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Regulation of Proliferation and Steroidogenesis in Human Granulosa Cells

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Background and Aims: Human follicles selected for further development are thought to receive precise signals from gonadotropins and locally produced growth factors for survival. In this experiment, the mechanisms of the regulation of proliferation and steroidogenesis by GDF-9 and Br-cAMP in human ovarian cells were investigated. **Methods**: SVOG cells were cultured and treated with GDF-9 and Br-cAMP. The expression of HOXA7 and cofactors were examined by Western blot analysis and/or RT-PCR. The cell proliferation was measured by thymidine incorporation assay. **Results**: Treatment with GDF-9 resulted in a significant increase of cell proliferation. Although the treatment with Br-cAMP increased the expression of StAR protein, this treatment resulted in a significant decrease of cell proliferation compared with the control and no change in HOXA7 or its cofactors. **Conclusion**: The present study demonstrated that GDF-9 had a stimulatory effect and cAMP had an inhibitory effect on the proliferation of SVOG cells. These results may reflect the characteristics of this human cell line as luteinized granulosa cell. Therefore, this culture system will be useful for studying the effects of local factors and gonadotropin on the regulation of human luteinized granulosa cells in the future. (Kitakanto Med J 2007；57：157～164)

Key Words：GDF-9, cAMP, SVOG cells and HOXA7

Introduction

In mammals, a single or small number of germ cell(s) will ovulate during an ovarian cycle, and follicles selected for further development are thought to receive precise signals from gonadotropins and locally produced growth factors for survival.[1] Throughout early antral follicular development, granulosa cells acquire functional characteristics, including the induction of steroidogenic enzymes and LH receptors.[2,3] It is well accepted that FSH controlls these changes via a cAMP pathway in the granulosa cells. However, granulosa cell proliferation and differentiation could also be influenced by oocyte factors, because granulosa cells in antral follicles display a distinct phenotype according to their distance from the oocytes.[4,5]

Growth differentiation factor-9 (GDF-9) is an oocyte-derived factor and a member of the TGF-superfamily, which includes TGF-β, activin, and bone morphogenetic proteins (BMPs).[6,7] GDF-9 is expressed in mammalian oocytes throughout follicular development.[8-11] Deletion of the *GDF-9* gene in mice blocked folliculogenesis at the primary stage, demonstrating the importance of this growth factor in early follicular development.[9] Subsequent studies have shown that GDF-9 stimulates granulosa cell proliferation,[12] preantral follicle growth,[10] and cumulus cell expansion,[13] whereas it suppresses FSH-induced cAMP production and steroidogenesis.[12]

The recent data suggest that HOXA7 protein expression is closely related to the proliferative activities of developing follicles, and that its expression is regulated, at least in part, by GDF-9.[14] The increase in HOXA7 protein in response to GDF-9 represents the first demonstration of a possible regulatory role of human oocytes in follicular HOX gene expression. On the other hand, FSH has been shown to stimulate cyclin D2 mRNA via a cAMP/PKA pathway in granulosa cells.[15,16] Therefore, in this experiment, I examined the effect of cAMP on cellular proliferation and the expression of HOXA7 and its cofactors in order to determine the relationship between local fac-

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tors and gonadotropin on cell proliferation and the activation of steroidogenesis.

Material and Methods

Materials

The antibody of FSHR was kindly provided by Dr. J.A. Dias (Wadsworth Center, David Axelrod Insititute for Public Health, Albany, NY) and the antibodies of StAR, PBX-2, cyclin D2 were purchased from Santa Cruz Biotechnology Ltd. (Santa Cruz, CA).

Human recombinant FSH was purchased from Dr. A.F. Parlow (National Hormone and Pituitary Program, CA). 8-Br-cyclic AMP (Br-cAMP) was obtained from Sigma-Aldrich (St. Louis, MO). HOXA7 antibody was kindly provided by Dr. Takayo Ota (University of British Colombia). GDF-9 was kindly provided by Dr. A.J.W. Hsueh (Stanford University).

Cell culture

Immortalized human granulosa cells (SVOG) were generated by transfection with the immortalizing simian virus 40 early genes into primary granulosa cells obtained from IVF department in Vancouver General Hospital.[17] Cells were cultured in M-199/MCDB105 (1 : 1)/10% fetal bovine serum (FBS). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were passaged when they reached about 90% confluence using a trypsin/EDTA solution (0.05% trypsin and 0.53mM EDTA).

RT-PCR

Total RNA was prepared by RNeasy kit (QIAGEN Inc., Mississauga, ON) following the manufacturers' instructions. Total RNA concentration and purity were determined using a spectrophotometer. Complementary DNA (cDNA) was synthesized from 1.5μg of total RNA using the First Strand cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ). The cDNA was used as a template for polymerase chain reaction (PCR). The following primers specific for HOXA7, MEIS1, PBX1 and PBX2 were used :

HOXA7
5'-CTTATACAATGTCAACAGCC-3' and
5'-TCCTTATGCTCTTTCCTTCCA-3'
MEIS1
5'- AAGGTGATGGCTTGGACAA and
5'-CGGAGAAGAATAGTGCAGCC
PBX1
5'-CCACGTGATGAATCTCCTGCGAGAG and
5'-TCAGCCAGACAGGAGGATACAGTGA
PBX2
5'-CTGGTTTGGCAACAAGAGGATTCGC and
5'-GGGAGTGTTCACTCTGATACCTCCA

The expected size of the PCR products using these sets of primers are 414 bp for HOXA7, 272 bp for MEIS1, 627 bp for PBX1 and 410 bp for PBX2. The PCR reaction mix (25μl) contained 1×PCR buffer, 0.2mM dNTP, 1mM $MgCl_2$, 50pmol primers, 1-1.5μl cDNA and 0.25 unit Taq polymerase. Each cycle consisted of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products were separated by gel electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

Proliferation assay

To see the effect of FSH, AMP and GDF-9 on immortalized granulosa cells, proliferation assay was performed using [^{3}H] thymidine incorporation assay. The cells were plated at 2.0×10^4 cells 24-well dish in 0.5ml medium supplemented with 10% FBS. After a preincubation of 48 h, the cells were starved in media containing 1 % FBS for 24 h. After 20 h incubation with FSH, AMP and GDF-9, the cells were incubated with medium containing 1μCi [^{3}H] thymidine (0.5 Ci/mmol ; Amersham Pharmacia Biotech Inc.) and collected after 4 h incubation. The cells were washed two times with PBS and precipitated with 0.5ml 10% trichloroacetic acid for at least 20 min at 4°C. The precipitate was washed in methanol twice and solubilized in 0.5ml of 0.1 N sodium hydroxide. The radioactivity was measured in the Tri-Carb Liquid Scintillation Analyzer (model 2100 TR, Packard Instrument Co., Meriden, CT).

Western blot

Immunoblot analysis was performed to investigate the expression of HOXA7, StAR and FSH receptor. The 4.0×10^4 cells/well were seeded into 6-well plates with M-199/MCDB105 (1 : 1)/1% FBS. After preincubation of 24 hours, the cells treated with each chemical were washed once with ice-cold PBS and lysed in 100μl of ice-cold RIPA buffer (150mM NaCl, 1 % Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50mM Tris (pH 7.5), 1mM phenylmethylsulfonyl fluoride, 10μg/ml leupeptin, and 100μg/ml aprotinin). The extracts were placed on ice for 10min, collected into a 1.5ml tube, and centrifuged for 15min at 14,000rpm. The supernatants were moved to new tubes, and the protein concentrations of supernatant were determined using Bradford assay (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Thirty micrograms of total protein were

mixed with 6x sample buffer (75mM Tries-HCl of pH 6.8, 15% SDS, 0.15% bromophenol blue, 15% glycerol, and 37.2% 2-mercaptoethanol) and boiled for 10 min. The sample mixtures were run on 10% SDS-PAGE gels (acrylamide : bisacrylamide. 29 : 1) in 1% gel running buffer (25mM Tris/250mM lysine, pH 8.3/ 0.1% SDS) at 100 V for 2.5 h and electrotransferred to a nitrocellulose membrane (Hybond C, Amersham Pharmacia Biotech Inc, Oakville, Ontario, Canada) at 100 v for 2 h. The membranes were immunoblotted using a rabbit polyclonal antibody for HOXA7, StAR, with protein molecular size markers (New England Biolabs, Inc., Ontario, Canada). After washing four times with TBS-T (0.1% Tween 20 in Tris-buffered saline) for 15 min, the signals were detected with a horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech Inc.) and visualized using the enhanced chemiluminescence system (Amersham Pharmacia Biotech Inc.). The intensity of the signals were quantitated by densitometry (BioDocAnalyze, Biometra, Germany).

Immunofluorescence

Cultured cells were grown on glass coverslips, fixed in freshly prepared 4% paraformaldehyde in PBS for 10 min at 4°C (HOXA7) or ice-cold methanol (PBX2), permeabilized by 0.1% Triton X-100 in PBS for 10 min at 25°C (HOXA7) or ice-cold methanol/ acetone (1 : 1) for 20min (PBX2). They were incubated with an anti-HOXA7 antibody (1 : 400) or an anti-PBX2 antibody at 1 : 50 dilution in Protein Block (DAKO, Mississauga, ON) for 1 h at 25°C, followed by a Texas Red-labeled goat anti-rabbit immunoglobulin antibody for 1 h at 25°C. Control experiments were done in the absence of primary antibodies or in the presence of pre-immune serum. For Hoechst staining, the cells were rinsed three times with PBS after serum removal and then incubated with 5μg/ml Hoechst 33342 in PBS for 15min, rinsed once with PBS, coverslipped, and then allowed to dry for several hours.

Statistical analysis

The data represents the mean$\pm$SE from three independent experiments. Comparisons between groups were performed by one-way ANOVA. The significance of the differences between the mean values of the control group and each treated group was determined by Duncan's multiple-comparison test.

Results

In order to characterize this cell culture system, the expression of FSH receptor and HOXA7 were examined by Western blot experiments. The FSH receptor was detected as an 80 kDa band in the Western blot using a specific receptor antibody, and HOXA7 was detected as a 27 kDa band (Fig. 1).

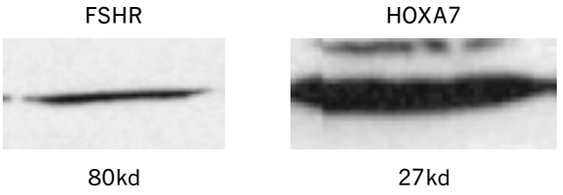

Fig. 1 Immunoblot analysis of SVOG cells extracts.
SVOG cells extracts were separated by SDS-PAGE, transferred to a PVDF-plus membrane, and incubated with the antibodies against FSH receptor and HOXA7.

Recent studies have identified a number of HOX genes in the oocytes, 18 including proteins that contain a short conserved amino acid motif required for interaction with members of the PBX protein family. Therefore, in this paper we examined the expression of HOX cofactor proteins in SVOG cells. The expected PCR products were detected, as shown in Fig. 2. The immunoflourescence data also showed that PBX-2 was present in the nucleus of SVOG cells (Fig. 3).

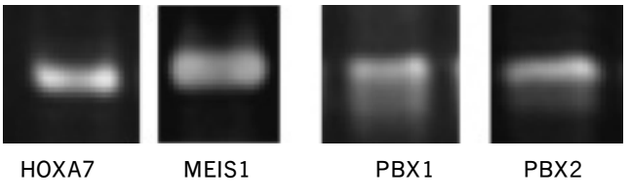

Fig. 2 RT-PCR analysis of the gene expression of HOXA7 cofactors in SVOG cells
The RT-PCR products were separated by gel electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

To examine the effect of GDF-9 on cell proliferation, the SVOG cells were treated with increasing doses of GDF-9 for 24 h. Treatment with GDF-9 resulted in a significant increase of cell proliferation compared with the control, except at the highest dose (400 ng/ml) (Fig. 4). In the ovaries, cyclin D2 expression is known to be localized to the glanulosa cells of the follicles, and its expression has been shown to be crucial for their proper growth. The effect of GDF-9 on the expression of cyclin D2 was investigated. Treatment with GDF-9 resulted in a dose-dependent increase of cyclin D2 (Fig. 5).

Since FSH has been shown to mediate its signal via a cAMP pathway in granulosa cell, I examined the effect of Br-cAMP on the proliferation of SVOG cells. The treatment with Br-cAMP resulted in a significant decrease of cell proliferation compared with the control (Fig. 6).

The cells were also treated with Br-cAMP in order to

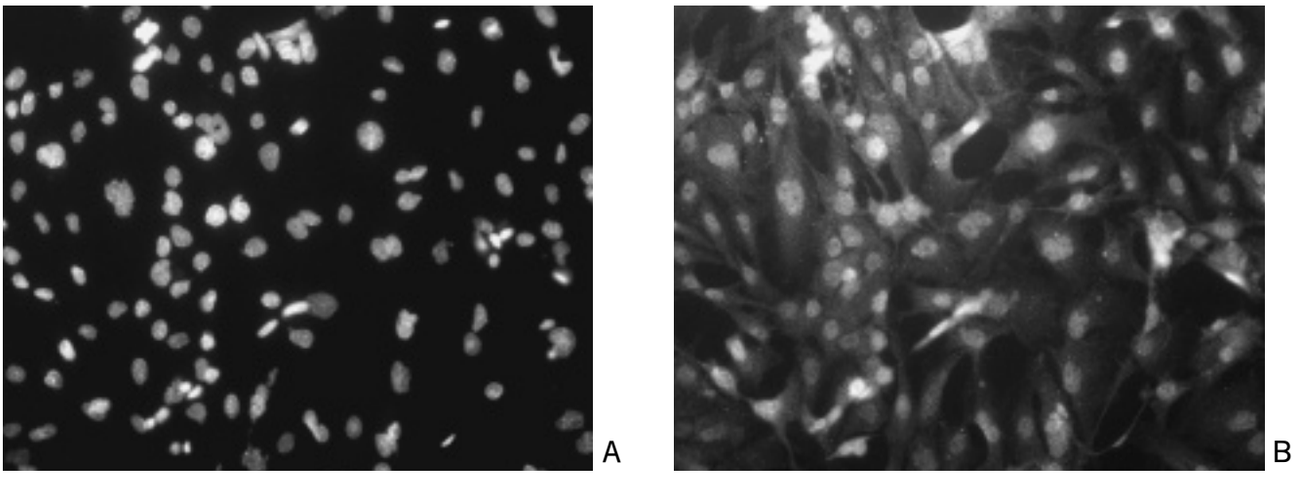

Fig. 3 PBX2 immunofluoresence in cultured SVOG cells.
SVOG cells were prepared from 60% confluent plates containing proliferating cells. They were incubated with an anti-PBX2 antibody at 1 : 50 dilution in Protein Block (DAKO, Mississauga, ON) for 1 h at 25°C, followed by a Texas Red-labeled goat anti-rabbit immunoglobulin antibody for 1 h at 25°C. For Hoechst staining (A), the cells were rinsed three times with PBS after serum removal and then incubated with 5μg/ml Hoechst 33342 in PBS for 15min, rinsed once with PBS, coverslipped, and then allowed to dry for several hours. (B) PBX-2 immonoreactivity appears in the nuclei of SVOG cells.

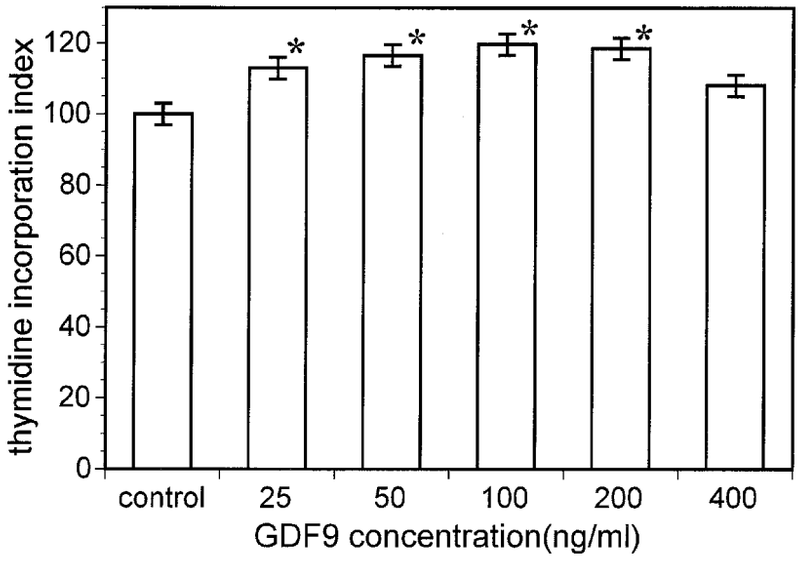

Fig. 4 Dose-dependent effect of GDF-9 on cell proliferation
After a preincubation of 48 h, the cells were starved in media containing 1% FBS for 24 h. After 20 h treatment with GDF-9, the cells were incubated with 1 μCi [^{3}H] thymidine for 4 h. Values are represented as mean $\pm$SE of three individual experiments. * indicates $p < 0.01$

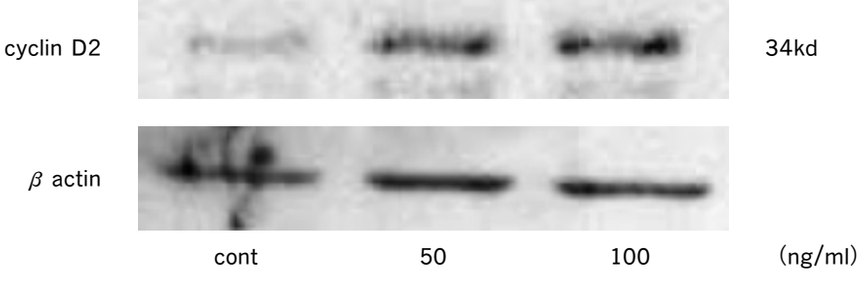

Fig. 5 Dose-dependent effect of GDF-9 on the cyclin D2 expression
SVOG cells were cultured for 24 h, and GDF-9 was added at the indicated concentrations for 24 h. The cyclin D2 protein levels were analyzed by immunoblot assay.

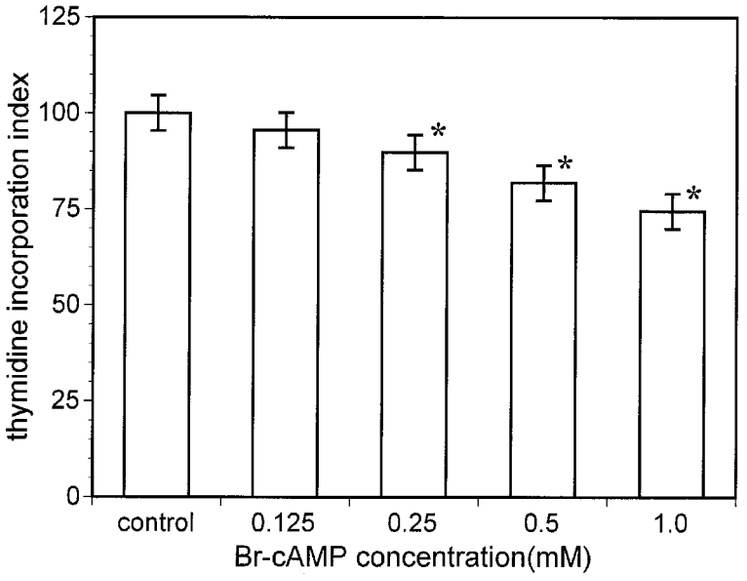

Fig. 6 The effect of Br-cAMP on the cell proliferation
After a preincubation of 48 h, the cells were starved in media containing 1% FBS for 24 h. After 20 h treatment with 1 mM of 8-Br-cAMP, the cells were incubated with 1 μCi [^{3}H] thymidine for 4 h. Values are represented as mean±SE of three individual experiments. * indicates $p<0.01$

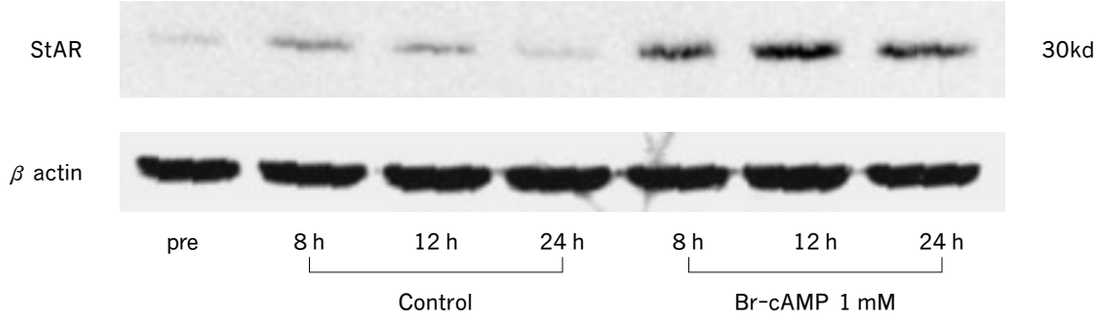

Fig. 7 The effect of Br-cAMP on the expression of StAR protein in SVOG cells
SVOG cells were cultured and treated with Br-cAMP at the indicated time course. The StAR protein levels were analyzed by immunoblot assay.

assess the effect of this factor on the expression of StAR in SVOG cells. Treatment with Br-cAMP (1 mM) induced a significant increase of StAR protein (Fig. 7). Treatment with 1 mM of Br-cAMP did not affect the expression of HOXA7 or its cofactors (data not shown).

Discussion

In this study, the effects of cAMP and GDF-9 on the function and proliferation of immortalized human granulosa cells (SVOG cells) were explored to further clarify the characteristic of this cell line. First, the stimulatory effects of GDF-9 on basal SVOG cell proliferation were observed. The results agreed with previous reports in which treatment with GDF-9 increased the numbers of rat granulosa cells as well as the cell proliferation measured by [^{3}H] thymidine incorporation.[12,19,20] In contrast, a recent report indicates that treatment with either 1 or 2 μg/ml recombinant ovine GDF-9 had no significant effect on [^{3}H] thymidine incorporation into ovine or bovine granulosa cells.[19]

Previous studies with rat granulosa cells have reported that 48-h[12,20] and 6-day[19] treatment with 30ng/ml GDF-9 inhibits progesterone production induced by FSH. McNatty *et al.*[21] recently reported that 6-day treatment with 2 μg/ml GDF-9 inhibited progesterone production by ovine and bovine granulosa cells cultured in the presence of FSH, IGF-I, and insulin. Yamamoto *et al.*[22] also observed that GDF-9 inhibits progesterone production induced by Br-cAMP in cultured human granulosa cells. In the present study, under basal conditions, GDF-9 had a weak inhibitory effect on progesterone production (data not shown). In previous studies with cultured mouse granulosa

cells, 50-100ng/ml GDF-9 treatment for 16-24h increased basal progesterone production but had no effect on FSH-induced progesterone production.[13,23] The discrepancies among these studies could be due to species differences and/or differences in culture conditions.

Although there is no direct evidence of GDF-9 activity in humans, GDF-9 has been shown to be absent in human primodial follicles and present in primary follicles after growth is initiated,[24] suggesting that GDF-9 may act as a mitogen during the initial, gonadotropin-independent period of follicular development. In addition, it has been reported that GDF-9 upregulated HOXA7 protein expression in SVOG cells.[14] Therefore, to investigate the effect of gonadotropin on the HOXA7 in this cell line, we examined the effect of cAMP on the expression of the HOXA7 protein in SVOG cells. In addition, because Hox proteins require a complex set of interactions with TALE proteins, I also studied the expression of HOX cofactor genes. The RT-PCR analysis revealed that HOX confactors, including PBX-2, were expressed in SVOG cells, and localized primarily in the nucleus (data not shown). This observation suggests that these cofactors are present in human glanulosa cells. However, there were no significant changes in the amount of HOXA7 protein following treatment with cAMP or FSH, and the expression of HOX cofactors did not change in the presence of GDF-9 or cAMP. Although no significant changes in HOXA7 or its cofactor proteins were detected in these experiments, further studies may be needed to investigate the transportation of these factors.

In the ovaries, cyclin D2 expression is localized to the granulosa cells of the follicles, and its expression has been shown to be crucial for their proper growth.[16] Female mice carrying a null mutation on the cyclin D2 gene are infertile because of impairment in granulosa cell proliferation in response to FSH, resulting in small follicles with trapped oocytes.[16] FSH has been shown to stimulate cyclin D2 mRNA via a cAMP/PKA pathway in granulosa cells.[15,16] However, a luteinizing dose of LH in hormonally primed hypophysectomized female rats results in a rapid decrease in cyclin D2 mRNA and protein levels.[15] It is unclear how cAMP mediates the actions of FSH and LH in producing their contrasting effects on granulosa cell growth and cyclin D2 expression.[25-29] One possibility might be related to the differential expression of the transcription factors that mediate the cAMP pathway. The CREM isoform, ICER, shares the DNA binding and dimerization domains with the other CREM isoforms but lacks the kinase and transactivation domains. Therefore, ICER functions as a dominant negative transcriptional repressor by binding as a homodimer or heterodimer with other CRE-binding family members.[30] This unique feature endows ICER with a key role in mediating the repression of cAMP-dependent transcription. In the ovaries of adult cycling rats, CREM mRNA levels of ICER isoforms have been shown to be selectively induced in the granulosa cells of preovulatory follicles in response to the ovulatory surge of LH.[31] Similarly, ICER expression was found to be induced in granulosa cells of eCG-primed immature rats injected with hCG, whereas eCG alone did not induce ICER expression.[31] This induction of ICER in response to LH/hCG has been proposed to mediate the suppression of FSH-inducible genes, such as inhibin α (Inha)[31] and cytochrome P450 (Cyp19a1).[32] Since the present experiment showed that cAMP reduced cell proliferation in SVOG cells, this established cell line may have developed not only the characteristics of granulosa cells but also the characteristics of luteinized granulosa cells. In addition, granulosa cells are controlled by FSH under physiological conditions, I confirmed the expression of FSH-R in granulosa cells. However, we could not detect any effect of FSH on the SVOG cells (data not shown). Furthermore, the SVOG cells used in this experiment were established from granulosa cells provided by patients who had undergone hCG treatment for ovulation induction. Previous studies have demonstrated that granulosa cell proliferation requires normal expression of the cell cycle regulator cyclin D2,[15,16] and that the expression of this regulator is induced by FSH in granulosa cells. We speculate that ICER contributes to the maintenance of the tight control of cyclin D2 after LH treatment, potentially inhibiting luteinized glanulosa cell growth.

Steroidogenic acute regulatory protein (StAR) plays a critical role in tropic hormone-stimulated steroid biosynthesis by facilitating the transfer of cholesterol across the mitochondrial membrane. Because FSH and LH are critically important regulators of estradiol synthesis in both granulosa and luteal cells, we evaluated the effect of cAMP on the StAR production. One mM of cAMP stimulated the production of StAR protein and had a negative effect on the proliferation of SVOG cells. These results might be related to the differential expression of the transcription factors that mediate the cAMP pathway in the SVOG cells, and may offer proof that the inhibitory effect induced by 1 mM Br-cAMP was not due to the toxic effect of Br-cAMP on the cells.

It has been well established that GDF-9 enhances follicle growth through the proliferation of granulosa cells ; however, the present data suggested that GDF-9 may also contribute to the proliferation of human

luteinized granulosa cells. Whereas the role of FSH and LH as primary triggers of folliculogenesis remains indisputable, the role played by local factors has become increasingly apparent over the past decade. Both granulosa and theca cells, and more surprisingly, the oocytes, produce a variety of peptides that act broadly to influence the action of gonadotropin either positively or negatively. In this respect, it is fascinating to note that a large number of follicular functions are controlled by a balance between activating and inhibiting factors. However, despite this improved understanding of human folliculogenesis and luteinization, many processes remain poorly understood.

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