Maintenance of In Vitro Granulosa Cell Function by Adenoviral Mediated Follicle Stimulating Hormone Receptor Gene Transduction

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Purpose: To maintain in vitro granulosa cell function by adenoviral-mediated FSHR gene transduction.

Methods: Rat granulosa cells were cultured and transduced with adenovirus carrying FSHR gene. The number of receptors and the rate of steroidogenesis were assessed.

Results: The number of FSHR on the granulosa cells was 4,874 per cell immediately after extraction, it was 2,176 by the third day, and had further reduced to 693 by the seventh day. On the third day of culture, the amount of production of estradiol by FSH stimulation also decreased to about one-quarter of the first day's quantity. Compared to the untransduced granulosa cells, when the cells contained FSHR gene, the FSHR expression and steroidogenesis were both enhanced (2,176 vs. 7,206 per cell ($p < 0.001$) and 192 vs. 5940 pg/mL ($p < 0.01$), respectively).

Conclusion: Granulosa cell functions can be maintained or increased by novel gene therapy. This can be a useful component of assisted reproductive therapy.

KEY WORDS: Adenoviral vector; FSH receptor; granulosa cell; in vitro maturation.

INTRODUCTION

In vitro maturation (IVM) of cumulus–oocyte complexes (COC) potentially offers many advantages in assisted reproductive technology, as it would reduce the cost of treatment; decrease the risk of ovarian hyperstimulation syndrome; and increase the number of matured oocytes for fertilization.

To date, several successful births of normal babies have been achieved by in vitro matured human oocytes (1), although the implantation rate of embryos derived from in vitro matured oocytes re-

mains low when compared with in vivo matured oocytes (2).

In mammalian species, oocytes retrieved from the antral follicles are capable of undergoing spontaneous nuclear maturation, but in general fail to complete cytoplasmic maturation (3). Establishment of the optimal culture conditions to induce synchronized nuclear and cytoplasmic maturation of oocytes is required.

Granulosa cells within the developing follicle play a crucial role in providing a suitable microenvironment for the maturation of oocytes; therefore coculture with granulosa cells may improve the developmental competence of in vitro maturing oocytes. Unfortunately, granulosa cells rapidly lose FSH receptor and steroidogenesis capability in tissue culture conditions (4).

In this study, we introduced the follicle stimulating hormone receptor (FSHR) gene into primary

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rat granulosa cells by replication-deficient adenoviral vector to maintain their response to FSH.

MATERIALS AND METHODS

Preparation of Culture Medium and Cell Culture

The primary culture of rat granulosa cells was done with Dulbecco's modified Eagle medium (DMEM, Immuno-Biological Laboratories, Fujioka, Japan) supplemented with 0.01% bovine serum albumin (BSA, Sigma, St. Louis, MO). Rat granulosa cells were cultured on plates coated with 20 µg/mL E-C-L Cell Attachment Matrix (Upstate biotechnology, Lake Placid, NY). To control for the influence of FSH and estrogen production, cells from COLO320 human colon adenocarcinoma cell line were used (5). These cells do not express the FSH receptor. They were cultured with DMEM supplemented with 10% fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS).

Both of the cells were cultured in a humidified atmosphere containing 5% carbon dioxide (CO₂) and 95% air at 37°C.

Laboratory Animals

Immature female Wistar rats (21 days old) were purchased from Charles River, Japan (Yokohama, Japan) and maintained under pathogen-free environment. These animals were given an injection of 2 mg diethylstilbestrol (Sigma) in 0.1 mL sesame oil (Kanto Chemical, Tokyo, Japan) once daily for 4 days to stimulate the development of multiple immature follicles. Two days later, the animals were sacrificed by CO₂ inhalation (6), followed by cervical dislocation. The ovaries were then rapidly removed for granulosa cell isolation.

Granulosa Cell Isolation and Culture

Granulosa cells were released by puncturing surface follicles on the ovaries with a hypodermic needle. The cell number was counted with a hemocytometer under a phase-contrast microscope and viability was assessed using trypan blue dye exclusion. The granulosa cells were then cultured in DMEM on E-C-L Cell Attachment Matrix coated plates in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C (7).

Receptor Binding Assay

Rat granulosa cells were cultured on Multiscreen 96-well filtration plates and (Millipore, Bedford, MA) coated with E-C-L. Each well contained 10⁵ cells in 0.1 mL DMEM with 0.01% BSA. After an appropriate incubation period, FSH binding sites were measured by Radio Receptor Assay (RRA) on whole cultured cells using ¹²⁵I-FSH, with a specific activity of 2732–5529 Ci/mmol, purchased from NEN Life Science Products (Boston, MA).

The granulosa cells were incubated for 1 h at 37°C in 100 µL assay buffer, which consisted of 0.1% BSA and 0.1% sodium azide in phosphate buffered saline (pH 7.4), with an increasing amount of ¹²⁵I-FSH. Fertinorm P (Serono Laboratories, Aubonne, Switzerland) was added as unlabeled FSH to determine nonspecific binding. The incubation was terminated by the addition of 150 µL of ice-cold assay buffer. The unbound ¹²⁵I-FSH was separated from bound ¹²⁵I-FSH by centrifuging the filtration plates at 2000 rpm. Then the cells were successively and rapidly washed three times with 200 µL of ice-cold assay buffer. The filters were punched out and the amount of radioactivity was measured with the Auto Well Gamma System ARC-2000 (Aloka Co., Ltd., Mitaka, Tokyo).

Total binding was defined as the binding of ¹²⁵I-FSH without unlabeled FSH, and nonspecific binding was defined as the binding of ¹²⁵I-FSH, which was nondisplaceable with 1000-fold excess of unlabeled FSH. The specific binding was defined as the difference between the total binding of ¹²⁵I-FSH and the nonspecific binding. Equilibrium saturation curves were determined by incubation with increasing amounts of ¹²⁵I-FSH (200–1600 pM). All samples were assayed in triplicate wells and the results were processed in a Scat chard plot. The parameters *K_d* and *B_{max}* were determined for each experiment by fitting the data to the single-site binding equation ($B = B_{\max} \times L / (K_d + L)$), where *B* is specific binding at free ¹²⁵I-FSH concentration (*L*) using nonlinear least-square regression method).

Assays

Concentrations of estradiol in the culture medium were measured by radioimmunoassay using DPC estradiol kit (Diagnostic Products Corporation, Los Angeles, CA) in the presence of 4-androstene-3,17-dione (Sigma) as the substrate. Concentrations of cAMP were measured by radioimmunoassay using

the YAMASA RIA kit (Yamasa Shoyu, Choshi, Japan).

Construction of hFSHR Recombinant Adenovirus

The cDNA encoding the human FSHR (2393 bp) was kindly provided by Dr. Takashi Minegishi (Gunma University, Gunma) (8). Replication-defective adenovirus vector containing FSHR cDNA (AxCAFSHR) under CAG promoter was used for this study. AxCAFSHR was constructed by the COS-TPC method using an Adenovirus Expression Vector Kit (Takara Shuzo, Otsu, Japan) according to the manufacturer's instruction (9). The infectivity of recombinant adenoviruses was measured by an end-point cytopathic effect assay, and the 50% tissue culture infection dose (TCID₅₀) was calculated (10). In our series, TCID₅₀ approximately corresponds to plaque forming units (pfu). The titer of AxCAFSHR after final propagation was 4.5×10^9 pfu/mL. These studies were permitted by Niigata University recombinant DNA experiments safety committee.

Adenovirus Infection

Granulosa cells and COLO320 cells were seeded into 96-well (10^5 cells per well) tissue culture plates and allowed to attach overnight. The next day, medium and unattached cells were removed and the remaining cells were exposed to adenoviruses at MOI 100 (COLO320 cells) or MOI 10 (granulosa cells) in 40 μ L of medium for 1 h at 37°C with occasional rocking. A total of 160 μ L of fresh medium was added to each well and the wells were appropriately incubated.

Preparation of Probes

Probe for FSHR was generated by polymerase chain reaction (PCR) using murine genomic DNA as a template. The sequence of forward primer was 5'-GCAGGCTGATGCTGCTGGC-3' and the sequence of reverse primer was 5'-GGCACCTTGAGGGAGGCAGAAAT-3'. The PCR product (418 bp) was separated by electrophoresis on 1% agarose gels, and extracted from the gel using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The purified products were labeled with ³²P using the Rediprime II DNA Labeling System (Amersham Pharmacia Biotech, Buckinghamshire, England). The cDNA probe of beta-actin was labeled in the same way.

RNA Blot Analysis

Total RNA was extracted from COLO320 cells or granulosa cells by the guanidinium acid-thiocyanate-phenol chloroform method (11) using ISOGEN (NIPPON GENE, Tokyo, Japan). Ten micrograms of total RNA was separated by electrophoresis on denaturing agarose gels for the Northern blot analysis. This was subsequently transferred onto a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech). Northern blots were hybridized overnight at 45°C with the ³²P-labeled FSHR or beta-actin probe. The membrane was rinsed three times in 3 $\times$ SSC and exposed to Kodak BioMax MS film using intensifying screens, TranScreen HE (Eastman Kodak, Rochester, NY).

RESULTS

In Vitro FSHR Expression and Estradiol Production of the Granulosa Cells

Rat granulosa cells immediately after extraction expressed 4874.2 ± 1753.8 /cell FSHR. The number of receptors decreased as time passed (2176.0 ± 1288.8 , 827.9 ± 351.7 , and 692.6 ± 188.8 on the third, fifth, and seventh day of culture, respectively) without any addition of hormones (Fig. 1).

Fig. 1. The number of FSHR expressed on rat granulosa cells on days 0, 3, 5, and 7 of culture. Data shown are the mean of three experiments.

Fig. 2. The amount of estradiol production of rat granulosa cells on days 0, 3, 5, and 7 of culture. After washing plate, 50 ng/mL of androstenedione (■), which was a substrate for aromatase, or androstenedione plus 100 mIU/mL of FSH (●) were added to the culture medium. The medium was collected 48 h after the addition of hormones for estradiol assay. Data shown are the mean of three experiments.

On day 0, FSH stimulated estradiol production in granulosa cells, but the production of estradiol decreased with time in culture and on day 5, the significant response of estradiol production was not observed by FSH stimulation (Fig. 2).

Expression of Functional FSHR in COLO320 Cells

To confirm the function of the FSHR transduced by adenoviral vector that we constructed, FSHR was introduced into COLO320 cells. Specific ^{125}I -FSH binding was observed in the FSHR transduced COLO320 cells. On the other hand, the labeled FSH did not bind to the cells that lacked the FSHR (Fig. 3). As shown in Fig. 4, RNA blot analysis indicated the expression of the 2.5 kb mRNA of FSHR in total RNA prepared from COLO320 transfected the FSHR gene.

Since there is no aromatase for the production of estrogen in COLO320 cells, the quantity of cAMP production was measured in order to evaluate the function of the introduced FSHR (Fig. 5). Non-transduced COLO320 cells did not produce cAMP in response to FSH because they lacked FSHR. In

Fig. 3. ^{125}I -FSH specific binding to COLO320 cells. COLO320 cells were seeded into 96-well (10^5 cells per well) tissue culture plates and allowed to attach overnight. The next day, FSHR was introduced into COLO320 cells by the above-mentioned method. Forty-eight hours after exposure to adenoviruses, RRA was performed (●). As a control, the cells were incubated with the virus free culture medium (■).

FSHR transduced COLO320 cells, FSH clearly stimulated cAMP production (1220.0 ± 99.0 pmol/mL).

FSH Binding Assay and RNA Blot Analysis for FSHR Infected Rat Granulosa Cells

Rat granulosa cells were seeded into 96-well (10^5 cells per well) E-C-L coated plates and allowed to attach overnight. Transfection and RRA were carried out as described for COLO320 cells above. The number of receptors was calculated from Scatchard analysis (Fig. 6). Although the number of receptors expressed on the surface of the noninfected cells was 2176.0 ± 1288.8 /cell, transfected cells expressed an increase in the number of receptors by the third day 7206.3 ± 2407.1 /cell. This increase was statistically significant ($p < 0.001$). As shown in Fig. 7, 2.4 kb of endogenous FSHR mRNA was detected in the total RNA prepared from rat granulosa cells immediately after sacrifice. Subsequently, the FSHR mRNA level was markedly reduced after 72 h. A remarkable increase of the 2.5 kb mRNA from introduced FSHR gene was noted. This increase was detected by the third day.

Fig. 4. RNA blot analysis of transduced FSHR gene expression in COLO320 cells. The samples were transferred to a nylon membrane and hybridized to probes for FSHR or beta-actin. The probes of FSHR were obtained by PCR from the murine DNAs. The migrating positions of the 28S and 18S ribosomal RNAs are indicated.

FSH-Stimulated Estradiol Production from FSHR Transfected Rat Granulosa Cells

Since there is aromatase for the production of the estrogen in granulosa cells, the function of the introduced FSHR gene in the presence of androstenedione (50 ng/mL) as substrates was assessed by measuring the quantity of estradiol production. The amount of estradiol production was significantly higher in the infected cells compared with uninfected cells on the third day of culture (5939.8 ± 2369.1 vs. 191.6 ± 205.5 pg/ml, $p < 0.01$) (Fig. 8).

DISCUSSION

In mammalian species, immature oocytes are capable of undergoing spontaneous nuclear maturation in vitro, and then normal fertilization and cleavage. Unfortunately, embryos derived from in vitro matured oocytes reveal the reduced implantation potential when compared with in vivo matured oocytes.

The establishment of a culture condition that supports cytoplasmic maturation is required for subse-

Fig. 5. cAMP production of COLO320 cells transfected the FSHR gene or cells without transfection. Cells were seeded to 96-well plates and incubated for 24 h at 37°C in the presence or absence of 100 mIU/mL FSH. All experiments were performed in the presence of IBMX. The data are mean $\pm$ SD of triplicate determinations. Only the cells by which FSHR gene was introduced were highly responsive to FSH.

quent fertilization. Many culture conditions for IVM of immature oocytes have been developed and published (12).

Janssenswillen *et al.* showed that co-culture with Vero cells improved the in vitro maturation of human GV-stage oocytes (13). Granulosa cells play a crucial role for complete maturation of the oocyte by secreting a wide variety of growth factors. Therefore, co-culture of granulosa cells could promote the synchronous nuclear and cytoplasmic maturation of oocytes. In the cumulus-intact oocytes matured in vitro, the rate of normal fertilization and cleavage were higher than similar rates in cumulus denuded oocytes (14). Dandekar *et al.* reported that 44% of immature oocytes were fertilized after co-culture with granulosa cells compared with 20% fertilization rate in immature oocytes cultured without granulosa cells (15). Shramm and Bavister demonstrated that the development into morulae and blastocysts was improved by co-culture with granulosa cells compared with no granulosa cells (16). Granulosa cells obtained from antral follicles rapidly lose the expression of FSHR and steroidogenesis function in primary culture conditions. In this study, we

showed that the number of FSHR on granulosa cells decreased with time in culture. In addition, by the fifth day, the estradiol production was not observed in response to FSH. Therefore co-culture with granulosa cells has a limited role in helping to achieve satisfactory rates of oocyte maturation for clinical application.

In this study, we introduced the FSHR gene into primary rat granulosa cells by adenoviral vector to maintain their response to FSH. We developed the replication-deficient adenoviral vector (AxCAFSHR) expressing FSHR under a constitutive promoter. We showed that COLO320 cells transduced FSHR gene by AxCAFSHR express functional FSHR on their cell surface. The adenovirus vector we constructed was able to efficiently direct the expression of exogenous protein in primary cultured granulosa cells confirmed by the expression of b-galactosidase in more than 80% of the cells.

When the granulosa cells were infected with AxCAFSHR on the first day of culture, the decrease of the expression of the FSHR was significantly restored in the infected cells compared with the uninfected cells on the third day of culture ($p < 0.001$) (Fig. 6). Also we confirmed the strong expression of the transduced FSHR gene in the infected cells by Northern

Fig. 6. Number of FSHR on rat granulosa cell on the third day of culture. The FSHR gene was introduced into cultured granulosa cells on the first day of culture, and then FSHR expression was assayed by RRA 48 h later.

Fig. 7. RNA blot analysis of FSHR gene expression in the cultured rat granulosa cells. Lane 1, rat granulosa cells cultured in vitro for 0 h; lane 2, 72 h; lane 3, rat granulosa cells transduced the FSHR gene. The 2.4 kb band indicates endogenous FSHR mRNA and the 2.4 kb indicates mRNA from transduced FSHR. The migrating positions of the 28S and 18S ribosomal RNAs are indicated.

blot analysis (Fig. 7). The uninfected cells had almost completely lost the expression of endogenous FSHR gene by the third day of culture. Furthermore, the amount of estradiol production was significantly higher in the infected cells than in the uninfected cells on the third day of culture (Fig. 8).

FSH induces the expression of aromatase and the production of estradiol in granulosa cells. cAMP is the principal mediator of FSHR signals. Indeed, 8-bromo-cAMP (Br-cAMP) is able to induce the expression of aromatase by itself (17). Therefore the function of granulosa cells could be maintained by the addition of Br-cAMP to the culture medium. But cAMP is not solely responsible for FSHR signal transduction (18). Hence we introduced the FSHR gene into granulosa cells to retain their function.

This is the first report to demonstrate that the primarily cultured granulosa cells could be efficiently transduced with FSHR gene by the adenovirus vector. The granulosa cells transfected with FSHR gene maintained the expression of FSHR on their cell surface and the response to FSH stimulation. Our

Fig. 8. Estradiol production of rat granulosa cells transfected FSHR gene. Ad-FSHR was introduced into cultured granulosa cells on the first day of culture, FSH and androstenedione were added to the medium 48 h after transfection. The medium was collected at 48 h after the addition of hormones and the estradiol was measured by radioimmunoassay.

study demonstrates that gene therapy can be used to sustain granulosa cell function in vitro. This may have reproductive implications. So we suggest that co-culture of the immature oocytes with FSHR transduced granulosa cells would improve their developmental competence.

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REFERENCES

1. Chian RC, Buckett WM, Tulandi T, Tan SL: Prospective randomized study of human chorionic gonadotrophin priming before immature oocyte retrieval from unstimulated women

- with polycystic ovarian syndrome. *Hum Reprod* 2000;15:165–170
2. De Vos A, Van de Velde H, Joris H, Van Steirteghem A: In-vitro matured metaphase-I oocytes have a lower fertilization rate but similar embryo quality as mature metaphase-II oocytes after intracytoplasmic sperm injection. *Hum Reprod* 1999;14:1859–1863
3. Trounson A, Anderiesz C, Jones G: Maturation of human oocytes in vitro and their developmental competence. *Reproduction* 2001;121:51–75
4. Lie BL, Leung E, Leung PC, Auersperg N: Long-term growth and steroidogenic potential of human granulosa-lutein cells immortalized with SV40 large T antigen. *Mol Cell Endocrinol* 1996;120:169–176
5. Quinn A, Moore GE, Morgan RT, Woods LK: II lines from human colon carcinoma with unusual cell products, double minutes, and homogeneously staining regions. *Cancer Res* 1979;39:4914–4924
6. Minegishi T, Kishi H, Tano M, Kameda T, Hirakawa T, Miyamoto K: Control of FSH receptor mRNA expression in rat granulosa cells by 3',5'-cyclic adenosine monophosphate, activin, and follistatin. *Mol Cell Endocrinol* 1999;149:71–77
7. Nakamura M, Minegishi T, Hasegawa Y, Nakamura K, Igarashi S, Ito I, Shinozaki H, Miyamoto K, Eto Y, Ibuki Y: Effect of an activin A on follicle-stimulating hormone (FSH) receptor messenger ribonucleic acid levels and FSH receptor expressions in cultured rat granulosa cells. *Endocrinology* 1993;133:538–544
8. Minegishi T, Nakamura K, Takakura Y, Ibuki Y, Igarashi M: Cloning and sequencing of human FSH receptor cDNA. *Biochem Biophys Res Commun* 1991;175:1125–1130
9. Miyake S, Makimura M, Kanegae Y, Harada S, Sato Y, Takamori K, Tokuda C, Saito I: Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. *Proc Natl Acad Sci USA* 1996;93:1320–1324
10. Niwa H, Yamamura M, Miyazaki J: Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 1991;108:193–199
11. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156–159
12. Durinzi KL, Wentz AC, Saniga EM, Johnson DE, Lanzendorf SE: Follicle stimulating hormone effects on immature human oocytes: In vitro maturation and hormone production. *J Assist Reprod Genet* 1995;14:199–204
13. Janssenswillen C, Nagy ZP, Van Steirteghem A: Maturation of human cumulus-free germinal vesicle-stage oocytes to metaphase II by coculture with monolayer Vero cells. *Hum Reprod* 1995;10:375–378
14. Hassan HA: Cumulus cell contribution to cytoplasmic maturation and oocyte developmental competence in vitro. *J Assist Reprod Genet* 2001;18:539–543
15. Dandekar PV, Martin MC, Glass RH: Maturation of immature oocytes by coculture with granulosa cells. *Fertil Steril* 1991;55:95–99
16. Schramm RD, Bavister BD: Granulosa cells from follicle stimulating hormone-primed monkeys enhance the development competence of in-vitro-matured oocytes from

- non-stimulated rhesus monkeys. Hum Reprod 1996;11:1698–1702
17. Lovekamp TN, Davis BJ: Mono-(2-ethylhexyl) phthalate suppresses aromatase transcript levels and estradiol production in cultured rat granulosa cells. Toxicol Appl Pharmacol 2000;172:217–224
18. Adashi EY, Resnick CE, Jastorff A: Blockade of granulosa cell differential by an antagonistic analog of adenosine 3',5'-cyclic monophosphate (cAMP): Central but non-exclusive intermediary role of cAMP in follicle-stimulating hormone action. Mol Cell Endocrinol 1990;72:1–11