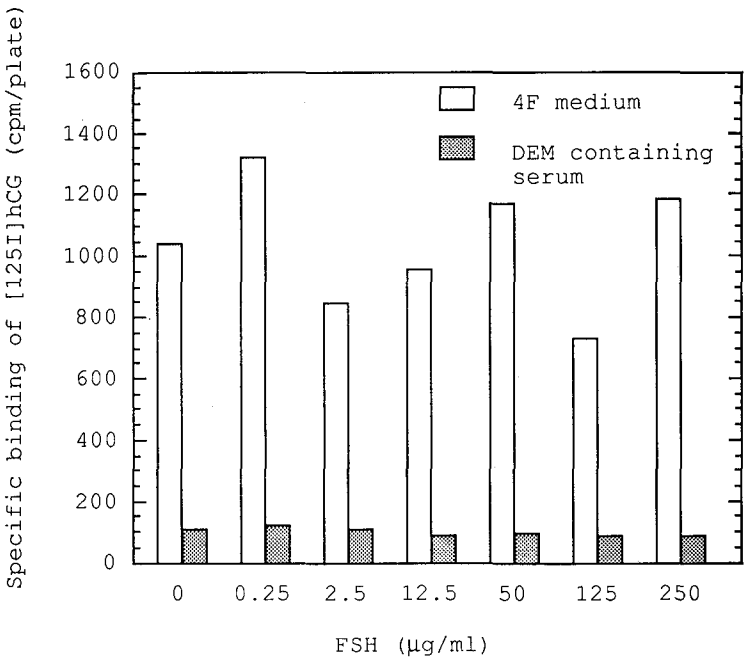

Fig.4 Effects of serum on specific binding of [^{125}I] hCG to porcine granulosa cells under the various concentrations of FSH. Granulosa cells ($1x10^6$ cells/plate) were cultured for 24 h in 4F medium (open bars) or serum-containing medium (closed bars) with FSH (0-250 μg/ml). Specific binding of ^{125}I-labeled hCG was determined as described in Materials and Methods.

in which copy numbers of an individual mRNA species were equalized.

Poly $(A)^+$ RNA was isolated at various times from granulosa cells cultured in 4F medium with or without FSH. A normalized cDNA library was constructed as described[22], and we obtained about $1x10^5$ independent cDNA clones. The extent of normalization procedures was examined by counting cDNA clones of abundant genes such as the actin or the ribosomal RNA gene before and after normalization. As a result, twelve out of five hundred plaques hybridized with the actin probe in prenormalized library, while no hybridization was detected in normalized library (Fig. 5), implying the achievement of significant equalization. On the other hand, since poly $(A)^+$ selected mRNA was used as the material, the ribosomal DNA probe gave no signal in both prenormalized and normalized libraries.

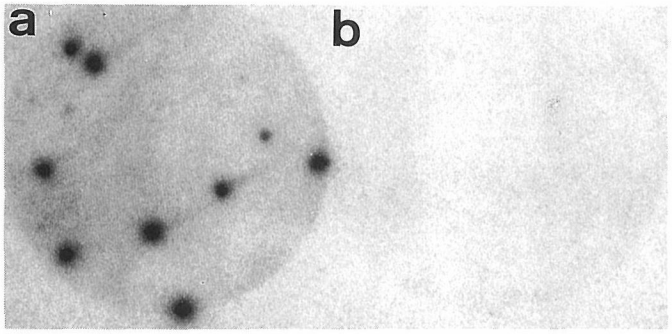

Fig.5 Effect of normalization procedures in constructing cDNA libraries. The extent of normalization was examined by plating about 500 clones of the prenormalized (a) and the normalized library (b) and counting the β-actin clones as described in Materials and Methods.

DISCUSSION

Defined primary culture systems have been inquired to elucidate the hormonal regulation of growth and/or differentiation of ovarian cells *in vitro* and that is an urgent goal in the related fields. Although both rats and pigs have been used as sources for granulosa cells, we have been more interested in the porcine system from points of view of reproductive biology as well as developmental biology. To this end, nutrient media supplemented with serum have been used in early studies to provide specific nutrients, growth factors, and hormones for facilitating cell attachment, maintenance, and growth. However investigations have been unsatisfactory as the cells grew to enter in proliferation, not in differentiation, and became to show morphological anomaly (Fig. 2a). Furthermore, serum has been known to contain a

variety of uncharacterized factors or substances which could function either positive or negative effectors on defined growth factors or hormones. In fact, induction of progesterone synthesis [23] or LH receptors [24] by FSH could be detected only in a serum-free medium. Therefore, we tried to establish an *in vitro* primary culture system without serum.

A plating efficiency of porcine granulosa cells was extremely low under serum-free conditions. Channing *et al.*[15] reported that the supplement of insulin together with hydrocortisone or thyroxine was required in a serum-free medium to allow FSH-induced luteinization. However, such hormones neither improved the low plating efficiency nor supported the cell growth in our hands. Furthermore, Buck *et al.*[14] reported a culture system consisting of fibronectin-coated plates and nutrient medium supplemented with low-density lipoprotein ,insulin, and thrombin; this culture condition has been successfully used to maintain epitheloid cells and has been shown to secrete progesterone and produce LH receptors after FSH stimulation. We tried these hormones in our systems and failed to maintain porcine granulosa cells; they rather caused cell death and no differentiation markers were observed (data not shown). On the other hand, Orly *et al.*[23] described that rat cells could remain healthy and steroidogenically responsive for at least 60 days in serum-free 4F medium supplemented with insulin, hydrocortisone, transferrin, and fibronectin. We incorporated the results of such observations and trials and finally established the culture conditions as described in the text.

Zhiwen *et al.*[25] demonstrated that FSH could stimulate the inhibin production in cultured rat cells. In this study, we also showed that FSH induced the inhibin mRNA in the cultured porcine cells two-fold as high as in control cultures after 24h, whereas FSH had no effect on the induction of the actin mRNA which was chosen as a representative of housekeeping genes. In addition, the inhibin mRNA decreased without FSH, suggesting that the inhibin mRNA had already been expressed in isolated cells and the continuous presence of FSH was required to maintain its level.

Champ *et al.*[27] showed by *in situ* hybridization that no LH receptor mRNA was observed in rat granulosa cells in small follicles (0.35-0.5 mm), while an abundant expression was detected in large follicles (more than 0.5 mm) after PMSG treatment, and also that the mRNA level changed during estrous cycle. Porcine granulosa cells isolated and cultured under the conditions as described above were shown to express LH receptors, suggesting the possibility that either some cells were derived from larger follicles or were already committed in luteinizing pathways. We examined the accumulation of progesterone in cultured porcine granulosa cells from small follicles (less than 1mm) by immunofluorescence and detected the product in a limited number of cells, implying that actually some cells were already in luteinizing processes even in small follicles (data not shown).

In order to isolate FSH-inducible or -repressible genes, we have established the culture system as described and constructed normalized cDNA libraries from the cultured cells. By applying differential hybridization techniques to the library, we have already obtained several candidates that could meet the criteria and started the characterization of structural and functional features of those genes.

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