ORIGINAL ARTICLE

Establishment of an immortalized porcine granulosa cell line (PGV) and the study on the potential mechanisms of PGV cell proliferation

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Abstract. In order to establish an immortalized granulosa cell line and to investigate the potential mechanisms of immortalized cell proliferation, simian virus (SV) 40 was used to infect porcine granulosa cells from small follicles (1–2 mm in diameter), and one colony was selected after four weeks of culture. The colony was digested with trypsin and the cells were cultured for more than 300 days (named PGV). The SV40 large T antigen gene and its products were confirmed in immortalized cells by Southern blotting and immunohistochemistry. Progesterone production was not detected in the conditioned culture media with follicle-stimulating hormone (FSH) and forskolin, possibly due to the lack of P450scc gene transcription as examined by Northern blotting. PGV cells responded significantly to the stimulation of sera (fetal bovine and horse sera) and protein kinase C (PKC) stimulators (PMA and OAG), while PKC inhibitors (staurosporine and calphostin C) blocked both sera and PKC stimulation. Phospholipase C (PLC) and phosphatidic acid phosphatase (PAP) inhibitors (U73122 and propranolol) significantly reduced PGV cell proliferation, while PMA restored PLC and PAP inhibition. These data suggest that diacylglycerol (DAG) is produced in PGV cells by PLD as well as by PLC, and that DAG then activates PKC stimulating the PGV cell cycle through yet unknown mechanisms. Thus, an immortalized granulosa cell line is very useful to study granulosa cells *in vitro*, as the cells are homogeneous and are a functionally defined population. (Keio J Med 54 (1): 29–38, March 2005)

Key words: granulosa, immortalization, PKC, PLC, PLD

Introduction

During ovarian follicle maturation from a primordial follicle to a preovulatory follicle, various events occur both morphologically and functionally inside the follicle. Among these events, the increase in the number of the granulosa cells plays a central role in follicle development.[1] If a developing human follicle is selected to ovulate it may grow 50-fold, increasing from 0.4 mm to 20 mm in diameter; the growth of the Graffian follicle is accompanied by granulosa cell proliferation and follicular fluid accumulation in the antrum. It is also well known that gonadotropins, especially follicle-stimulating hormone (FSH), govern follicle growth *in vivo*. Although studies on steroidogenesis in the follicles *in vivo* or in cultured granulosa cells *in vitro* have been performed extensively to date, the exact mechanisms of granulosa cell proliferation are still poorly understood.

Since granulosa cells luteinize spontaneously *in vitro* and do not undergo more than one cell doubling in culture,[2] it is difficult to study the mechanisms of granulosa cell proliferation using cell cultures. Moreover, in order to study granulosa cells *in vitro*, ovaries must be collected from animals whenever a study is set up. In addition, granulosa cell populations obtained from ovaries are heterogeneous and less than 50% of the cells are viable. Thus, it is difficult to obtain pure, functionally-defined populations. Hence, it is advantageous to develop a homogeneous population of granulosa cells.

In this study an immortalized porcine granulosa cell line (named PGV) was established with simian virus (SV) 40 infection, and was used to investigate the potential mechanisms underlying the regulation of gran-

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ulosa cell proliferation. PGV cell numbers increased with sera and also with protein kinase C (PKC) stimulators, but decreased with PKC inhibitors. Neither PKA stimulators nor growth factors increased PGV cell numbers. Both phospholipase C (PLC) and phosphatidic acid phosphatase (PAP) inhibitors reduced PGV cell proliferation, while PKC stimulator restored PLC and PAP inhibition. Based upon these data, it may be concluded that the PKC pathway is stimulated by diacylglycerol (DAG) generated through the PLD as well as PLC pathways and that the PKC pathway may play an important role in PGV cell proliferation as well as in proliferation of primary granulosa cells.

Materials and Methods

Porcine granulosa cell cultures

Porcine ovaries were collected at a local slaughterhouse in saline with penicillin (50 μg/ml) and streptomycin (50 μg/ml, Gibco, USA). Porcine granulosa cells were aspirated from small follicles (1–2 mm in diameter) with a 23-gauge needle and syringe, centrifuged three times, and then re-suspended with TCM199 containing 10% fetal bovine serum (FBS, Gibco, USA), penicillin (50 μg/ml), streptomycin (50 μg/ml), fungizone (0.25 μg/ml, Gibco) and gentamycin (50 μg/ml, Essex Japan, Japan) according to the methods by Channing *et al.*[3,4] Aliquots of cells (5×10^5 viable cells/well) were plated to 35 mm dishes and incubated at 5% CO_2 in air.

Infection of SV40 and establishment of a cell line

After two days of initial culture, SV40 was used to infect the cultured porcine granulosa cells at 10^4 plaque forming units (pfu)/ml, 10^5 pfu/ml and 10^6 pfu/ml; two dishes of mock infection were used as controls. Two hours following infection, cells were vigorously washed several times with fresh medium; thereafter, media were changed every two days. Four weeks after infection one colony was selected in a dish with 10^6 pfu/ml of SV40. The colony was digested with trypsin and the cells were transferred to another dish. When cells reached sub-confluence, cells were treated with trypsin and cell numbers were counted with a hemocytometer. Population doublings (PDS) were calculated at every cell passage. The cell line was named PGV (porcine granulosa cell-virus transformed).

Confirmation of the existence of SV40 large T antigen in PGV cells

The SV40 genome encoding the large T antigen was confirmed by Southern blotting. To create a probe, a plasmid containing the SV40 whole genome was digested with restriction enzymes (Stu I and BamH I) and the fragment (2657 bp) corresponding to large T antigen was purified and radiolabeled with ^{32}P. Total genomic DNA was extracted from PGV cells and digested with HindIII. Southern blotting was carried out with the radiolabeled probe after agarose-gel electrophoresis.

To confirm the existence of large T antigen products immunohistochemically, PGV cells were grown on cover glasses for two days, fixed with ethanol and stained with large T antigen-specific primary antibody (Wako, Japan) followed by FITC-labeled secondary antibody against mouse IgG (Wako, Japan).

Measurement of progesterone concentrations in conditioned culture media and cyclic adenosine 3′,5′-monophosphate (cAMP) in PGV cells and primary granulosa cells

PGV cells (1×10^6 cells/well) and primary granulosa cells (1×10^6 viable cells/well) were inoculated to 35 mm dishes and incubated for two days with 100 ng/ml FSH (AFP2844B, provided by National Hormone and Pituitary Program, NIH, USA) and forskolin (0.5 mM) with IBMX (3-isobutyl-1-methylxanthine, 10^{-5} M). Progesterone concentrations in conditioned media and cAMP in the cells were determined by their specific radio-immunoassays (RIA) (DPC Japan, Japan, and Amersham, UK, respectively). The inter-assay and intra-assay coefficients of variations in progesterone and cAMP were 8.5% and 6.2%, and 8.7% and 6.8%, respectively. All chemicals used in this study were purchased from Sigma, USA except where otherwise noted.

Determination of the P450scc mRNA by Northern blot analysis

In order to investigate the expression of the P450scc (cytochrome P450 side-chain cleavage enzyme) gene in PGV cells, Northern blotting was carried out using bovine P450scc cDNA (provided by Prof. Yoshiaki Fujii, Tohoku University) after extraction of total RNA from PGV cells and agarose-gel electrophoresis using a standard protocol.

Reversibility of steroidogenic activity in PGV cells with retinoic acid

As PGV cells possess no steroidogenic activity, PGV cells were cultured with retinoic acid (10^{-13} M$\sim 10^{-5}$ M) for up to 2 weeks and then challenged with FSH (30 ng/ml), luteinizing hormone (LH) (AFP4345B, provided by NIH, 10 ng/ml) and forskolin (0.5 mM) (+IBMX,

10^{-5} M). Progesterone concentrations in conditioned media were measured by RIA after 2 days of culture.

PGV cell proliferation investigations

In order to investigate the effects of intracellular signal transduction mechanisms of PGV cells on cell proliferation, various concentrations of sera (FBS and horse serum), gonadotropins (FSH and LH), growth factors (insulin-like growth factor (IGF)-I and -II (provided by Fujisawa Pharmaceutical Co., Japan), insulin and epidermal growth factor (EGF), tyrosine kinase inhibitor (genistein), PKA stimulators (forskolin, 8-bromo-cAMP, and dibutyryl cAMP), PKC stimulators (phorbol-12-myristate-13-acetate (PMA) and 1-oleoyl-2-acetyl-glycerol (OAG), PKC inhibitors (staurosporine and calphostin C), calcium ionophore (A23187), calmodulin antagonists (W5 and W7), PLC inhibitor (U73122), and PAP inhibitor (propranolol) were added to PGV cells (1×10^5 cells/well) in 24 multi-well dishes in replicate conditions. After three days of culture, except where otherwise stated, cell numbers were counted with hemocytometer using 0.066% trypan blue. As maximum doses of respective vehicles in the cultures did not influence PGV cell numbers, only medium containing 10% horse serum was used as a control.

Each experiment was performed at least three times and representative data were shown. Results are expressed as the mean $\pm$ standard error of mean (SE).

Statistical analysis

One-way analysis of variance (ANOVA) followed by Duncan's new multiple range tests was used to test for statistical significance ($p < 0.05$) between treatments.[5] Different superscripts in both table and figures denote the significant difference ($p < 0.05$) between the treatments.

Results

Establishment of an immortalized cell line (PGV)

Porcine granulosa cells from small follicles were infected with SV40. After four weeks of culture one colony was selected in a dish with 10^6 pfu/well of SV40. The colony was digested with trypsin, and maintained in culture. When cell numbers and PDS were calculated at each passage up to 300 days, cell numbers increased about sixteen times during one week. PGV cells exhibited an elongated and fibroblastic shape with relatively large nuclei as shown in Fig. 1.

Primary porcine granulosa cells and PGV cells were maintained in culture; although the primary cells were still viable on the 27th day of culture, many vacuoles appeared within the cells by the 43rd day, and almost all primary cells were not viable by the 45th day (data not shown) in contrast to the fact that all PGV cells were viable even on the 300th day.

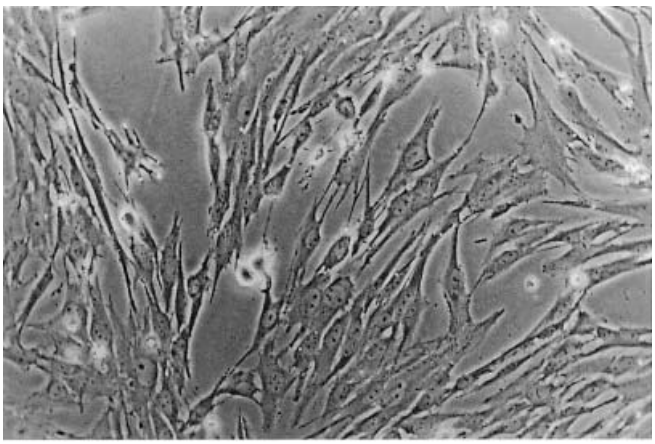

Fig. 1 Morphology of immortalized porcine granulosa cells (PGV) infected with SV40. Cells were elongated with fibroblast-like cell contours with relatively large nuclei.

Confirmation of large T antigen in PGV cells

Southern blotting was performed to confirm the presence of SV40 large T antigen DNA in PGV cells. The hybridized DNA fragments (1768 bp, 1169 bp, and 526 bp) with HindIII digestion were compatible in length to those predicted with HindIII digestion of SV40.

SV40 large T antigen products could also be visualized immunohistochemically after treatment with anti-large T antigen antibody, followed by FITC-labeled second antibody (data not shown).

Measurement of progesterone concentrations in conditioned culture media and cAMP in cells

Progesterone concentrations in conditioned culture media and cAMP contents in cells were determined by their specific RIAs. Although progesterone concentrations when challenged with FSH and forskolin were significantly higher than control levels in primary granulosa cell cultures, progesterone concentrations in PGV cell cultures were not detectable even by addition of FSH and forskolin as shown in Table 1. cAMP contents in both primary cells and PGV cells were slightly higher when treated with 100 ng/ml FSH than controls, but the difference was not significant. However, cAMP contents

when cultured with forskolin (+IBMX) were significantly higher than controls in both cell types.

Determination of P450scc mRNA in PGV cells

To confirm the expression of the P450scc (rate-limiting enzyme of steroidogenesis) gene in PGV cells, Northern blotting was carried out utilizing P450scc cDNA. However, P450scc mRNA could not be detected in PGV cells (data not shown).

Table 1 Effect of FSH and forskolin on progesterone and cyclic AMP synthesis by primary granulosa cells and PGV cells. After 2 days of culture with FSH (100 ng/ml) and forskolin (0.5 mM) (+IBMX, 10^{-5} M), progesterone concentrations in conditioned culture media and cAMP contents in cells were determined by their specific RIAs. *Different superscripts* show significant differences ($p < 0.05$ by ANOVA and Duncan's new multiple range test).

Addition	Cell type	
	Primary	PGV
	⟨Progesterone synthesis⟩	n mol/ml · 10^6 cells
None	212 ± 17[a]	ND
FSH (100 ng/ml)	2073 ± 166[b]	ND
Forskolin (0.5 mM)	4654 ± 372[c]	ND
	⟨cAMP formation⟩	p mol/ml · 10^6 cells
None	1.2 ± 0.2[a]	1.4 ± 0.3[a]
FSH (100 ng/ml)	1.7 ± 0.3[a]	1.7 ± 0.3[a]
Forskolin (0.5 mM)	7.2 ± 0.7[b]	11.8 ± 0.8[b]

mean ± SE, ND: Not Detectable.

Reversibility of steroidogenic activity with retinoic acid

To investigate whether PGV cells could restore steroidogenic activity, retinoic acid (10^{-13} M∼10^{-5} M) was added to PGV cells for up to 2 weeks. The cells were then incubated with FSH (30 ng/ml), LH (10 ng/ml) and forskolin (0.5 mM) (+IBMX, 10^{-5} M), and progesterone concentrations were measured in conditioned culture media by RIA. No progesterone could be detected in all culture dishes (data not shown).

PGV cell proliferation investigations

Serum: In order to see whether FBS and horse serum (HS) stimulated PGV cell numbers, FBS (1%∼20%) and HS (10%) were added to PGV cell cultures. Cell numbers were counted with hemocytometer every two days up until six days.

Although PGV cell numbers in serum-free medium (control) increased on day 4, they decreased on day 6 as shown in Fig. 2. FBS significantly stimulated PGV cell numbers in a dose- and time-dependent manner. Cell numbers in 1% FBS were not significantly different

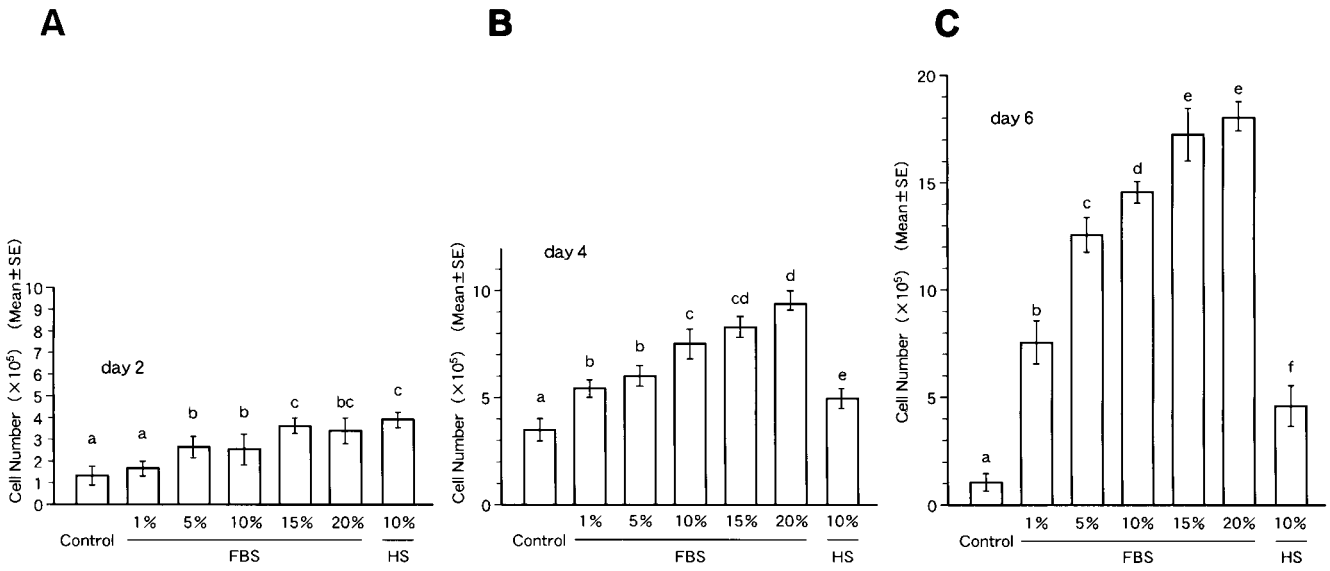

Fig. 2 Effects of increasing concentrations of fetal bovine serum (FBS) and horse serum (HS) on PGV cell number (/well). PGV cells (1×10^5 cells/well) were cultured in 24 multi-well dishes with FBS (1%∼20%) and HS (10%) for 2 to 6 days, and cell numbers were counted with hemocytometer using 0.066% trypan blue as described in *Materials and Methods*. Each *column* and *bar* represent the mean ± SE, and *different superscripts* (a∼f) show significant differences between groups ($p < 0.05$ by ANOVA and Duncan's new multiple range test). *Panel A*, on day 2 PGV cell numbers with greater than 5% FBS were significantly higher than controls (serum-free media). Cell numbers with 10% HS were equal to those with 15% and 20% FBS. *Panel B*, on day 4 PGV cell numbers with 1% FBS were significantly higher than controls. Cell numbers with all concentrations of FBS were higher on day 4 than on day 2. Cell numbers with 10% HS were significantly less than those at 1% FBS. *Panel C*, on day 6 FBS further stimulated PGV cell proliferation. There was no significant difference in cell numbers between 15% and 20% FBS even on day 6. Control cell numbers were less than those on day 4.

from controls on day two (Fig. 2-A), while they were significantly higher on days 4 and 6 (Fig. 2-B and 2-C). There was no significant difference in cell numbers between 15 and 20% FBS on each day. PGV cell numbers in 10% HS were nearly equal to those with both 15 and 20% FBS on day 2 (Fig. 2-A), while on days 4 and 6 they were significantly less than those with 1% FBS (Fig. 2-B and 2-C).

Gonadotropins: FSH (30 ng/ml) and LH (10 ng/ml) were added to PGV cell cultures and cell numbers were counted with hemocytometer after 3 days. There was no significant difference between controls and treated cells (data not shown).

Growth factors and tyrosine kinase inhibitor: EGF (0, 1, 10, 100 ng/ml), IGF-I (0, 1, 10, 100 ng/ml) and -II (0, 1, 10, 100 ng/ml), insulin (0, 0.5, 1, 2, 5, 10 ng/ml), and the tyrosine kinase inhibitor genistein (10^{-10}, 10^{-8}, 10^{-6}, 10^{-5} M) were added to PGV cells for three days.

PGV cell numbers were not different at all concentrations of EGF, IGF-I and -II or insulin from controls (data not shown). Similarly, genistein had no effect on cell numbers (data not shown).

PKA stimulators: PKA stimulators, forskolin (10^{-8} M ~10^{-4} M) (+IBMX, 10^{-5} M), 8-bromo-cAMP (10^{-8} M ~10^{-4} M) and dibutyryl cAMP (10^{-8} M~10^{-4} M) were added to PGV cells and cell numbers were counted after 3 days with hemocytometer.

All PKA stimulators had no effects on PGV cell numbers (data not shown).

PKC stimulators and inhibitors: PKC stimulators, PMA (10^{-12} M~10^{-5} M) and OAG (10^{-8} M~10^{-4} M), were added to PGV cells and cell numbers were counted after 3 days. As shown in Fig. 3, both significantly stimulated PGV cell proliferation at concentrations of greater than 10^{-6} M PMA (Fig. 3-A) and at greater than 10^{-5} M OAG (Fig. 3-B).

PKC inhibitors, staurosporine (10^{-12} M~10^{-8} M) and calphostin C (10^{-12} M~10^{-8} M) containing 10% HS, were added to PGV cells. As shown in Fig. 4-A, cell numbers with greater than 10^{-10} M staurosporine were significantly less than controls (10% HS only). Cell numbers with greater than 10^{-10} M calphostin C were also significantly less than controls (data not shown). PMA (10^{-6} M) and staurosporine (10^{-12} M~ 10^{-8} M) were simultaneously added to PGV cells. As shown in Fig. 4-B, 10^{-6} M PMA significantly stimulated cell numbers compared with controls (10% HS alone), while staurosporine significantly inhibited cell numbers in a dose-dependent manner, with a significant difference at greater than 10^{-10} M staurosporine. Cell numbers with 10^{-9} M and 10^{-8} M staurosporine were equal to controls. PGV cells were not viable at 10^{-7} M staurosporine.

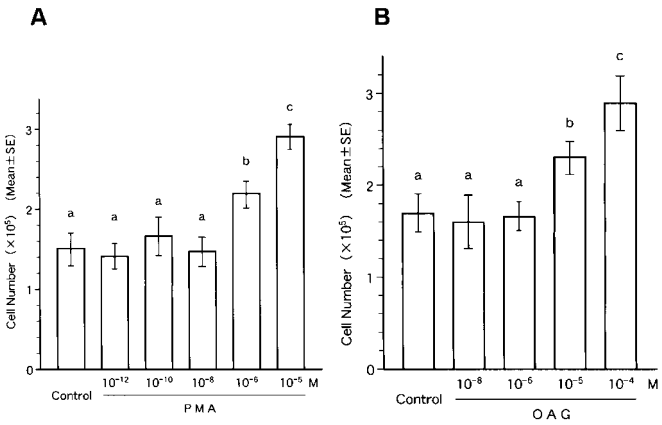

Fig. 3 Effects of PKC stimulators (PMA; 10^{-12} M~10^{-5} M, and OAG; 10^{-8} M~10^{-4} M) on PGV cell number. PGV cells were cultured for 3 days, and cell numbers were counted with hemocytometer. a, b and c denote significant difference between groups ($p < 0.05$). *Panel A*, cell numbers with 10^{-6} M and 10^{-5} M PMA were significantly higher than controls (10% HS alone). *Panel B*, cell numbers with 10^{-5} M and 10^{-4} M OAG were significantly higher than controls.

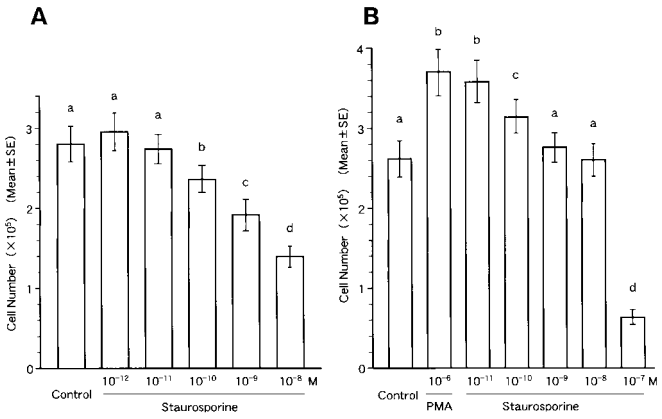

Fig. 4 Effects of PKC inhibitor staurosporine (10^{-12} M~10^{-8} M) on proliferation of PGV cells. a, b, c and d denote significant difference between groups ($p < 0.05$). *Panel A*, after 3 days of culture staurosporine significantly inhibited cell numbers in a dose-dependent manner, with significant difference with 10^{-10} M or greater compared to controls (10% HS only). *Panel B*, PGV cells were cultured for 3 days with PMA (10^{-6} M) alone or in combination with increasing concentrations of staurosporine (10^{-12} M~10^{-8} M). PMA (10^{-6} M) again significantly stimulated PGV cell numbers, while staurosporine dose-dependently inhibited PMA stimulation. PGV cells were not viable at 10^{-7} M staurosporine.

Calcium ionophore and calmodulin antagonists: Calcium ionophore A23187 (10^{-10} M~10^{-5} M) and calmodulin antagonists, W5 (10^{-8} M~10^{-5} M) and W7 (10^{-8} M~10^{-5} M), were added to PGV cells and cell numbers were counted after 3 days.

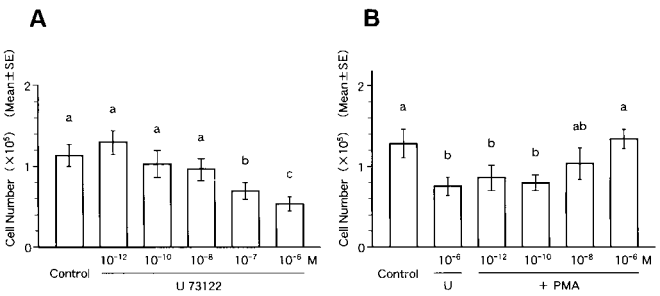

Fig. 5 Effects of PLC inhibitor U73122 (U; 10^{-12} M∼10^{-6} M) on PGV cell number. a, b and c denote significant difference between groups ($p < 0.05$). *Panel A*, after 3 days of culture PGV cell numbers with 10^{-7} M and 10^{-6} M U were significantly less than controls (10% HS alone). *Panel B*, PGV cells were grown for 3 days with U (10^{-6} M) alone or U (10^{-6} M) plus PMA (10^{-12} M∼10^{-6} M). U alone (10^{-6} M) again significantly inhibited PGV cell proliferation, while PMA (10^{-6} M) restored inhibition by U.

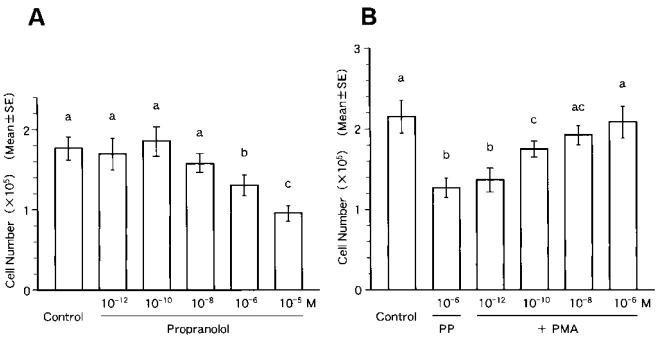

Fig. 6 Effects of phosphatidic acid phosphatase inhibitor propranolol (PP; 10^{-12} M∼10^{-5} M) on PGV cell number. a, b and c denote significant difference between groups ($p < 0.05$). *Panel A*, after 3 days of culture PGV cell numbers with 10^{-6} M and 10^{-5} M PP were significantly less than controls (10% HS alone). *Panel B*, PP alone (10^{-6} M) or PP (10^{-6} M) plus PMA (10^{-12} M∼10^{-6} M) were added to PGV cell cultures. Cell numbers with PP alone were similarly significantly less than controls, whereas those with 10^{-10} M PMA plus PP (10^{-6} M) were significantly higher than those with PP alone.

A23187, W5 and W7 had no effects on cell numbers (data not shown).

Phospholipase C inhibitor: PLC inhibitor U73122 (10^{-12} M∼10^{-6} M) was added to PGV cells and cell numbers were counted after 3 days. As shown in Fig. 5-A, PGV cell numbers significantly decreased with 10^{-7} M and 10^{-6} M U73122. When PMA (10^{-12} M∼10^{-6} M) was simultaneously added to PGV cells with 10^{-6} M U73122, cell numbers with U73122 alone were similarly significantly less than controls, while cell numbers with 10^{-8} M PMA were slightly higher than those with U73122 alone, but the difference was not significant (Fig. 5-B). Cell numbers with 10^{-6} M PMA were significantly higher than those with U73122 alone and compatible with controls (10% HS only).

Phosphatidic acid phosphatase inhibitor: Phosphatidic acid phosphatase (PAP) inhibitor propranolol (10^{-12} M∼10^{-5} M, PP) was added to PGV cells and cell numbers were counted after 3 days. PGV cell numbers with PP decreased in a dose-dependent manner, with a significant difference at 10^{-6} M and 10^{-5} M (Fig. 6-A).

When PMA (10^{-12} M∼10^{-6} M) was simultaneously added to cells with PP (10^{-6} M), cell numbers increased gradually according to the PMA doses shown in Fig. 6-B. PGV cell numbers with PP alone were similarly significantly less than controls (10% HS only), while cell numbers with 10^{-10} M PMA were significantly higher than those with PP alone, but still significantly lower than controls. PMA at 10^{-8} M or greater concentrations restored PGV cell proliferation that was blocked by PP.

Discussion

Our results indicate that an immortalized porcine granulosa cell line (PGV) has been successfully established using SV40 large T antigen. Although PGV cells were able to produce cAMP with response to forskolin, PGV cells had no steroidogenic activity (Table 1) possibly due to the lack of transcription of P450scc mRNA. However, this cell line significantly responded to stimulation by sera (Fig. 2) and also by PKC (protein kinase C) stimulators (Fig. 3) in terms of cell proliferation. This prompted us to investigate signaling pathways in PGV cells in terms of cell proliferation, with emphasis on the diacylglycerol (DAG)-producing pathways.

Granulosa cell surface-specific antibodies were used to immunologically stain PGV cells. PGV cells did not stain with OG-1 antibody[6] that reacts specifically to human granulosa cells, whereas PGV cells stained with POG-1 antibody[7] that reacts with porcine granulosa cells, theca interna cells and some stromal cells (unpublished observation, 1995). Since greater than 99.9% of aspirated cells from porcine ovarian follicles in culture stained with oil red O (unpublished observation, 1985), they are steroid-producing cells (possibly granulosa cells). These data may strongly suggest that porcine granulosa cells are the origin of PGV cells.

Neither PKA stimulators nor growth factors, including insulin and tyrosine kinase inhibitor, influenced PGV cell numbers. However, PKC stimulators significantly increased PGV cell numbers (Fig. 3), while PKC inhibitors significantly reduced PGV cell proliferation that were stimulated by both sera and PKC stimulators (Figs. 4-A and 4-B, respectively). Inhibitor of

PLC (phospholipase C), that catalyzes PIP_2 (phosphatidylinositol 4, 5 bisphosphate) to DAG and IP_3 (inositol 1, 4, 5-triphosphate), significantly reduced PGV cell proliferation (Fig. 5-A). Moreover, inhibitor of phosphatidic acid phosphatase (PAP), that converts phosphatidic acid (PA) to DAG, also significantly reduced PGV cell numbers (Fig. 6-A). PMA restored PGV cell proliferation that was reduced by both PLC and PAP inhibitors (Figs. 5-B and 6-B, respectively).

These data strongly suggest that both DAG and PKC play an important role in PGV cell proliferation and that DAG may be generated by PLC and also by PAP in PGV cells. It is generally known that there are two peaks of DAG production in many cells following agonist stimulation, the first peak being transient and the later sustained.[8–10] The first peak of DAG production is generated by PLC from PIP_2, and the second is produced by PAP from PA. Moreover, the later sustained DAG peak in cells activates PKC.[11] PA is known to be converted from phosphatidylcholine (PC) by PLD.[8] Thus, our data also suggest that both PLC and PLD pathways may work in PGV cells to produce two peaks of DAG from PIP_2 and PC, respectively. Therefore, it may be concluded that DAG, generated first by PLC and later by PLD, stimulates PKC in PGV cells similarly as in many other cells. The sustained PKC activity may then stimulate PGV cell proliferation through yet unknown mechanisms. Although PLA_2 may be involved in DAG and arachidonic acid generation in PGV cells, this has not been confirmed from our preliminary data using the PLA_2 inhibitor 4-bromophenacyl bromide (unpublished observation, 1998).

Further studies are necessary to clarity whether or not FSH actually activates Gα, PLC, PLD, and then PKC in PGV cells including mRNA expressions. DAG production must also be determined temporally in PGV cells. Since FSH did not stimulate PGV cell proliferation in this study, it is inpossible to determine the time course of DAG, PLC, PLD or PKC activity in PGV cells after addition of FSH. This activity must be determined using other FSH-sensitive immortalized granulosa cells or primary granulosa cells following FSH stimulation. In addition, the signaling pathways from PKC to cyclins/cyclin-dependent kinases (cdk) must also be clarified.

When FSH binds to its receptors, adenylate cyclase is activated and generates cAMP from ATP (adenosine triphosphate). The PKA pathway(s) does not regulate cell proliferation nor steroidogenic activity in the PGV cells, while PKA systems do regulate production of steroids (progesterone and estrogen) in the primary granulosa cells, as already shown by many investigators including us.[4] Although DAG concentrations have not yet been reported in the granulosa cells, DAG may be generated in granulosa cells, as IP_3 was detected after LH stimulation.[12,13] Using tissue cultures Peluso *et al.*[14] showed stimulation of granulosa cell proliferation by addition of PMA to rat granulosa cells, but this stimulation was inhibited by 8-Br-cAMP. On the other hand, cAMP promoted the proliferation of chicken granulosa cells in culture.[15] Sicinski *et al.* reported that FSH stimulated granulosa cell numbers through a cAMP-dependent pathway using granulosa cells from cyclin D2-deficient mice.[16] However, other cyclin Ds (D1 and D3) were shown to be regulated by different signaling pathways. Thus, there are still controversy concerning whether the PKA or PKC pathways regulate cell proliferation of primary granulosa cells. However, there may be cross-talk between the A-kinase and C-kinase pathways.

IP_3, the other component catalyzed from PIP_2, releases calcium ion from the endoplasmic reticulum. Although it has been reported that FSH evokes an increase in intracellular free calcium ion concentrations in single swine granulosa cells,[17] calcium ion may not play an important role in terms of PGV cell proliferation as PGV cell numbers were influenced neither by calcium ionophore nor by calmodulin antagonists in this study.

FBS stimulated PGV cell proliferation, whereas neither EGF nor IGF stimulated PGV cell proliferation. As FBS contains many unknown factors, other growth factors or cytokines in FBS that have not yet tested in this study may stimulate PGV cell proliferation. EGF has been shown to modulate FSH-induced DNA synthesis in cultured granulosa cells.[18] IGF-I is also known to stimulate proliferation of granulosa cells.[19] Zhou *et al.* showed using IGF-I knockout mice that FSH does not affect IGF-I expression in granulosa cells, but that IGF-I augments granulosa cell FSH receptor expression.[20] As PGV cell numbers were not influenced by EGF, IGF and genistein (tyrosine kinase inhibitor), growth factor-tyrosine kinase-mitogen-activated protein kinase (MAPK) pathway(s) may not function in the regulation of PGV cells. Our preliminary data suggest that MAPK activities were detected 5 to 10 minutes after FSH stimulation in cultured primary granulosa cells (unpublished observation, 1999). MAPK activity in granulosa cells after FSH stimulation was also reported by Das *et al.*[21] and Babu *et al.*[22] Das *et al.* showed that cAMP-directed pathways by which FSH initiated granulosa cell differentiation included activation of MAPK, while Babu *et al.* showed that the cAMP independent growth promoting effects of FSH were activated by calcium ion and MAPK-dependent pathways. FSH receptor may have intrinsic growth factor-like activity.[23] Recently, phosphatidylinositol 3 (PI3) kinase-PKB pathway has been shown to be present in granulosa cells[24] and to be an essential component of FSH-mediated granulosa cell differentiation.[25] Our preliminary data demonstrate that the PI3

kinase inhibitor wortmannin had no effect on PGV cell numbers (unpublished observation, 1999). Thus among the pathways tested in this study, with the exception of PKC, other signaling pathways may not regulate cell proliferation in PGV cells.

The Jun family (c-Jun, Jun-B, and Jun-D), a basic region leucine zipper family of transcription factors, forms homodimers or heterodimers among Jun family members and Fos family members (c-Fos, Fos-B, Fra-1, and Fra-2). The dimer binds the consensus DNA sequence (TGAC/GTCA) sites called AP-1 (activation proten-1) found in a variety of promoters.[26,27] The PMA-responsive element, namely TRE (TPA-responsive element), is recognized by AP-1.[28] AP-1 has been shown to be involved in granulosa cell proliferation in response to tumor necrosis factor alpha.[29] Serum response element (SRE) is also present in granulosa cells[30] and SRE is involved in the expression of c-fos by serum[31] and is involved in the response to PKC in many cells.[32] Moreover, PKC is shown to directly phosphorylate and activate Raf-1, part of the MAPK kinase kinase (MAPKKK).[33] Thus, there is cross-talk between known major signaling pathways and possibly between other yet unknown signaling pathways leading from FSH stimulation to granulosa cell proliferation.

In PGV cells, progesterone was not detected even though it was stimulated by FSH or forskolin, whereas primary granulosa cells responded to FSH and forskolin in terms of progesterone production. One reason may be the absence of P450scc mRNA (a rate-limiting enzyme of steroidogenesis) in PGV cells as evidenced by Northern blotting. Retinoic acid was tested to determine whether it would restore steroidogenic activity, but it failed to stimulate progesterone production in PGV cells, although retinoic acid is able to block or reverse the carcinogenic process in various malignancies including hematologic malignancies. It has also been reported that in primary granulosa cells retinoic acid enhances the ability of FSH to induce LH-receptors and to stimulate the formation of cAMP and progesterone.[34] Further studies are necessary to investigate why the P450scc mRNA is not expressed in PGV cells, and also whether PGV cells could restore steroidogenic activity by means of transfection of P450scc gene to PGV cells. Although PGV cells could not produce steroids, some cell lines including spontaneously established granulosa cell lines[35,36] can secrete progesterone and estrogen. These cells may be useful to investigate the mechanisms of steroidogenesis in granulosa cells.

Immortalized granulosa cell lines were initially established by Amsterdam *et al.*[37] followed by several researchers. To immortalize granulosa cells, SV40 large T antigen alone,[37,38] in combination with Ha-RAS oncogene,[37] or in triple transfection with Ha-ras oncogene and FSH receptor expression plasmid,[39] or Kirsten-ras oncogene[40] have previously been used to transfect rat, porcine or human granulosa cells. Immortalized granulosa cells have also been obtained from transgenic mouse ovaries.[41] However, all these papers focused on the mechanisms of steroidogenesis in immortalized cells.

The intracellular signal transduction pathways in immortalized granulosa cells regulating cell proliferation may differ from those of primary granulosa cells inside follicles. However, it is not possible to replicate *in vitro* the exact conditions of granulosa cells in follicles, as the granulosa cells luteinize spontaneously in culture and as cultured granulosa cells lose the capacity to produce estrogens and to proliferate. Furthermore, the steroidogenic and proliferative heterogeneity in granulosa cells renders it difficult to obtain pure, functionally defined populations. Hence, cultured primary granulosa cells are not appropriate to study the FSH signaling pathways in granulosa cells inside the follicle, although luteinized granulosa cells are often used to characterize granulosa cells *in vitro*. Therefore, it is advantageous to develop a homogeneous population of granulosa cells. Thus, our immortalized granulosa cell line (PGV) can serve as a useful model to study the potential mechanisms of granulosa cell proliferation.

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