Characterization of a Stable Steroidogenic Caprine Luteal Cell Line Transformed by a Temperature-Sensitive Simian Virus 40

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Abstract

A caprine luteal cell line (tsCLC-D) that synthesizes progesterone (P_4) was established following by transformation with a temperature-sensitive A209 (tsA209) mutant of simian virus 40 (SV40). The transformed cells have temperature-sensitive for morphology, cell propagation and progesterone steroidogenesis. At the permissive temperature of 34°C, these cells were spindle-shaped and grew with a similar rapidity as tumor cells. However, at the nonpermissive temperature of 40°C, the cells have exhibited a round shape and ceased to proliferate because the gene for maintenance of transformation was not expressed. The tsCLC-D cell line responds to 8-Br-cyclic AMP, 22-hydroxycholesterol and pregnenolone treatment with an increase in progesterone biosynthesis. This cell line still express StAR protein, 3β-HSD and P450scc enzyme of three kinds of steroidogenic protein and enzymes, this characteristic is similar to normal luteal cell. However, the addition of any doses oLH did not increase progesterone secretion. We speculate that tsCLC-D might lose the responsiveness to gonadotropins during the immortalization process, while retaining steroidogenic enzyme activity and progesterone production. To our knowledge, this is the first report of a stable cell line derived from corpus luteum of ruminant. The tsCLC-D retains steroidogenic capacity, which will make this cell line useful for the studies of regulation of steroidogenesis.

Key Words: caprine, luteal cells, cell line, steroidogenesis, simian virus

Introduction

The corpus luteum plays a critical role in the maintenance of pregnancy in mammals. During the estrous cycle, the cells of preovulatory follicles undergo rapid morphological and functional changes to become luteinized. Although primary luteal cell culture is a useful tool for the study of molecular mechanisms of lutenization and luteolysis *in vitro*, their isolation is time-consuming, and there is considerable heterogeneity between preparations. In addition, primary cells can be subcultured only for a finite number of times because of their limited life span *in vitro* (18, 22).

Sugino *et al*. (1998) have reported that the establishment of an immortalized rat luteal cell line (GG-CL) by infection of luteal cells with the SV-40 *ts*A209 mutant virus (20). This cell line lacks steroidogenic capability, gonadotropin response and steroidogenic enzyme expression. The establishment of a luteal cell line which shows both active growth and the inherent function of steroidogenesis would be most beneficial (9-11, 21). In recent years, our laboratory has attempted to circumvent the primary

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culture limitations by establishing stable steroidogenic luteal cell lines.

A temperature-sensitive mutant of SV40 allows the expression of large T antigen to be controlled by changes in temperature (11, 15, 16). We report here the successful establishment of such a cell line, which has been designated tsCLC-D. The cell line exhibited temperature-sensitive characteristics in growth and morphology at nonpermissive temperatures and retained steroidogenic capacity. The present data confirms that the observations made in the primary luteal cell cultures and attest to the great potential of a temperature-sensitive luteal cell line in the study of luteal cell differentiation and mechanisms of steroidogenesis.

Materials and Methods

Reagents and Hormones

Medium 199, Hank's balanced salt solution (without Ca^{2+} and Mg^{2+}), penicillin G, trypsin-EDTA, streptomycin sulfate, fetal calf serum (FCS) and trypan blue stain were purchased from Invitrogen (Carlsbad, CA, USA). Collagenase (type I) was obtained from Worthington Biochemical Corporation (Lakewood, NJ). Ovine luteinizing hormone (NIDDK-oLH-I-3) was kindly provided by the National Institutes of Health, Bethesda, MD, USA. 8-bromo-cyclic AMP (8-Br-cAMP) and other general chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pregnenolone (Δ_5P) and 22-hydroxycholesterol (22R-OHC) were purchased from Steraloids, Inc. (Newport, RI, USA).

Animals and Tissue Preparation

Adult (1-3 years) female crossbred Taiwan native goats were maintained in the controlled environment. The goats were in sound state of health and demonstrated normal pattern of estrous cycle. Blood samples were obtained from each animal daily. Serum was stored at -20°C. Adult females with regular estrous cycles were also checked daily to define the phases of the cycle as detailed subsequently. Serum progesterone concentration was measured by enzyme immunoassay. Day 1 of the estrous cycle was defined as the first day of low serum progesterone (less than 2 ng/ml) following by the midcycle peak progesterone. Ovaries (n = 3) were surgically obtained from goats in spontaneous estrus at estrous cycle (days 9-12). Corpora lutea were dissected out, washed thoroughly with cold PBS to remove excess blood and placed immediately into cold Medium 199 (M199) containing 100 U/ml of penicillin and 100 μg/ml of streptomycin. One gram of corpus luteum was sliced and dispersed with mild stirring in 5 ml of Hank's balanced salt solution using collagenase (400 U/ml) and DNase I (200 U/ml). The dispersed cells were washed three times with medium, filtered through a nylon mesh (diameter 63 μm) to remove undigested clumps, and centrifuged at 200 × g for 5 min at 4°C. Cell pellets were gently resuspended in M199 supplemented with glutamine (single-strength), a double-strength antibiotic-antimycotic solution, 0.1% insulin and 5% fetal bovine serum. Cell viability was approximately 85-90% as determined by the trypan blue dye exclusion method. The yield of luteal cells from 1 g of tissue ranged from approximately $13–15 \times 10^7$ cells depending on the degree of digestion.

Preparation and Culturing of the Transformed Cell Line

A primary culture of goat luteal cells was grown in T-25 cell culture flasks (Costar, Cambridge, MA, USA). Monolayers were incubated in M199 containing 5% fetal bovine serum at 37°C until the cells attached to the flasks (approximately 24 h). Non-adherent cells were removed by aspiration. The cultures were rinsed once with medium and infected with 0.5 ml of medium containing tsA209 at a multiplicity of infection of 10. After exposure to the virus for 2 h at 34°C, 5 ml of medium was added to the flask. The infected cells were further incubated at 34°C and the medium was changed twice weekly until the cell clones were formed. Clones identified by visual observation were isolated with sterile, stainless steel cylinders. Trypsin-EDTA was added to the cylinders to remove the clones. The cells from each clone were grown in separate T-25 flasks until a sufficient number of cells were obtained for freezing, further screening, and characterization. The cell lines were further cloned by serial dilution to obtain a single, pure cell line. After initial screening for positive responsiveness to 22R-OHC, a clone designated "tsCLC-D" was subjected to further characterization.

Morphology and Cell Growth Properties at Different Temperatures

Cell culture morphology was determined at 400 x magnification using an inverted phase-contrast light microscope. Six T-25 flasks were seeded with tsCLC-D cells and cultured at 34°C. Photomicrographs were taken 2 days later. After having attained 80% confluence, all the flasks were shifted to 40°C for 3 days and then back to 34°C for 3 days, with daily changes of medium. Microscopic assessment was again conducted at that time.

Culture morphology was compared for cells grown at 34°C or 40°C to demonstrate the presence and maintenance of transformed phenotype. Cells

were routinely maintained in M199 containing 5% fetal calf serum plus 100 units/ml penicillin and 100 μg/ml streptomycin in the presence of 5% CO_2 at 34°C. After reaching confluence, cells were treated with 0.25% trypsin-EDTA. Cell growth rates at two culture temperatures 34°C and 40°C were also determined. Equal amounts of cells were seeded from stock cultures into 90 T-25 culture flasks. The flasks were assigned randomly to be incubated at either 34°C or 40°C. Each day, three flasks from each group were used to generate cell counts. After seven days of incubation, half of the flasks at 40°C were shifted to 34°C. Cell counting was continued, with three flasks from each of the four groups counted daily. The culture medium in the remaining flasks was changed daily.

Treatment of Cells with Pharmacological Agents

Half milliliter of the culture medium with equal distribution of the cells were plated in 48-well culture plates (10^5 cells/ml) at 34°C for 24 h and then shifted to 40°C. Vehicle, 8-Br-cAMP, 22R-OHC or pregnenolone at different dose were added to wells at the time of the temperature shift. All measurements for each treatment was performed in triplicate and the medium was collected after incubation for 24 h. The accumulation of progesterone in the culture medium of tsCLC-D cells was determined by ELISA.

Immunoblot Analysis

Primary goat luteal cells and tsCLC-D cells were harvested following incubation at appropriate temperatures for 24 h. Then they were re-suspended in cold lysis buffer and whole cell extracts were prepared. Samples containing 50 μg proteins were individually loaded on a 12% SDS-poly-acrylamide gel for electrophoresis (SDS-PAGE). Following electrophoresis, the separated proteins were electro-transferred to a polyvinylidene fluoride membrane. The membrane was pre-incubated overnight at 4°C in PBS containing 0.05% Tween-20 (PBST) and 5% fat-free milk, prior to incubation with appropriate amounts of steroid acute regulatory (StAR) protein polyclone antibodies (12), P450 side-chain cleavage enzyme (P450scc) and 3β-hydroxysteroid dehydrogenase (3β-HSD) antibodies (6) in PBST-fat-free milk at room temperature for 1 h. After four washes with 15 ml PBST, the membrane was incubated with a 1:5000 dilution of corresponding antimouse peroxidase-conjugated IgG for 1 h. The membrane was washed again with PBST, and bound antibodies were visualized by the ECL system (Amersham Pharmacia, Piscatawy, NJ, USA) using Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY, USA). Monoclonal mouse anti-large T antigen, monoclonal mouse anti-goat β-actin and secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Progesterone Assay

The progesterone assay was modified from a direct enzyme immunoassay (23). The G7 monoclonal antibody against progesterone was prepared as previously described (8). G7 is an IgM with specific affinity of $1.1 \times 10^{10} \times M^{-1}$. Its cross-reactivity is less than 0.01% with bovine serum albumin or other steroids including pregnenolone, testosterone, estradiol, and estrone.

For the assay, the medium collected after the 24 h treatment period was stored at -20°C until assayed. Aliquots (50 μl) of diluted medium and horseradish-peroxidase-linked-progesterone conjugate (150 μl) were added to microtiter plates coated with 200 μl of G7 (representing a 1 : 40,000 dilution). After incubation at room temperature with gentle shaking for 15 min and three washes with Tween-20 in 0.01 M phosphate buffer, pH 7.0, the color was developed with 200 μl of 3.7 mM o-phenylenediamine in 0.03% H_2O_2 for 15 min. The reaction was stopped by the addition of 50 μl of sulfuric acid. The absorbency of samples, read with a dual wavelength reader (Dynatech) (490/630 nm), was compared with that of the progesterone standard curve. The coefficients of variation within and between assays were 7% and 12%, respectively. The sensitivity of the assay was 0.3 pg/ml.

Statistical Analysis

Each experiment was repeated for a minimum of three times and the mean and standard error of the mean (SEM) of one experiment with identical results was used to express the concentration for each determination. Measurements of progesterone were expressed as ng/ml/10^5 cells. The effect of hormonal stimulation and dose response of oLH, 8-Br-cAMP, 22R-OHC and pregnenolone on progesterone content were analyzed using analysis of variance (ANOVA), followed by Duncan's multiple comparison (5). $P < 0.05$ values greater than this to be significantly.

Results

Growth Curve of tsCLC-D Cells at the Permissive (34°C) and Nonpermissive (40°C) Temperatures

We observed the colonies (A to Z) after transfection with a temperature-sensitive mutant of SV40. After initial screening for positive responsiveness to 22R-OHC, one of the clone (tsCLC-D) was chosen and

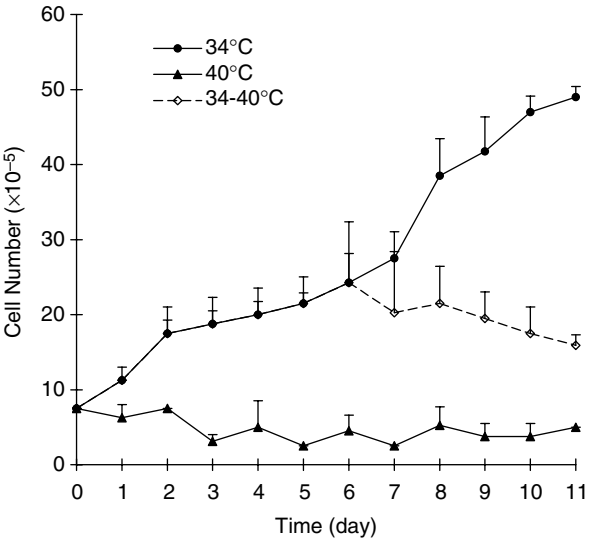

Fig. 1. Growth curve of tsCLC-D cells at the permissive (34°C) and nonpermissive (40°C) temperatures. At 34°C, cells rapidly divided and became confluent, whereas the cells moved to 40°C ceased to divide and were able to grow only as a monolayer.

grown at 34°C for further characterization. Propagation to 150 passages did not affect the cell growth rate at 34°C, confirming the stability of this cell line. To demonstrate that the tsCLC-D cells were indeed temperature-sensitive, cells were plated at a density of 7.5×10^5 cells per flask in a 25-cm^2 tissue culture flask and cultured at 34°C for 12 days. Cells were also cultured at 34°C for 4 days and were than shifted to 40°C to be cultured for 8 days. Cells were harvested at each time point and counted by the trypan blue dye exclusion method.

The results show that cells grow rapidly at 34°C and cell numbers continuously increase. However, after temperature shift to 40°C, cells cease to divide (Fig. 1).

Morphology of tsCLC-D Cells at the Permissive (34°C) and Nonpermissive (40°C) Temperatures

As shown in Fig. 2, tsCLC-D cells exhibited remarkable morphological differences when grown at 34°C compared with those grown at 40°C. At the permissive temperature (34°C) the cells assumed a spindle shape. After culture for two days at nonpermissive temperature (40°C), the cells resumed a rounded shape.

Immunoblot of Primary Caprine Luteal Cells and the Transformed Cell Line

The representative expression of SV40 large T antigen, StAR protein, P450scc and 3 β-HSD enzymes

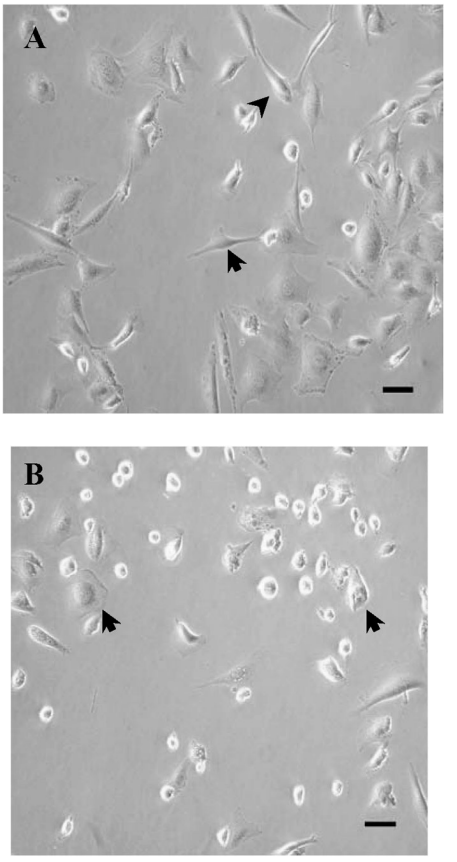

Fig. 2. Morphology of tsCLC-D cells at permissive (34°C) and nonpermissive (40°C) temperatures. At the permissive temperature (A) the cells assumed a spindle shape. After culturing for two days at nonpermissive temperature (B), the cells resumed a rounded shape (— 45 μm).

by tsCLC-D cells is depicted in the Western blot shown in Fig. 3. The levels of expression of large T antigen were analyzed at both nonpermissive (40°C) and permissive (34°C) temperatures. At 34°C, the level of large T antigen protein was higher than that at 40°C. In the results of steroidogenic protein expression, the expression of StAR protein or P450scc is all obviously lower than that of the primary luteal cell, no matter under the culture temperature of 34°C or 40°C. However, the 3β-HSD does not have significant difference.

Effects of Different Regents and Hormones on Progesterone Secretion by tsCLC-D Cells

Progesterone secretion by tsCLC-D cells during 24 h of incubation at 40°C with oLH, 8-Br-cAMP, 22R-OHC and pregnenolone is shown in Figs. 4 and 5. In the absence of hormonal stimulation (control), basal progesterone concentration was 5.4 ± 0.6 ng/ml. Furthermore, either 8-Br-cAMP (10-100 μM), 22R-

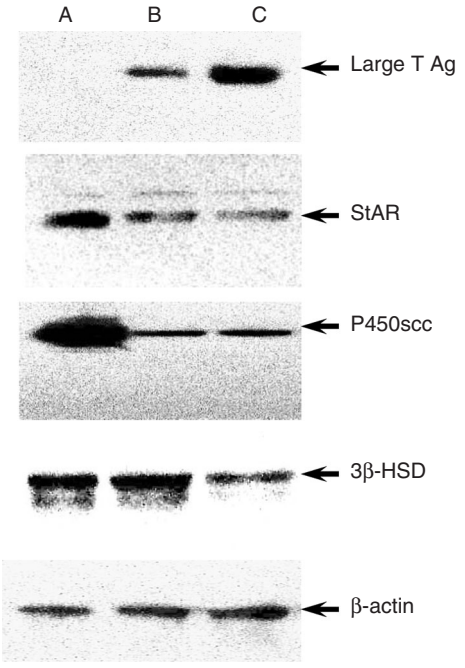

Fig. 3. Western blot of immunoreactive large T antigen, StAR, P450scc and 3β-HSD from primary caprine luteal cells and the transformed cell line (tsCLC-D) incubated at different temperatures (34°C/40°C) for 24 h. A: primary carpine luteal cells; B: tsCLC-D at 40°C for 24 h; C: tsCLC-D at 34°C for 24 h.

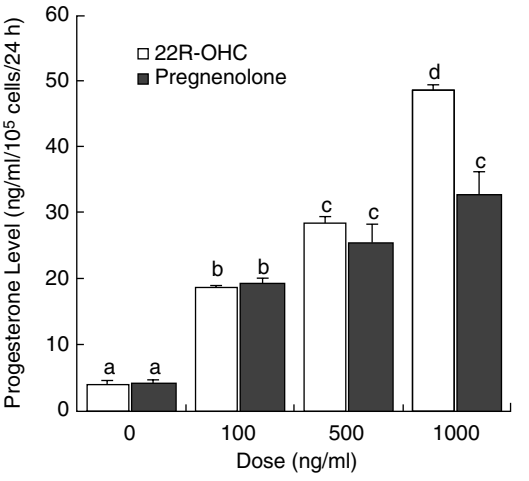

Fig. 4. Effect of different doses of 22-hydroxycholesterol and pregnenolone on progesterone secretion by tsCLC-D cells. Cells were cultured for 24 h in the presence or absence of 22-hydroxycholesterol or pregnenolone at the nonpermissive temperature (40°C). Data are expressed as ng of progesterone per ml of culture medium. Each bar represents the means ± SEM of triplicate samples. Bars with different letters are significantly different from each other ($P < 0.05$).

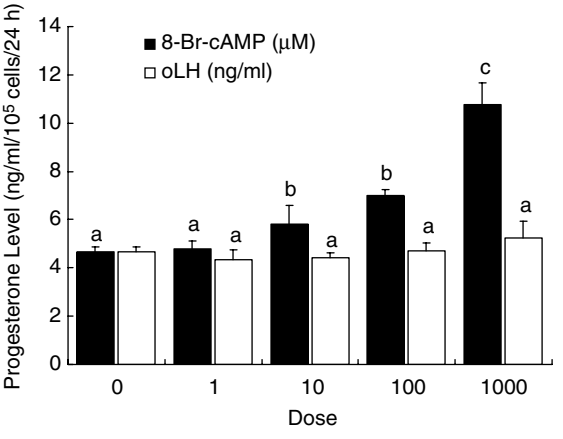

Fig. 5. Dose response of 8-Br-cAMP or ovine LH on progesterone secretion by tsCLC-D cells. Cells were cultured for 24 h in the presence or absence of 8-Br-cAMP or ovine LH at the nonpermissive temperature (40°C). Data are expressed as ng of progesterone per ml of culture medium. Each bar represents the means ± SEM of triplicate samples. Bars with different letters are significantly different from each other ($P < 0.05$).

OHC (100-500 ng/ml) or pregnenolone (100-500 ng/ml) to the cells in culture all showed various degree of stimulated progesterone secretion above control levels. However, the addition of any doses oLH did not increase progesterone secretion. Even if the cell line puts and still stimulates the result which secretes progesterone to 8-Br-cAMP incubation at 34°C. This result shows that temperature has not totally changed its steroidogenic ability.

The effect of the addition of 22R-OHC and pregnenolone as a substrate for progesterone synthesis by tsCLC-D is shown in Fig. 4. High levels of progesterone were found in tsCLC-D cells, after 24 h in culture with either 22R-OHC or pregnenolone. The addition of 100, 500 and 1000 ng/ml 22R-OHC elevated the levels of progesterone 4–10 folds versus control. The addition of 100, 500 and 1000 ng/ml pregnenolone induced a 4–6 folds increase versus control.

The effect of cAMP on progesterone synthesis by tsCLC-D is shown in Fig. 5. cAMP at 10, 100 and 1000 μM induced 2–2.5 folds increases in progesterone synthesis, respectively, versus control after 24 h of incubation.

Discussion

In view of the increasing need for goats in biomedical research, it is desirable to develop appropriate caprine-specific cell culture models that could prevent or significantly reduce the increasing use of primary cultures and experiments with living animals (13, 19). Luteal cells not only are essential for the control of hormone-dependent processes, such

as the ovarian cycle and pregnancy, but also linked with many reproduction disorders. For this reason it is of great interest to know more about control mechanisms existing in these luteal cell types and the effect of pharmacological or toxicological agents on them.

To our knowledge, the present study reports about the first successful development of a cell line originating from the luteal cells of the caprine corpus luteum that retains steroidogenic function. Luteal cells are terminally differentiated and fail to proliferate in the primary culture. Their transfection with a temperature-sensitive mutant of SV40 endows these cells with the ability to grow in an unrestricted manner (1, 17). The temperature-sensitive mutants of SV40 are defective in the gene required for the maintenance of the transformed phenotype in mammalian cells (14). Therefore, the ts-SV40 infected luteal cells are conditionally transformed cells, and express the transformed phenotype and large-T antigen accumulation at the permissive temperature (34°C). At the nonpermissive temperature (40°C), these cells revert to their normal morphology and produce high level progesterone. This marked morphological and functional difference allows the use of these cells (4).

Our data show that a very high proportion of SV40 Tag-immortalized cell lines retained steroidogenic functions such as basal and cAMP-stimulated progesterone secretion and also exhibit the characteristic rounding response of steroidogenic cells to 22R-OHC and pregnenolone. Interestingly, treatment with ovine luteinizing hormone did not increase progesterone biosynthesis in this cell line. Although the tsCLC-D cells did not respond to oLH, the cells were responsive to larger doses of cAMP, 22R-OHC and pregnenolone and increased rapid in the accumulation of progesterone. Sugino *et al*. (1998) have reported a temperature-sensitive SV40 transformed rat luteal cell line that also lacks of gonadotropin receptors but retains many of the cell-specific elements encountered in the primary cells of origin (20). It is unclear why this cell line loses gonadotropin receptors (2, 9). Perhaps this is a species-specific difference relative to the replication capacity of the cells. This unique feature demands further investigation, which the cell line after further scrutinizing can be used as a model to study steroidogenesis and regulation of progesterone biosynthesis *in vitro*.

In conclusion, we have established a stable caprine luteal cell line. The tsCLC-D are the stable cell line since it has been grown continuously for at least 150 passages in culture. Characterization showed this cell line has lost the ability to respond to gonadotropins but has retained basal and regulated progesterone biosynthetic capability. Although this cell line does not express a number of desirable differentiation properties, it does exhibit steroidogenic enzyme activity and maintains steroidogenic potential. We believe that this cell line will provide a valuable tool for the investigation of the regulation of steroidogenesis in a non-rodent system, and may prove useful for studies of the mechanisms that control steroidogenesis in luteal cells. Immortalization protocols for ovarian cell types of pigs (3) and rats (7) have been developed. All cell lines established so far have been examined with regard to the maintenance of known, tissue-specific features (*e.g.* hormone responsiveness and enzyme expression). The results obtained indicate that it is worthwhile cloning and characterizing the cell lines and more detail investigation is necessary so that they may be used as a defined test system both for basic research, and for pharmacological or toxicological screening.

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References

1. Amsterdam, A., Zauberman, A., Meir, G., Pinhasi-Kimhi, O., Suh, B.H. and Oren, M. Cotransfection of granulosa cells with simian virus 40 and *Ha-RAS* oncogene generates stable lines capable of induced steroidogenesis. *Cell Biol.* 85: 7582-7586, 1988.
2. Ascoli, M. Characterization of several clonal lines of cultured leydig tumor cells: gonadotropin receptors and steroidogenesis responses. *Endocrinology* 108: 88-95, 1998.
3. Chedrese, P.J., Rodway, M.R., Swan, C.L. and Gillio-Meina, C. Establishment of a stable steroidogenic porcine granulosa cell line. *J. Mol. Endocrinol.* 20: 287-292, 1998.
4. Chen, H.D., Chen, H.W., Chou, J.Y., Gillis, K.D. and Chen, C.L. Characterization of a prolactin-synthesizing rat pituitary cell line immortalized by the temperature-sensitive mutant SV40. *Chinese J. Physiol.* 38: 201-209, 1995.
5. Duncan, D.B. Multiple range and multiple F test. *Biometrics* 11: 1-42, 1995.
6. Fei, C.Y., Chiu, C.H. Chen, C. Wu L.S. and Guo, I.C. Production of polyclonal antibodies against 3β-hydroxysteroid dehydrogenase of cattle, pig, and goat. *J. Chin. Soc. Anim. Sci.* suppl. 30: 198, 2001.
7. Fitz, T.A., Wah, R.M., Schmidt, W.A. and Winkel, C.A. Physiologic characterization of transformed and cloned rat granulosa cells. *Biol. Reprod.* 40: 250-258, 1988.
8. Guo, I.C., Wu, L.S., Lin, J.H. and Meng, C.L. Production of monoclonal antibodies to progesterone. *Memoirs of the College of Agriculture, Natl. Taiwan Univ.* 31: 10-21, 1991.
9. Keren-Tal, I., Dantes, A., Sprengel, R. and Amsterdam, A. Establishment of steroidogenic granulosa cell lines expressing follicle stimulating hormone receptors. *Mol. Cell. Endocrinol.* 95: R1-R10, 1993.
10. Kilgore, M.W. and Stocco, D.M. Initial characterization of a subclone of the MA-10 mouse leydig tumor cell line. *Endocrinology* 124: 1210-1216, 1989.
11. Kitamura, Y.M., Michikwa, M., Tanigawa, T., Kastumoto, T., Morita, T., Nanba, E., Ohama, E. and Terada, T. Establishment and

characteristic of practical and useful astrocyte cell line transformed by a temperature-sensitive mutant of simian virus 40. *Brain Res.* 759: 295-300, 1997.

12. Liao, C.H., Chiu, C.H., Fei, C.Y., Guo, I.C. and Wu, L.S. Preparation of antiserum against steroidogenic acute regulatory protein (StAR). *J. Chin. Soc. Anim. Sci.* suppl. 31: 220, 2002.
13. Lie, B., Leung, E., Leung, P.C.K. and Auersperg, N. Long-term growth and steroidogenic potential of human granulosa-lutein cells immortalized with SV40 large T antigen. *Mol. Cell. Endocrinol.* 120: 169-176, 1996.
14. Logan, S.K., Hansell, E.J., Damsky, C.H. and Werb, Z. T-Antigen inhibits metalloproteinase expression and invasion in human placental cells transformed with temperature-sensitive simian virus 40. *Matrix Biol.* 15: 81-89, 1996.
15. Martin, R.G. and Chou, J.Y. Simian virus 40 functions required for the establishment and maintenance of malignant transformation. *J. Virol.* 15: 599-612, 1975.
16. O'Neill, F.J., Hu, Y., Chen, T. and Carney, H. Identification of p53 unbound to T-antigen in human cells transformed by simian virus 40 T-antigen. *Oncogene* 14: 955-965, 1997.
17. Rao, I.M., Gadson, P.F., Jr., Anderson, E., Hornsby, P.J. and Mahesh, V.B. Characterization of progesterone biosynthesis in a transformed granulosa cell line. *Mol. Cell. Endocrinol.* 94: 121-128, 1993.
18. Simard, J., Durocher, F., Mebarki, F., Turgeon, C., Sanchez, R., Labrie, Y., Couet, J., Trudel, C., Rheaume, E., Morel, Y., Luu-The, V. and Labrie, F. Molecular biology and genetics of the 3-beta-hydroxysteroid dehydrogenase/DELTA-5-DELTA-4 isomerase gene family. *J. Endocrinol.* Suppl. 150: S189–S207, 1996.
19. Srivastava, R.K., Gu, Y., Zilberstein, M., Ou, J.S., Mayo, K.E., Chou, J.Y. and Gibori, G. Development and characterization of simian virus 40-transformed, temperature-sensitive rat antimesometrial decidual cell line. *Endocrinology* 136: 1913-1919, 1995.
20. Sugino, N., Zilberrstein, M., Srivastava, R.K., Telleria, C.M., Nelson, S.E., Risk, M., Chou, J.Y. and Gibori, G. Establishment and characterization of a simian virus 40-transformed temperature-sensitive rat luteal cell line. *Endocrinology* 139: 1936-1942, 1998.
21. Suh, B.S. and Amsterdam, A. Establishment of highly steroidogenic granulosa cell lines by cotransfection with SV-40 and Ha-ras oncogene: induction of steroidogenesis by cyclic adenosine 3'-5'-monophosphate and its suppression by phorbol ester. *Endocrinology* 127: 2489-2500, 1990.
22. Thomson, M. Molecular and cellular mechanisms used in the acute phase of stimulated steroidogenesis. *Horm. Met. Res.* 30: 16-28, 1998.
23. Wu, L.S., Wang, H.L., Fang, S.W., Janung, R.H., Cheng, S.C., Hwang, S.Y. and Lin, J.H. The establishment of microtiter plate enzyme immunoassay for progesterone in bovine milk. *Memoirs of the College of Agriculture, Natl. Taiwan Univ.* 29: 173-183, 1989.