# Establishment of a Human Nonluteinized Granulosa Cell Line that Transitions from the Gonadotropin-Independent to the Gonadotropin-Dependent Status

Bayasula, Akira Iwase, Tohru Kiyono, Sachiko Takikawa, Maki Goto, Tomoko Nakamura, Yoshinari Nagatomo, Tatsuo Nakahara, Tomomi Kotani, Hiroharu Kobayashi, Mika Kondo, Shuichi Manabe, and Fumitaka Kikkawa

Departments of Obstetrics and Gynecology (B., A.I., S.T., M.G., T.Nakam., Y.N., T.Nakah., T.Ko., H.K., S.M., F.K.) and Maternal and Perinatal Medicine (A.I., M.G.), Nagoya University Graduate School of Medicine (M.K.), Nagoya 466-8550, Japan; and Division of Virology (T.Ki.), National Cancer Center Research Institute, Tokyo 104-0045, Japan

The ovary is a complex endocrine organ responsible for steroidogenesis and folliculogenesis. Follicles consist of oocytes and two primary steroidogenic cell types, the granulosa cells, and the theca cells. Immortalized human granulosa cells are essential for researching the mechanism of steroidogenesis and folliculogenesis. We obtained granulosa cells from a 35-yr-old female and immortalized them by lentivirus-mediated transfer of several genes so as to establish a human nonluteinized granulosa cell line (HGrC1). We subsequently characterized HGrC1 and investigated its steroidogenic performance. HGrC1 expressed enzymes related to steroidogenesis, such as steroidogenic acute regulatory protein, CYP11A, aromatase, and gonadotropin receptors. Stimulation with FSH increased the mRNA levels of aromatase, which consequently induced the aromatization of androstenedione to estradiol. Activin A increased the mRNA levels of the FSH receptor, which were synergistically up-regulated with FSH stimulation. HGrC1 also expressed a series of ligands and receptors belonging to the TGF-$\beta$ superfamily. A Western blot analysis showed that bone morphogenetic protein (BMP)-4, BMP-6, and BMP-7 phosphorylated small mother against decapentaplegic (Smad)1/5/8, whereas growth differentiation factor-9 phosphorylated Smad2/3. BMP-15 and anti-Müllerian hormone phosphorylated Smad1/5/8 while also weakly phosphorylating Smad2/3. These results indicate that HGrC1 may possess the characteristics of granulosa cells belonging to follicles in the early stage. HGrC1 might also be capable of displaying the growth transition from a gonadotropin-independent status to gonadotropin-dependent one. **(*Endocrinology* 153: 2851–2860, 2012)**

The ovaries are complex and precisely regulated endocrine organs that are responsible for the production of the sex steroids and fertilizable ova. A greater understanding of the mechanisms of folliculogenesis and steroidogenesis may lead to improvements in the success rates of assisted reproductive technologies or the development of novel treatments for reproductive disorders. A follicle consists of the oocyte itself and two kinds of somatic cells surrounding the oocyte, the granulosa cells and the theca cells, which play main roles in steroidogenesis. Therefore, the ovarian function can be considered to be dependent upon the follicle functions, in other words,

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Abbreviations: ActRII, Activin receptor II; ALK, activin receptor-like kinase; AMH, anti-Müllerian hormone; BMP, bone morphogenetic protein; BrdU, bromodeoxyuridine; CDK4, cyclin-dependent kinase 4; ER, estrogen receptor; FCS, fetal calf serum; FOXL2, forkhead box protein L2; FSHR, FSH receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDF, growth differentiation factor; hCG, human chorionic gonadotropin; HGrC1, human non-LGC line; HPV16, human papillomavirus type 16; hTERT, human telomerase reverse transcriptase; IVF, *in vitro* fertilization; LGC, luteinized granulosa cell; LHR, LH receptor; HRas, v-Ha-ras Harvey rat sarcoma viral oncogene homolog; SF-1, steroidogenic factor-1; Smad, small mother against decapentaplegic; StAR, steroidogenic acute regulatory protein.

steroidogenesis, oogenesis, folliculogenesis, atresia, and luteinization.

To understand the mechanisms of the follicle function at the molecular level, materials which are suitable for *in vitro* studies are needed. Granulosa cells proliferate during folliculogenesis and then undergo differentiation during the terminal follicular growth and luteinization (1). Cross talk using gap junctions and paracrine signals, such as bone morphogenetic proteins (BMP), between oocytes and granulosa cells is mainly involved in follicle growth. In addition, the apoptosis of granulosa cells is induced by an imbalance between cell death and survival signals (2). Therefore, it is thought that if the proliferation or differentiation of granulosa cells does not occur appropriately, follicles fall into atresia. Therefore, human granulosa cells obtained from *in vitro* fertilization (IVF) programs have often been used to study the mechanisms underlying the proliferation and differentiation of granulosa cells (1, 3–6). However, they are only obtainable in small numbers, which thus makes it difficult to perform detailed analyses at the cellular and molecular levels. In addition, these cells are typically obtained during luteinization due to the administration of human chorionic gonadotropin (hCG), which causes granulosa cells to decrease the expression of FSH receptor (FSHR) and exit the cell cycle (7).

Due to these obstacles, the use of granulosa cell lines for *in vitro* experiments has become an attractive option, and immortalization of primary cultured granulosa cells has been performed. Consecutively, immortalized cell lines derived from rodent, bovine, and porcine granulosa cells have been established, which have been demonstrated to be useful for examining steroidogenesis and the production of growth factors (8). Although this has been accomplished, with some success in humans, the problem still remains that the immortalized human cell lines are derived from granulosa cell tumors or luteinized granulosa cells (LGC) obtained in IVF. The HTOG, COV434, and KGN lines were established from granulosa cell tumors (9–11). On the other hand, the HGL5, HO-23, and HGP53 were established from the LGC obtained from IVF (12–14). Only KGN cells show a FSH-dependent increase of aromatase activity (10). However, KGN has a missense mutation in the Forkhead transcription factor gene, *FOXL2* (15), which is involved in the proliferation and differentiation of granulosa cells.

It is challenging to establish cell lines from non-LGC, because the cell number obtained from human ovaries is very limited, and the cultured cells soon stop proliferation. Normal human cells undergo a limited number of divisions in culture and then enter a nondividing state referred to as senescence. Ectopic expression of telomerase alone bypasses replicative senescence in certain cell types, such as human foreskin fibroblasts. However, cellular stress, including artificial culture conditions, activates the $p16^{INK4a}$/pRB pathway resulting in premature cell cycle arrest before telomere attrition (16). The forced expression of human papillomavirus type 16 (HPV16) E7, which binds and inactivates pRB or components of physiological kinase of Rb protein, cyclin-dependent kinase 4 (CDK4) and cyclin D1 enabled human telomerase reverse transcriptase (hTERT) to immortalize several types of primary human cells, including human mammary epithelial cells, mesenchymal stem cells, and umbilical cord blood-derived cells (16–18). However, because abrupt inactivation of the $p16^{INK4a}$/pRB pathway activates p53, inactivation of p53 by HPV16 E6, which enhances degradation of p53, helps efficient immortalization of some cell types (19, 20). In the present study, combined expression of cyclin D1, a mutant CDK4 (CDK4R24C), which cannot be bound by $p16^{INK4a}$ and hTERT together with additional expression of HPV16 E6 and E7, efficiently immortalized the cells to establish a new cell line derived from human non-LGC, which underwent the transition from gonadotropin-independent to gonadotropin-dependent status.

## Materials and Methods

### Preparation of the primary cultured human granulosa cells

A 35-yr-old female with a left ovarian tumor was admitted to our department and underwent a surgery at Nagoya University Hospital in August 2008. Because she was diagnosed with a mucinous adenocarcinoma by a histological analysis of the left ovary by examination of the frozen tumor tissue sections, a total hysterectomy, bilateral salpingo-oophorectomy, and omentectomy were performed. Immediately after the resection of the right ovary, the antral follicles, approximately 3–5 mm in diameter, were dissected from the ovary under a surgical dissecting microscope. Each follicle was opened using fine watch-maker forceps, and the granulosa layers were removed. The right ovary showed no metastasis in the permanent section. The granulosa layers were resuspended in DMEM (Sigma, St. Louis, MO) containing 10% fetal calf serum (FCS) (Sigma), 100 IU/ml penicillin, 100 $\mu$g/ml streptomycin, 25 mg/liter amphotericin, and L-glutamine. The cells were seeded onto six-well collagen-coated dishes (Biocoat; Becton Dickinson and Co., Franklin Lakes, NJ). This study was approved by the Ethics Committee of Nagoya University School of Medicine.

### Lentivirus-mediated gene transfer

Lentiviral vector plasmids were constructed by recombination using the Gateway system (Invitrogen, Grand Island, NY). The tTA-advanced segment from the pTet-Off Advanced plasmid (CLONTECH, Mountain View, CA) was recombined with a lentiviral vector, CSII-CMV-RfA, by the LR reaction (Invitrogen) to generate CSII-CMV-Tet-Off. The elongation factor 1 $\alpha$ promoter (EF) in CSII-EF-MCS was replaced with a tet-respon-

**TABLE 1.** The primers used for RT-PCR

| | | |
|---|---|---|
| StAR | F | CGTGACTTTGTGAGCG |
| | R | GCCACGTAAGTTTGGT |
| CYP17 | F | GTGACCGTAACCGTCT |
| | R | ATGAACTGATCCGGCT |
| CYP11A | F | AGAGTTGAAATCCAACA |
| | R | TGGGACAGACGACTGA |
| Aromatase | F | CCATAAAGACCCGATTCCACCA |
| | R | GCTGAGGCATAAATCGACAGAC |
| FSHR | F | GCGGAACCCCAACATCGTGTC |
| | R | TGAAGAAATCTCTGCGAAAGT |
| LHR | F | TCAATGTGGTGGCCTTCTTCATA |
| | R | TTGGCACAAGAATTGATGGGATA |
| ERα | F | ATGACCATGACCCTCACAACACCAA |
| | R | CTTGGCAGATTCCATAGCCATAC |
| ERβ | F | TACAGCATTCCCAGCAATGTCAC |
| | R | GCCCTGGAGGTAGGACAGGCC |
| FOXL2 | F | GAGAAGGCTACGCTGTCCGG |
| | R | CCCATAGAGCATGCATCAT |
| GATA4 | F | GGACCATGTATCAGAGCTTG |
| | R | GCCCTGGAGGTAGGACAGGCC |
| SF-1 | F | GGAGTTTGTCTGCCTCAAGTTCA |
| | R | CGTCTTTCACCAGGATGTGGTT |
| GAPDH | F | TGAACGGGAAGCTCACTGGCATGGCCTT |
| | R | GTGTGGTGGGGGACTGAGTGTGGCAGGGAC |
| BMP-2 | F | ATGGATTCGTGGTGGAAGTG |
| | R | GTGGAGTTCAGATGATCAGC |
| BMP-4 | F | AGCATGTCAGGATTAGCCGA |
| | R | TGGAGATGGCACTCAGTTCA |
| BMP-6 | F | CAGCCTGCAGGAAGCATGAG |
| | R | CAAAGTAAAGAACCGAGATG |
| BMP-5 | F | AAGAGACAAGAAGGACTAAAAATAT |
| | R | GTAGAGATCCAGCATAAAGAGAGGT |
| BMP-7 | F | AGCCCGGGTAGCGCGTAGAG |
| | R | GCGCGGTGGATGAAGCTCGA |
| BMP-15 | F | GGGGGATTTGGGTTTTAGCC |
| | R | GGTAAAGCAGAAAGAGGTCCCG |
| AMH | F | ACATCAGGCCCAGCTCTATCAC |
| | R | TGTTTGTGCAGGACAGACCC |
| GDF-9 | F | CTCTTCACCCCCTGTACCC |
| | R | CAGTTCCACTGATGGAAGGAT |
| Inhibin α | F | GTCTCCCAAGCCATCCTTTT |
| | R | TGGCAGCTGACTTGTCCTC |
| Inhibin βA | F | CTCGGAGATCATCACCGTTTG |
| | R | CCTTGGAAATCTCGAAGTGC |
| Inhibin βB | F | ATCAGCTTCGCCGAGACA |
| | R | GCCTTCGTTGGAGATGAAGA |
| ActRIIA | F | TCATGAATTTGGCTTTTGGA |
| | R | TTTGGCAGTGTGACGCTTAC |
| ActRIIB | F | ATATTGGGAGATTGCTCGAAGA |
| | R | GGCAGCTGATATTCTTCATGG |
| BMPRII | F | AGCAGCAGAACCCTTCCCAAG |
| | R | CCAGAGAATTAGGCCTCTGT |
| AMHRII | F | TGTGTTTCTCCCAGGTAATCCG |
| | R | AATGTGGTCGTGCTGTAGGC |
| TGFRII | F | CGCTTTGCTGAGGTCTATAAGGC |
| | R | GATATTGGAGCTCTTGAGGTCCCT |
| ALK 2 | F | ACTACAGCCTGGAGCATTGGTAAG |
| | R | ATCTGCCCACAGTCCTTCAAGC |
| ALK 3 | F | TCATTTGGGAGATGGCTCGTC |
| | R | TGTGAGTCTGGAGGCTGGATT |
| ALK 6 | F | CGTCCAAAGGTCTTGCGTTG |
| | R | CCCGACACTGAAAATCTGAGCC |
| The primers used for SYBR Green | | |
| GAPDH | F | CAGCCTCAAGATCATCAGCA |
| | R | GTCTTCTGGGTGGCAGTGAT |
| FSHR | F | TTCAAGAACAAGGATCCATTCC |
| | R | CCTGGCCCTCAGCTTCTTAA |
| Aromatase | F | TGAATATTGGAAGGATGCACAGAC |
| | R | TGGAATCGTCTCAGAAGTGTAACGAG |

F, Forward; R, reverse; AMHRII, AMH type II receptor; BMPRII, BMP type II receptor; TGFRII, TGF type II receptor.

sive promoter (TRE-Tight) from pTRE-Tight (CLONTECH) followed by a modified RfA fragment (Invitrogen) to produce a tet-responsive lentivirus vector, CSII-TRE-Tight-RfA. The entry vectors containing cDNA for hTERT, human cyclinD1, human mutant CDK4 ($CDK4^{R24C}$), which cannot bound by $p16^{INK4a}$ (21, 22), HPV16 E6E7, E6, and the C-terminal 234 amino acids of p53 (p53C234) were recombined with CSII-TRE-Tight-RfA by the LR reaction (Invitrogen) to generate CSII-TRE-Tight-hTERT, CSII-TRE-Tight-CyclinD1, CSII-TRE-Tight-CDK4R24C, CSII-TRE-Tight-16E6E7, CSII-TRE-Tight-16E6, and CSII-TRE-Tight-p53C234. The production and infection of recombinant lentiviruses were performed as described previously (21, 23).

## Reverse transcription-polymerase chain reaction

Total RNA was isolated from LGC in 35-mm dishes using an RNeasy Mini kit (QIAGEN, Inc., Valencia, CA) following the manufacturer's protocol. A RT reaction with 1 μg of total RNA was carried out with a first strand cDNA synthesis kit (ReverTra Ace α; Toyobo Co., Ltd., Osaka, Japan). Thereafter, 1-μl aliquots of the RT reaction products were used for PCR to amplify the steroidogenic acute regulatory protein (StAR), CYP17, CYP11A, aromatase, FSHR, LH receptor (LHR), estrogen receptor (ER)α, ERβ, forkhead box protein L2 (FOXL2), steroidogenic factor-1 (SF-1), GATA binding protein 4 (GATA4), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-15, anti-Müllerian hormone (AMH), growth differentiation factor (GDF)-9, inhibin α, inhibin βA, inhibin βB, activin receptor II (ActRII)A, ActRIIB, BMP type II receptor, AMH type II receptor, TGF type II receptor, activin receptor-like kinase (ALK) 2, ALK 3, and ALK 6 mRNA. The sets of oligonucleotide primers used are shown in Table 1. Amplification was performed using *Taq* polymerase (PerkinElmer, Waltham, MA) over 35 cycles. Each cycle consisted of denaturation at 94 C for 1 min, annealing at 54 C for 1 min, and extension at 72 C for 1 min. We compared the expression levels determined by RT-PCR with primary cultured LGC obtained from IVF, KGN cells derived from a granulosa cell tumor (10), and MRC5 human fetal lung fibroblasts as a negative control.

## Quantitative RT-PCR

To detect the aromatase expression, the cells were cultured in DMEM with 5% charcoal-stripped FCS for 24 h. The cells were then stimulated with 0.05, 0.5, or 5 IU/ml human recombinant FSH (follitropin β; Merck & Co., Inc., Kenilworth, NJ) or 50 μM forskolin (Sigma) for 48 h. To detect of the FSHR expression, the cells were stimulated with 50 ng/ml activin A (R&D Systems, Inc., Min-

neapolis, MN) and/or 0.05, 0.5, or 5 IU/ml human recombinant FSH. Thereafter, real-time PCR was performed in 96-well 0.2-ml thin-wall PCR plates using the Thermal Cycler Dice (Takara Bio, Inc., Tokyo, Japan) and SYBR Premix Ex Taq (Takara Bio, Inc.). The real-time PCR mixture contained 1× SYBR Premix Ex Taq, 10 μM PCR primers, and 1 μg of cDNA in a total volume of 25 μl. The sets of oligonucleotide primers for glyceraldehyde-3-phosphate dehydrogenase, FSHR, and aromatase are shown in Table 1. The PCR reaction consisted of an initial incubation at 95 C for 10 sec followed by 40 cycles of denaturation at 95 C for 5 sec and annealing and extension at 60 C for 30 sec.

### Assay for estradiol and progesterone in the culture medium

To detect the presence of estradiol in the culture medium, the cells were cultured in DMEM with 5% charcoal-stripped FCS for 24 h. Then, 10 μM androstenedione (4-androstene-3,17-dione; Sigma) was added in the culture medium, and the cells were stimulated with 0.05, 0.5, or 5 IU/ml human recombinant FSH or 50 μM forskolin for 48 h. The concentrations of estradiol and progesterone in the medium were determined with a chemiluminescent immunoassay (Estradiol II CalSet II, Elecsys, Cobas and Progesterone II CalSet, Elecsys, Cobas; Roche Diagnostics, Indianapolis, IN). The intraassay coefficients of variation were less than 10%.

### Cell count and bromodeoxyuridine (BrdU) assay

Human non-LGC line (HGrC1) cells of twelfth and thirtieth passage were seeded at $60 \times 10^3$ on each well of 24-well plates and then cultured in DMEM containing 10% FCS for 48 h. The number of cells was counted every 8 h. The rate of DNA synthesis was determined from the incorporation of BrdU into cells (BrdU cell proliferation assay; Oncogene Research Products, San Diego, CA) as previously described (6). HGrC1 cells were seeded at $10 \times 10^3$ on 96-well plates and cultured with 1, 10, or 100 IU/ml for 24 h to allow BrdU incorporation. The cells were fixed and incubated with anti-BrdU antibody for 1 h, followed by horseradish peroxidase-conjugated goat antimouse IgG. Next, 100 μl of substrate (tetramethylbenzidine) were added. The absorbance at dual wavelengths of 450–540 nm was then determined using a microplate reader.

### Western blot analysis and the effects of BMP

To detect of phosphorylated small mother against decapentaplegic (Smad), the cells were cultured in DMEM without FCS for 24 h. Then, the cells were stimulated with 100 ng/ml BMP-4 (R&D Systems, Inc.), 1 μg/ml BMP-6 (BioVision, Mountain View, CA), 1 μg/ml BMP-7 (ProSpec-Tany TechnoGene Ltd., Rehovot, Israel), 500 ng/ml BMP-15 (R&D Systems, Inc.), 2 μg/ml GDF-9 (BioVision), and 1 μg/ml AMH (R&D Systems, Inc.) for 30 min. The cells were lysed, resolved by SDS-PAGE, and transferred as described above. Membranes were immunoblotted with an anti-Smad1/5/8 Ab (N-18-R, 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), antiphospho-Smad1/5/8 Ab (no. 9511, 1:1000; Cell Signaling Technology, Inc., Danvers, MA), anti-Smad2/3 Ab (C-8, 1:1000; Santa Cruz Biotechnology, Inc.), or antiphospho-Smad2/3 Ab (sc-11769, 1:1000; Santa Cruz Biotechnology, Inc.). Antibodies against CDK4 (Transduction lab; 1:1000), cyclinD1 (clone G124–326, 1:1000; PharMingen, San Jose, CA), p16INK4a (clone G175–405, 1:50; PharMingen), p53 (clone DO-1, 1:1000; Merck KGaA, Darmstadt, Germany), and 16E6 (clone 46A4; in house, hybridoma sup; 1:5) were used for immunoblotting to confirm the expression of transgenes regulated by Tet-Off system in HGrC1 cells treated with 1 μg/ml of doxycycline or vehicle (70% ethanol) for 2 d.

### Statistical analysis

A one-way ANOVA with the Dunnet's *post hoc* test was used to determine the differences in the results of the quantitative RT-PCR and concentrations of estradiol and progesterone. All statistical analyses were performed using the SigmaPlot 11 software program (Systat Software, Inc., San Jose, CA).

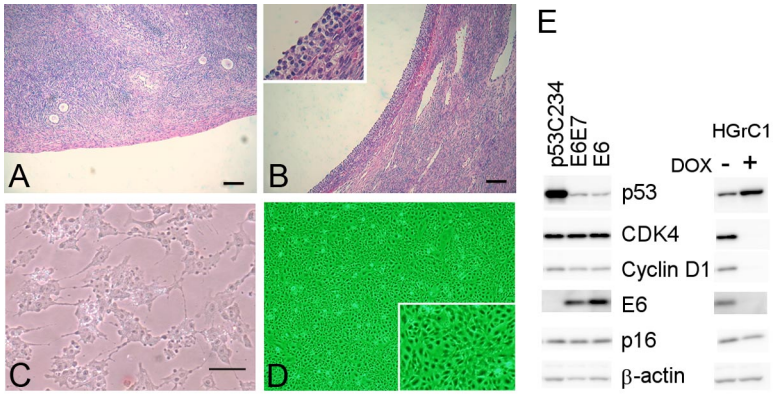


**FIG. 1.** Establishment of the HGrC1 cells. A normal ovarian cortex, including primordial, primary, and secondary follicles (A) and a developing follicle with healthy granulosa cell layers (B) of the ovary from which the HGrC1 cells were established. The primary cultured human granulosa cells before oncogenic transformation (C) and the HGrC1 cells after immortalization (D). *Scale bars*, 25 μm. E, Western blotting confirmed expression of the transgenes in the resultant cell populations and showed down-regulation and accumulation of endogenous p53 by HPV16 E6 and dominant-negative p53 (p53C234), respectively. HGrC1 cells treated with 1 μg/ml of doxycycline or vehicle (70% ethanol) for 2 d were similarly subjected to Western blotting. DOX, Doxorubicin; FK, forskolin.

## Results

### Immortalization of primary human granulosa cells

Human non-LGC were obtained from the intact contralateral ovary of an ovarian cancer patient. Figure 1 shows that the ovary from which the cells are derived contains a normal ovarian cortex (Fig. 1A) and developing follicles with healthy granulosa cell layers (Fig. 1B). The primary cells seeded in a six-well dish (Fig. 1C) were infected with CSII-CMV-Tet-Off, CSII-TRE-Tight-hTERT, CSII-TRE-Tight-CyclinD1, and CSII-TRE-Tight-CDK4R24C lentiviruses at the multiplicity of infection of more than 5 on the third day. Growing cells were split into 12 wells of a 24-well plate on d 7 and subsequently infected with CSII-TRE-Tight-16E6E7, CSII-TRE-Tight-16E6, or CSII-TRE-Tight-p53C234. On d 20 when only a few colonies consisting of fibroblastic cells were ob-

served in the wells inoculated with viruses expressing CDK4R24C, cyclin D1, and hTERT alone, many healthy colonies consisting of small round-shaped cells emerged in these cultures and were passaged into larger dishes. At passage 4, cells were harvested, and the expression of representative transgenes was confirmed by immunoblotting (Fig. 1E, *left panel*). Among them, the cells expressing 16E6E7 were named HGrC1 and then were further characterized. Because these transgenes are expressed under the control of tet-responsive promoter, addition of doxycycline terminated their expression and induced growth arrest of the cells, indicating that HGrC1 cells are indeed immortalized by these transgenes (Fig. 1E, *right panel*). The HGrC1 cells grew as an adherent monolayer until confluence and then proliferated in multilayers after they reached confluence. The cells demonstrate a round or polygonal shape (Fig. 1D) like epithelial cells, and colony formation was noted during the low-cell density state. The estimated doubling time of the cells was approximately 40 h *in vitro*. Further experiments were performed during the tenth to thirtieth passages unless otherwise noted.

## Expression of the steroidogenesis-related molecules

We next performed RT-PCR using HGrC1 cells to assess the expression of the steroidogenesis-related genes (Fig. 2). RT-PCR demonstrated that the HGrC1 cells express the enzymes related to steroidogenesis in the ovaries, including StAR, CYP11A, and aromatase. CYP17, which catalyzes the conversion of 17$\alpha$-hydroxy pregnenolone and 17$\alpha$-hydroxy progesterone to dehydroepiandrosterone and androstenedione, respectively, was expressed in primary cultured LGC obtained from oocytes retrieved during the IVF program but not in the HGrC1 cells or granulosa cell tumor-derived KGN cells. Gonadotropin receptors and ER were also expressed by HGrC1 cells. FOXL2, GATA4, and SF-1, which are significant transcriptional factors in granulosa cells, were expressed by HGrC1 cells.

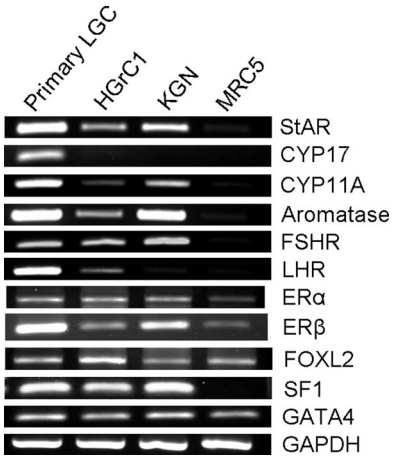


**FIG. 2.** The expression levels of steroidogenesis-related enzymes determined by RT-PCR in HGrC1 cells, primary cultured LGC, KGN cells, and MRC5 human fetal lung fibroblasts (as a negative control). StAR, CYP11A, aromatase, the FSHR, LHR, ER$\alpha$, ER$\beta$, FOXL2, SF-1, and GATA4 were expressed by the HGrC1 and KGN cells. CYP17 was expressed by primary LGC cells but not the HGrC1 cells.

## Induction of steroidogenic activity and aromatase expression in HGrC1 cells

The steroidogenic activities of the HGrC1 cells were evaluated by examining the sex steroid concentration in the culture medium and performing a quantitative analysis of the aromatase mRNA expression. With the addition of

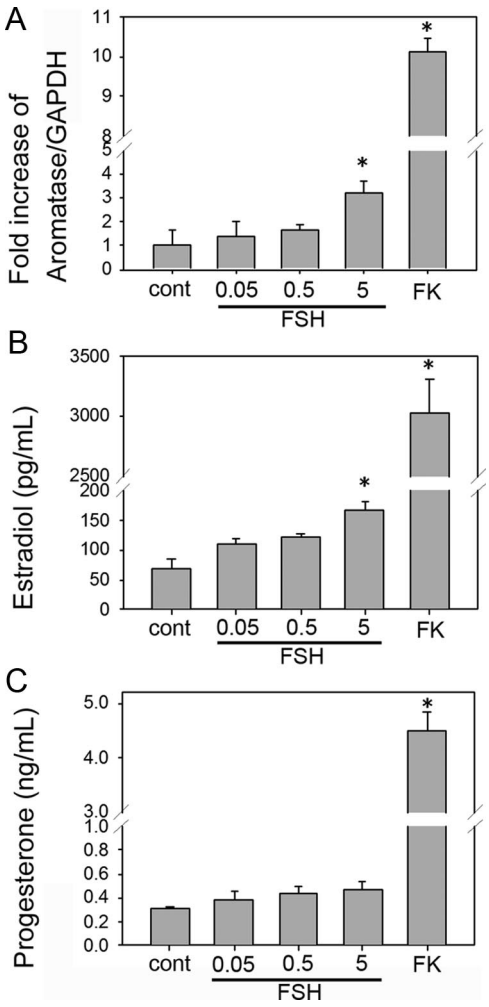


**FIG. 3.** The steroidogenic activities of the HGrC1 cells. A, The quantitative analysis of the mRNA expression showed that FSH stimulated the expression of aromatase in a dose-dependent manner. B and C, The concentrations of sex steroids in the culture medium. After 10 $\mu$M androstenedione was added in the culture medium, the cells were stimulated with 0.05, 0.5, or 5 IU/ml human recombinant FSH or 50 $\mu$M forskolin for 48 h. The concentrations of estradiol and progesterone in the medium were determined with a chemiluminescent immunoassay. The data are shown as the means $\pm$ SD. Four independent experiments showed similar results. *, $P < 0.05$ *vs.* control. FK, Forskolin.

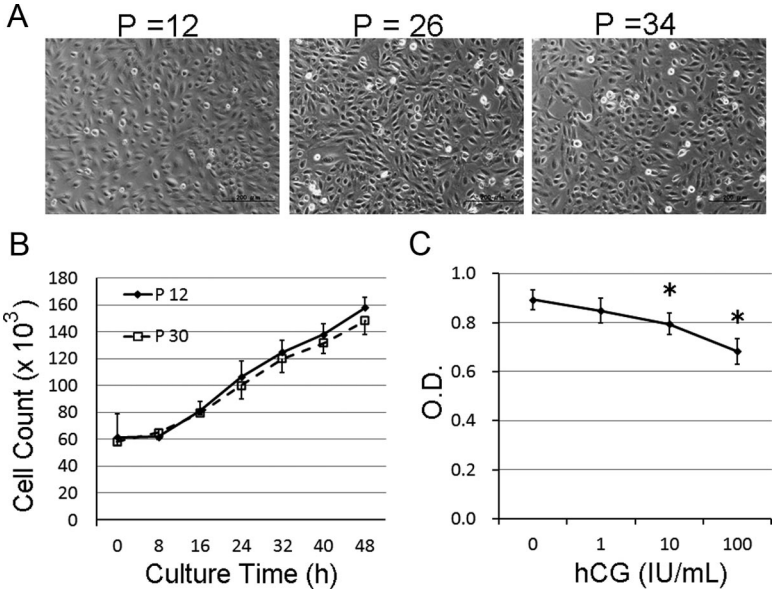


**FIG. 4.** A, A morphological analysis of the HGrC1 cells during extended culture. No remarkable morphological differences was found among the twelfth ($P = 12$), twenty-sixth ($P = 26$), and thirty-fourth ($P = 34$) passages. B, An analysis of the number of cells by counting every 8 h in HGrC1 cells of twelfth and thirtieth passages. No significant differences were found in the cell proliferation. C, BrdU proliferation assay. The incorporation of BrdU was measured using an ELISA system. Treatment with hCG attenuated the DNA synthesis in a dose-dependent manner. The data are presented as the means ± SD. Three independent experiments showed similar results. *, $P < 0.05$ *vs.* control.

androstenedione in the culture medium, estradiol was detectable in the medium of the HGrC1 cells after 48 h of culture, and the concentration was increased by stimulation with FSH and forskolin (Fig. 3A). The aromatase mRNA levels were also increased by FSH and forskolin (Fig. 3B). The production of progesterone from HGrC1 cells was induced by forskolin, whereas FSH stimulation did not lead to any significant increase in the progesterone levels (Fig. 3C).

### Analysis of the changes in the morphology, proliferation, and differentiation of HgrC1 cells

HGrC1 cells have been cultured for over 40 passages and for 1 yr. No obvious morphological changes were found during the extended culture (Fig. 4A). The proliferation assay demonstrated no significant changes between the twelfth passage and the thirtieth passage (Fig. 4B). The forskolin-induced production of progesterone from HGrC1 cells did not show any significant changes (data not shown).

Human granulosa cells gradually lose their proliferation capacity and then differentiate during the luteinization process. Therefore, this study explored whether hCG stimulation affects proliferation in HGrC1 cells. Stimulation with 1 or 10 IU/ml hCG decreased the DNA synthesis by approximately 11 or 24% in HGrC1 cells, respectively (Fig. 4C).

### Induction of the FSHR with activin and FSH

During folliculogenesis, follicular growth develops from the gonadotropin-independent stage to a gonadotropin-dependent stage. This transition is considered to be important for the changes in the expression levels of the FSHR in the granulosa cells. Therefore, we investigated the induction of the FSHR in HGrC1 cells. Activin A is thought to be one of the key molecules responsible for the induction of the FSHR (24, 25). The quantitative RT-PCR analysis demonstrated that activin A induced the expression of the FSHR mRNA in a time-dependent manner (Fig. 5A). In addition, FSH stimulation after pretreatment with activin A led to a synergistic induction of the FSHR mRNA (Fig. 5B).

### Expression of TGF-$\beta$ superfamily members and their receptors, and Smad signaling

Recent studies have demonstrated that the TGF-$\beta$ superfamily is involved in folliculogenesis, including the assembly of primordial follicles and follicle growth by autocrine and paracrine regulation under bidirectional communication among oocytes and somatic cells (26, 27). Therefore, we examined the expression of the ligands in the TGF-$\beta$ superfamily and their receptors, as well as the signaling pathways downstream of some ligands in the TGF-$\beta$ superfamily, to further characterize our immortalized cells. The RT-PCR analysis showed that the TGF-$\beta$ superfamily members and their receptors, such as BMP, GDF-9, AMH, inhibin subunits, and type I and II receptors of the TGF-$\beta$ superfamily, were expressed by HGrC1 cells (Fig. 6A). A Western blot analysis using an antibody to phosphorylated Smad revealed that BMP-4, BMP-6, and BMP-7 phosphorylated Smad1/5/8, whereas GDF-9 phosphorylated Smad2/3. AMH weakly phosphorylated both Smad1/5/8 and Smad2/3. BMP-15 strongly phosphorylated Smad1/5/8 and weakly phosphorylated Smad2/3 (Fig. 6B).

## Discussion

Recently, CDK4R24C and cyclin D1, which phosphorylate pRB so as to inactivate it, and hTERT have been used to immortalize human ovary surface epithelial cells, myoblasts, and tongue and dermal keratinocytes without inducing genomic abnormality (21, 28, 29). Based on the

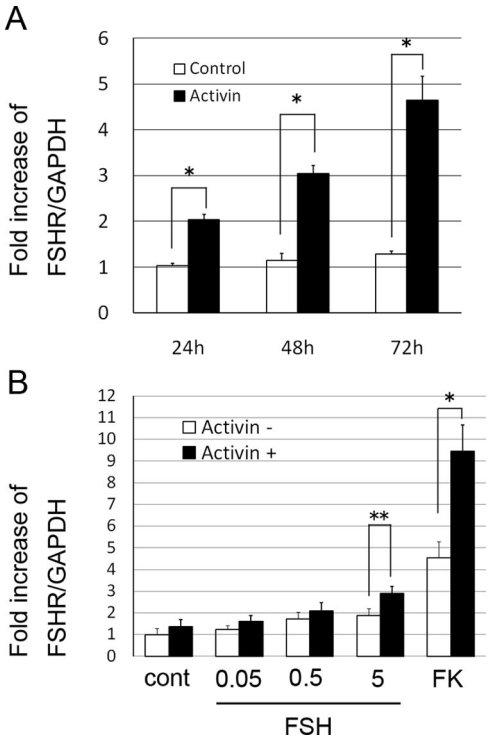


**FIG. 5.** The expression levels of the FSHR. A, The expression of the FSHR was increased in a time-dependent manner by stimulation with (*black bars*) 50 ng/ml activin A for 24, 48, and 72 h. Cells not stimulated with activin A are indicated by *white bars*. B, Stimulation with 0, 0.05, 0.5, or 5 IU/ml human recombinant FSH with (*black bars*) or without (*white bars*) pretreatment with 50 ng/ml activin A showed that FSH stimulation with activin A pretreatment had synergistic effects on the induction of FSHR mRNA, as determined by RT-PCR. The data are shown as the means ± SD. Four independent experiments showed similar results. *, $P < 0.01$; **, $P < 0.04$.

results, we first transduced CDK4R24C, cyclin D1, and hTERT into primary non-LGC, but it was not sufficient to immortalize them. With additional transduction of HPV16 E6E7, E6, or p53C234, we could extend their life span. Although we further characterize HGrC1 cells that express HPV16 E6E7, we speculate that inactivation of the p53 pathway was required to immortalize the cells in our culture conditions. It is not ideal to inactivate p53 to immortalize normal human cells, because it could induce genetic instability. However, unlike the cells immortalized by simian virus 40 and/or v-Ha-ras Harvey rat sarcoma viral oncogene homolog (HRas), HGrC1 cells are nontransforming and well retain functional features of original human non-LGC.

HGrC1 cells express steroidogenesis-related enzymes, such as StAR, CYP11A, and aromatase. On the other hand, CYP17, the expression of which is predominantly limited to theca cells, was not expressed by HGrC1 cells. These results are consistent with the properties of granulosa cells of developing follicles *in vivo*. The RT-PCR analysis also demonstrated that the HGrC1 cells expressed FSHR and LHR. In addition, we found that FSH and forskolin up-regulated the expression of aromatase mRNA and induced the aromatization of androstenedione to estradiol in the HGrC1 cells. Taken together, our data indicated that FSHR and steroidogenesis-related enzymes are all functional in HGrC1 cells. With regard to progesterone, forskolin increased the secretion of progesterone in the culture medium, whereas FSH and hCG stimulation were associated with a very low level of progesterone production (data not shown). The proliferation capacity of HGrC1 cells decreased with hCG stimulation. Human granulosa cells decrease proliferation capacity and then differentiate into luteal cells during the luteinization process, which is mainly regulated by LH. The decrease in the proliferation of HGrC1 cells with hCG might be weakly associated with the low expression level of LHR. All things considered, these results suggest that HGrC1 cells, which are not luteinized, have the potential to cease proliferation and produce progesterone, once luteinization occurs.

Another characteristic of the HGrC1 cells is that the FSHR was induced by stimulation with activin A. FSH has an obligatory role in the production of oocyte and sex steroid hormones in the ovary. In the absence of FSH, follicular growth is arrested, and the female is infertile. Follicle growth therefore acquires FSH dependency during folliculogenesis (30), and FSH acts exclusively on granulosa cells via the FSHR (31). Therefore, the expression and induction of FSHR in granulosa cells has been considered to be significant for the transition from FSH-independent to FSH-dependent growth. We demonstrated that the FSHR was induced by activin A in the HGrC1 cells and that this was enhanced by the presence of FSH. It is not fully understood how the FSHR in granulosa cells is expressed and induced. So far, activin A has been considered to be one of the key molecules responsible for the induction of the FSHR (24, 25). Furthermore, previous reports demonstrated the possible involvement of estradiol and FSH itself in induction of the FSHR (32, 33). HGrC1 cells might be a helpful tool to study the expression and induction of the FSHR in human granulosa cells.

For humans, the granulosa cells available for *in vitro* studies were derived from patients undergoing IVF. Although the cells obtained from IVF are useful to some extent, there exist some limitations to the experiments that can be performed using these cells. First, the cells obtained from IVF patients are fully luteinized or in the luteinization process due to the administration of hCG before oocyte retrieval. Although the effect of hCG might be eliminated by means of culture in medium without hCG, hCG has a major influence on the differentiation of granulosa cells into luteinized cells, which includes effects on significant molecules related to intracellular signaling pathways and

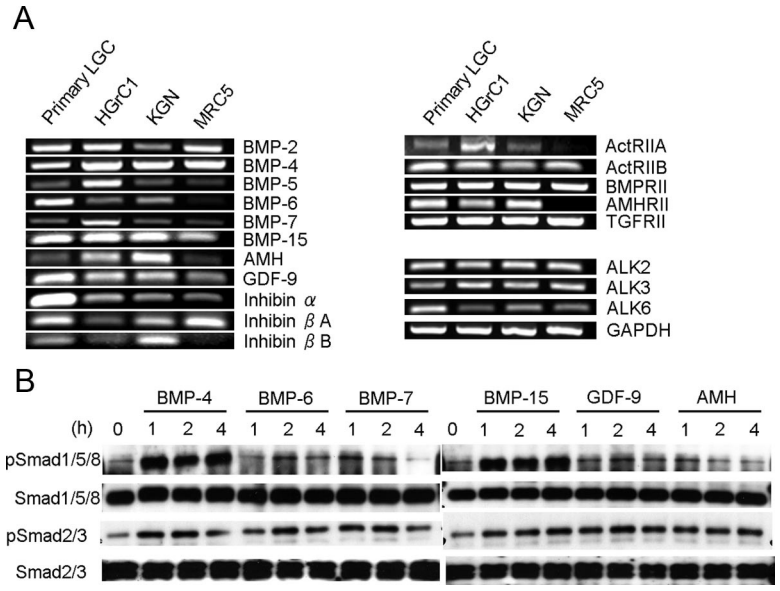


**FIG. 6.** A, The RT-PCR analysis for the expression of BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-15, AMH, GDF-9, inhibin subunits, and type I and type II receptors of the TGF-$\beta$ superfamily mRNA by the HGrC1 cells. B, The effects of BMP-4, BMP-6, BMP-7, BMP-15, GDF-9, and AMH on the levels of Smad2/3 and Smad1/5/8 phosphorylation. The HGrC1 cells were cultured with BMP-4 (100 ng/ml), BMP-6 (1 $\mu$g/ml), BMP-7 (1 $\mu$g/ml), BMP-15 (500 ng/ml), GDF-9 (2 $\mu$g/ml), and AMH (1 $\mu$g/ml) for 0, 1, 2, or 4 h. Samples were subjected to SDS-PAGE and an immunoblot analysis using an antiphospho-Smad1/5/8 Ab, anti-Smad1/5/8 Ab, antiphospho-Smad2/3 Ab and anti-Smad2/3 Ab. BMP-4 mainly phosphorylated Smad1/5/8. BMP-6 and BMP-7 also phosphorylated Smad1/5/8, which is rather weak. AMH weakly phosphorylated both Smad1/5/8 and Smad2/3. GDF-9 phosphorylated Smad2/3. BMP-15 strongly phosphorylated Smad1/5/8 and weakly phosphorylated Smad2/3.

cell cycle regulation (34). Second, each follicle contains only a limited number of granulosa cells. Therefore, granulosa cells are usually pooled from several follicles, which might contain granulosa cells of different profiles. In addition, the limited number of cells makes it impossible to repeat experiments, making it necessary to perform experiments in cells from different patients. Another source of granulosa cells used for *in vitro* studies has been experimental animals. However, it should be noted that there may be species-specific differences in the granulosa cell function, *e.g.* nonfunctioning mutations of the BMP-15 gene cause infertility in sheep (35), whereas mice with null mutations of the BMP-15 gene show normal follicle development (36). Taken together, the use of homogenous granulosa cells derived from human ovarian follicles, which are not atretic or luteinized, is highly desired to improve research on steroidogenesis and folliculogenesis.

The immortalization of primary cultured human granulosa cells would be one solution to overcome these problems. After the establishment of immortalized granulosa cells of rodent, bovine, and porcine origin, several human granulosa cell lines were established. The HTOG cell line was first established from a granulosa cell tumor. However, the gonadotropin responsiveness was not tested, although the cells possessed aromatase activity (9). Other cell lines derived from granulosa cell tumors, such as COV434 and KGN, showed responses to FSH. In particular, the KGN cells showed a strong and dose-dependent response to FSH, whereas the cells lacked the response to hCG (10). Therefore, KGN cells have been widely used for experiments about steroidogenesis. However, there may be some problems associated with the use of KGN cells for experiments that investigate the proliferation and apoptosis of granulosa cells during normal folliculogenesis because of its origin of their neoplastic origin. This may have occurred because KGN showed a missense mutation in *FOXL2* (15), which is involved in the proliferation and differentiation of granulosa cells.

The other source of immortalized human granulosa cells is from oocyte retrieval during IVF. HGL5 and HO-23 cells isolated from such oocytes showed progesterone synthesis. However, these two cell lines were not responsive to FSH or hCG (12, 13). The HGP53 cell line, which was established by transfection of cells with mutated *p53* and *HRas* genes, showed a FSH-responsive increase in progesterone synthesis (14). However, these cells failed to demonstrate a FSH-responsive increase of aromatase expression or estradiol synthesis. Therefore, it is not clear whether the HGP53 cell line is truly useful as an experimental model of preovulatory follicles. In addition, the LGC obtained from IVF would have the same problems. It would be difficult to verify that our HGrC1 cells possess all of the characteristics of human non-LGC at each stage of developing follicles. However, our immortalization, which was achieved by avoiding the oncogenic transformation by simian virus 40 and/or HRas, and our results showing both proliferation and steroidogenesis under gonadotropin regulation suggest that HGrC1 cells retain the original granulosa cell character and function.

Accumulating evidences suggests that the TGF-$\beta$ superfamily members expressed by ovarian somatic cells and oocytes are important for the intraovarian control mechanisms, including the recruitment and progression of follicles (26). However, the intracellular signals downstream BMP, GDF-9, and other TGF-$\beta$ superfamily molecules have not been fully elucidated. We herein demonstrated that Smad1/5/8 and/or Smad2/3 were phosphorylated with BMP-4, BMP-5, BMP-7, BMP-9, BMP-15, GDF-9, and AMH in the HGrC1 cells. The subclasses of Smad phosphorylated by TGF-$\beta$ superfamily in HGrC1 cells are mostly consis-

tent with previous reports (37, 38). However, our results showed that Smad2/3 was weakly phosphorylated with BMP-15 and AMH. The signaling pathways under type I and II receptors of TGF-$\beta$ superfamily should therefore be explored in HGrC1 cells by comparison with primary-cultured granulosa cells and other cell lines. On the other hand, recent progress in the research on the intracellular signaling pathways has revealed several significant characteristics of granulosa cells. For example, we previously demonstrated that phosphatase and tensin homolog deleted on chromosome 10 induced with hCG or insulin attenuated the phosphorylation of serine-threonine kinase, but not ERK, and thus attenuated the IGF-I-induced cell proliferation (5, 6). Moreover, Fan *et al.* (39) reported that the selective disruption of phosphatase and tensin homolog deleted on chromosome 10 in mouse granulosa cells increased the granulosa cell proliferation and ovulation, whereas the selective disruption of ERK1/2 attenuated the LH-induced oocyte resumption of meiosis and luteinization (40). All things considered, the HGrC1 cells that we established might be useful to explore the effects of the TGF-$\beta$ superfamily and intracellular signals of human granulosa cells during folliculogenesis.

In conclusion, we established an immortalized human granulosa cell line, which shows FSH-responsive aromatase activity, inducible FSHR expression, and responsiveness to the TGF-$\beta$ superfamily. Further *in vitro* studies using this cell line would be helpful to reveal the intrafollicular mechanisms of the human ovary, including the recruitment, progression, and atresia of follicles.

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Address all correspondence and requests for reprints to: Dr. Akira Iwase, Department of Obstetrics and Gynecology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. E-mail: akiwase@med.nagoya-u.ac.jp; or Dr. Tohru Kiyono, Division of Virology, National Cancer Center Research Institute, 5-1-1 Tsukiji, Tyuo-ku, Tokyo 104-0045, Japan. E-mail: tkiyono@ncc.go.jp.

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