ISSN: 2278-4632
Vol-12 Issue-10 No.01 October 2022

A COMPREHENSIVE REVIEW ON DEVELOPMENT OF GRANULOSA CELL LINES BY IMMORTALIZATION

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Abstract

The condition that occurs most frequently during folliculogenesis is polycystic ovarian syndrome (PCOS). PCOS occurs due several reasons like increased level of androgens in females, insulin resistance, low grade inflammation, heredity, etc. Both in vivo and in vitro procedures can be used to test for PCOS; one of the in vitro methods is to assess activity by cell line. Granulosa cells (GCs) plays a crucial role in folliculogenesis because of their secretive activity. GC culture has a very limited capacity to spread. Therefore, primary cells immortalization is a method to get over these limitations. GC culture has a very limited capacity to spread. Therefore, primary cells immortalization is a method to get over these limitations. Different immortalization procedures using various viral vectors by transfection approach can be used to develop PCOS in healthy granulosa cells. After a successful transfection, the virus with the desired DNA should be inserted into the host cells using the transduction technique to obtain immortalised cells with PCOS condition. Using various molecular biology techniques, several biological markers, such as oestradiol and progesterone, were estimated.

INTRODUCTION

One of the most prevalent endocrine metabolic illnesses, at reproductive age approximately 5% of women affected by PCOS (1). A malfunction of granulosa secreted cells is one of the causes that can lead to this condition (2). The intricate process of ovarian follicle maturation, or folliculogenesis, necessitates a precise timetable and hormonal control directed at the pituitary, ovaries, and central nervous system. Only 1% of follicles in female mammals reach ovulation, resulting in atresia in the remaining follicles (1). Follicular atresia is thought to be caused by granulosa cell apoptosis and theca cell degradation (3). Additionally, granulosa cells are essential for the release of sex-stimulants such oestrogen and progesterone.

Polycystic ovary syndrome is the most common disorder during folliculogenesis (PCOS). Folliculogenesis, is a multifaceted process that involves specific timing and endocrinal regulation of the CNS, pituitary gland, and ovaries, any syndrome that develops during folliculogenesis will result in infertility because folliculogenesis is crucial to fertility. Due to their secretory activity, Granulosa cells (GCs) play an important role in development of follicular. GC culture very less capability to proliferate. As a result, primary cells immortalization is a technique for evading these constraints. In this review, we will look at the techniques used to generate immortalised PCOS cells, specifically granulosa cells (4), (5).

Keywords: *Immortalization, Granulosa cells, polycystic ovary syndrome, folliculogenesis, Follicular atresia.*

Why Do We Need Cells with Immortality?

First of all, initial cells experience limited generation before entering senescence. Frequently establishing new cultures from explanted tissue is time-consuming and might result in significant variance from the original culture. First of all, initial cells experience limited generation before entering senescence. Regularly re-establishing fresh cultures from explanted tissue is time-consuming and can lead to considerable variations in preparations. Utilizing immortalised cells can ensure the consistency of the test materials.

In addition to having the ability to proliferate for an extended period of time, immortalised cells also have genotypes and phenotypes that are comparable to or identical to those of their parent tissue. Additionally, hTERT immortalised cells have been used in numerous studies to induce the

differentiation of distinct cell types, resulting in the development of structures that mirror those created in vivo and the appearance of tissue-specific characteristics and differentiation-specific proteins.

STRATAGIES

Techniques involved in immortalization by viral transduction

- Simian virus 40 (SV40),
- hTERT,
- Epstein Barr virus (EBV),
- Adenovirus E1A and E1B,
- Human Papillomavirus (HPV) E6 and E7.
- PSV
- C-myc

Due to the absence of some essential transcription factors or the presence of cancer-causing proteins brought on by transformation, the majority of malignant cell lines that overexpress for immortalization are unable to maintain their normal physiological functions. It has been established that the most reliable technique for converting a variety of cell lines is the SV40 T antigen. All viral genes promote immortalization by inhibiting senescence-inducing tumour suppressor genes (p53, Rb, and others).

1. Simian virus 40 (SV40)

SV40 has been extensively used as a model for mammalian system replication and gene expression. It is an effective "probe" for cellular activities. viral gene expression restricted to only two proteins, large T antigen (LT) and small T antigen (ST), which can lead to altered cell proliferation and the development of numerous "transformed" phenotypes associated with tumours. Immortalization of granulosa cells by SV 40 can be accomplished in a variety of organisms, including mice, pigs, and humans. Immortalization of granulosa cells by SV 40 can be accomplished in a variety of organisms, including mice, pigs, and humans (6).

a) IN MOUSE

Primary granulosa cells preparation of mouse.

At the age of 20 days, 8 IU of pregnant mare serum gonadotropin was intraperitoneally given into female outbred NMRI mice. The ovaries were taken out after 48 hours, the follicles were pierced with a 25-gauge needle, and the ovaries were triturated with medium using a 30-ml plastic pipette. The media containing the flushed cells were then collected, cleaned, and counted before being seeded at 1 X 10^5 cells/cm^2 in Ham's F12 and Dulbecco's modified Eagle's medium (HD; 1:1, vol/vol) with 10% foetal bovine serum (FBS). By using this technique, each pair of ovaries had about 5 X 10^5 cells selected.

Cell culture and transfection procedures

At 90% confluence the culture was divided into two culture vessels, 2 g of pSVv-myc were transfected into one culture vessel using either the2 g of pSVv-myc were transfected into one culture vessel using either the calcium phosphate method or the modified lipofection (lipofectin 10 µl/µg) DNA method, and DNA were dissolved in serum free medium (10 µl/µg lipofectin or 1µg DNA /ml medium). Other second culture vessel served as a control. Before being added to the culture flask, the diluted samples were combined. For 5 hours, the co-precipitates were left on the cells.

Transfected cultures were compared with the control culture on a daily basis. Following passage (dilution 1: 2), the cells were grown in HD supplemented with fatty acid-free bovine serum albumin (5 mg/litre), bovine insulin (5 mg/litre), sodium selenite (5 mg/litre), and human transferrin (10 mg/litre) (7).

Characterization

- Cell morphology and cytochemistry
- Biological assay

ISSN: 2278-4632
Vol-12 Issue-10 No.01 October 2022

- Inductions
- Tumorigenicity
- Radioimmunoassay
- Analysis of the concentration of prostaglandins

b) IN PORCINE

Culture of porcine granulosa cells

Pig ovaries were removed and placed in saline containing 50 mg/ml each of penicillin and streptomycin. A 23-gauge needle and syringe were used to separate granulosa cells from ovarian follicles (1-2 mm in diameter), the material was centrifuged three times, and re-dissolved in TCM199, which contains gentamycin (50 mg/ml), penicillin (50 mg/ml), streptomycin (50 mg/ml), fungizone (0.25 mg/ml), and 10% foetal bovine serum. According to the procedures described by Channing et al.3,4, aliquots of cells (5105 viable cells/well) were seeded to 35 mm dishes and incubated at 5 percent CO2.

SV40 Infection and development of a cell line.

Granulosa cells were infected with SV40 at concentrations of 10^4, 10^5, and 10^6 plaque forming units per millilitre; two dishes with fake infection served as the control, cells were washed with fresh media several times after two hours of incubation, and media were then changed every two days after that. In a dish with 10^6 pfu/ml of SV40, one colony was chosen after four weeks of infection. When the cells reached sub-confluence, the chosen colony was trypsinized and transferred to a new plate. The confluency of the cells was then measured using a hemacytometer. Every generation, population doublings were calculated, and the PGV cell line was used (porcine granulosa cell-virus transformed (8).

Characterization

- SV40 big T antigen in PGV cells has been verified to exist.
- Progesterone concentrations in conditioned culture media and primary granulosa cell (PGV) and cyclic adenosine 30, 50 - monophosphate (cAMP) levels were measured.
- Using Northern blot analysis, the P450scc mRNA was identified.
- Retinoic acid can reverse the steroidogenic activity in PGV cells.
- PGV cell proliferation studies

c) IN HUMAN

Human Granulosa Cell Preparation

For IVF, oocyte and hGC cells were collected from the follicular fluid of eight women and suspended in M-199/10% FBS. Each of the eight preparations contained cells from several follicles, and samples were centrifuged for 5 minutes at 1000 rpm, after being resuspended in the same mix M-199/10% FBS and seeded in 35 mm culture dishes (0.5- 1 x105), cell survival was 90-95 percent after tryphan blue exclusion. The author obtained information from the University of British Columbia's In Vitro Fertilization (IVF) Program.

Transfection and culture

Before transfection Each patient's primary hGL was cultured independently for 4 - 6 days. To boost the possibilities of immortalization, EGF (20 ng/ml) was added daily. For 15 minutes, 14.3 l lipofectin (1 mg/ml), 3.4 g plasmid P-X8 DNA containing the SV-40 early genes, and 1.4 ml M-l99 were combined in 1.4 ml M-l99 per culture. The plasmid P-X8 was used to immortalise human ovarian surface epithelial cells.

The cells were treated with serum-free M-199 and with the lipofectin-DNA solution for 24 hours before being replaced with M-199/10% FBS and 20 ng/ml EGF, every two days following transfection, 40 ng EGF/20 1 PBS/2 ml culture media was included, and once a week, the media was changed. 3 - 10 weeks after transfection, colonies of morphologically distinct transformed cells were identified and isolating cells with a cloning ring or a cell scraper. The isolated clones were developed in M-199/MCDB 105 (1:1)/10% FBS/0.4 g/ml hydrocortisone because they developed more efficiently than

ISSN: 2278-4632
Vol-12 Issue-10 No.01 October 2022

in M-199/10% FBS. The cells had passed through a 0.06 percent trypsin (1:250)/0.01% EDTA solution.(9).

Characterization

- Biochemical analysis
- Analysis of growth potential
- Immunofluorescence microscopy
- Statistical analysis

2. Human telomerase reverse transcriptase (hTERT) h TERT

During cell division Telomerase is an enzyme that can re-establish the DNA base pairs lost from telomeres. It is made up of two parts: an RNA component (hTERC or hTR) and a catalytic component (hTERT). Cells with active telomerase continue to divide and maintain chromosomal length without becoming senescent (10) .

Human telomerase reverse transcriptase (hTERT) has been known as a key protein involved in the majority of tumours' abnormal cell proliferation, immortalization, metastasis, and stemness preservation, in spite of having slight or there is no expression in normal somatic cells.

When hTERT is transfected into human primary cells, it causes chromosome telomere elongation and maintenance. In many cases, forcing hTERT expression alone allows cells to suppress replicative senescence and overcome the growth crisis in order to achieve efficient immortalization (11) (12) (13).

a) IN PORCINE

Granulosa Cell Isolation and Culture

With a 10 mL syringe and three washes of warm sterile saline, GCs were isolated from 2-3 mm ovarian follicles. Following that, the primary GCs were collected and centrifuged for 5 minutes at 1000 rpm to remove white blood cells, followed by three PBS washes. In a 10 cm culture dish, the primary GCs were cultured in vitro in DMEM medium containing 10% foetal bovine serum, 1% supplement, and 20 ng/mL epidermal growth factor. The primary Cells GCs were given a change in culture medium on the second day of isolation, followed by three PBS washes.

Packaging, Infection, and Cell Screening of Lentiviruses

70 percent confluent transfection 10 cm plates of 293FT cells with plasmids of lentiviral gene transfer 10 g pLVX-Tet3G-Large T-T2A-mCherry-Puror, in addition to packaging plasmids of 10.4 g psPAX2 and 3.5 g pMD2. The recombinant lentiviral particles discovered in the supernatant were collected and filtered (0.45 m) into sterile 50 mL centrifuge tubes at 48 and 72 hours. An ultrafiltration device was used to extract the high-concentration lentivirus (by centrifuging at 4000 rpm, 4 C for 30 minutes, and lentivirus aliquots were stored at -80° C until use). The viral particles were then suspended in 500 L of GC complete medium containing 6 g/mL polybrene. The multiplicity of infection (MOI) of the lentiviral solution was 20. Infected GCs were cultured for 48 hours more before being screened with 1g/mL puromycin and inducted for 14 days with 50 ng/mL Dox. The infection outcomes were examined for mCherry expression using a fluorescent microscope (IX73, Olympus, Tokyo, Japan). CIPGCs were cultivated in DMEM supplemented with 10% FBS, 1% and 50 ng/mL Dox in successive passages for more than six months. CIPGCs were cultured for six days with or without Dox before testing for cell cycle, cell proliferation, immunofluorescence, mRNA expression, and steroid hormone production. (14).

Characterization

- Cell Cycle Analysis
- Cell Proliferation Assays
- Immunofluorescence
- RNA isolation and Quantitative Reverse Transcriptase (RT)-PCR
- Morphology and Phenotypic Analysis of CIPGCs with or without Dox
- Determination of Steroid Hormones' Secretion

ISSN: 2278-4632
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- Statistics Analysis

b) IN GOAT

Isolation and culture of goat primary granulosa cells

Cells of the Granulosa were extracted from healthy follicles of medium size using a fine needle (4-6 mm). If the follicles were consistently translucent and vascularized, they were considered healthy, the granulosa cells were then isolated and modified. GCs were grown in M-199 medium containing 10% foetal calf serum and incubated in a humidified incubator with 5% CO2 at 37°C, for every three days, the culture medium was changed.

Cell transfection and selection:

Once goat granulosa cells (GCs) reached 50% confluence, they were immortalised by transducing them with pCI-neo-hTERT turbofect transfection reagent. After 24 hours, transfected cells were selected from the whole culture medium containing 400 µg/ml G418, which confined 400 µg/ml G418. Drug-resistant monoclonal cells were selected After two weeks. Three positive colonies were propagated and cultured for additional study, and the cells were dubbed hTERT goat granulosa cells (hTERT-G) (15).

Characterization

- RNA isolation and real-time PCR
- Immunofluorescence
- Oestradiol and progesterone measurement
- Proliferation assay
- Telomere length measurement
- Karyotype assay
- Cell cycle analysis
- Western blot analysis

c) IN HUMAN

Collection of follicular fluid cells.

Transvaginal ultra sound guided aspiration was used to extract all oocytes from follicles with diameters greater than 10 mm. After removing the fresh follicular fluid, oocyte corona cumulus complexes were centrifuged for 5 minutes at 524 x g and 4° C, PBS was used to wash the cell pellet three times. Cells were then extracted using density gradient centrifugation for 30 minutes at 2,095 x g and 4 C with 2 ml Percol. Cells from the interphase layer was collected and three times washed with Dulbecco's modified Eagle's medium.. Cells were cultured in 100mm tissue culture dishes with MEF conditioned medium. 5 to 10 patients' follicular fluid was pooled into a single primary cell culture to achieve the required number of cells (16).

After resuspending the hGCs in Tyrode's Salt Solution, they were layered on a Sil-Select Plus gradient and centrifuged at 700 g for 10 minutes, hGCs were collected and washed in DMEM: Hams F12 medium with 10% foetal bovine serum (FBS). The PCOS cells were grown in DMEM/F-12 Ham's medium enriched with 17.5% foetal bovine serum, 2.5% horse serum, 4 mM L-glutamine, 2 mM non-essential amino acids, and 100 IU/ml penicillin, Streptomycin 100 mg/ml, r FSH 100 ng/ml, bFGF 100 ng/ml, EGF 25 ng/ml, 0.5. Normal cells were grown in DMEM/F-12 Ham's medium containing 20% FBS, 4 mM L-glutamine, 2 mM non-essential amino acids, 100 IU/ml penicillin, 100 mg/ml streptomycin, 100 ng/ml bFGF, 25 ng/ml EGF, 0.5 g/ml hydrocortisone, 50 g/ml ascorbic acid, and 100 mg/ml(1).

Retroviral-mediated gene transfer

Passage of platinum-A cells at a rate of 2106 cells per 60 mm dish. The retroviral vectors pBABE-hTERT and pMXs-hcMyC were introduced into Platinum-A cellsUsing the X-treme HP DNA transfection reagent, follow the manufacturer's instructions. After 48 hours, The viral particles in the supernatant were filtered using a 0.45 m syringe filter. Finally, the produced retroviruses were infected at a concentration of 6 g/ml into both PCOS and normal hGCs Polybrene (1).

Cell culture conditions

A mixture of follicular fluid cells was cultured of 70% basic culture medium and 30% ME conditioned medium, with the basic medium being DMEM. The cells were seeded at 1.5x106 cells/100 mm2 bacterial culture dish and incubated at 37°C with 5% CO2. Every 3-4 days, cells were passaged after 24 hours of treatment, capture primary passages 1, 2, 4, 50, and 99 using inverted light microscope, fresh FCs were cultured in DMEM After 24 hours, the medium was changed every two days (1).

Characterization

- Growth curve of human GCs
- Colony formation assay
- Flow cytometric analysis
- Reverse transcription (RT)-semi-quantitative PCR
- Measurement of intracellular cyclic adenosine monophosphate (cAMP)
- Estimation of the amount of steroid secreted in cell medium.
- Cell labelling.
- Intra-ovarian transplantation
- Oestradiol, progesterone, and anti-Mullerian hormone estimation (AMH)
- Immunofluorescence staining
- Immuno blot analysis
- GCP and GCN cell line quality control and confirmation
- Chromosome analysis

3. HUMAN PAPILLOMA VIRUS

HPVs have the ability to extend life and contribute to the immortalization of squamous epithelial cells. The DNA of 'low-risk' HPVs lacks this immortalization property. The immortalization of normal human mammary epithelial cells (76N) by plasmid pHPV18 or pHPV16, both of which contain the linearized viral genome. For efficient immortalization of 'high-risk' individuals' cloned DNAs, E6 and E7 genes of 'high-risk' HPV are both compulsory and sufficient.

Transduced HFKs had more double strand DNA breaks than controls, but there was no statistically significant variance between types.

However, immortal offspring of HPV-transduced HFKs who had earlier gone through a crisis period (HPV45/51/59/66/70-transduced HFKs) had far more chromosomal abnormalities than crisis. The hTERT locus at 5p was discovered only in cells previously experienced crisis, and it was associated with amplified expression. After eight multiple chromosomal rearrangements Chromothripsis in a single cell line resulted in MYC gain. The number of chromosomal aberrations increased during HPV-induced immortalization is discovered to be inversely related to viral immortalization capacity (17).

a) IN HUMAN

Primary cultures of Human Granulosa cells

The follicular cells were isolated of women whose eggs had been extracted for IVF. Women were given GnRH agonists before and during follicular stimulation with human menopausal gonadotropins. Luteinized HG cells larger than 15 mm in diameter were isolated following follicular aspiration. To separate luteinized HG cells, they had been washed twice with Dulbecco's Modified Eagle's and Ham's F-12 (DME/F12) medium and incubated at 37°C for 30 minutes containing 0.1% hyaluronidase and the cells had been resuspended in 20 ml medium prior to transmission to 250 mL tubes with 3.5 ml Histopaque 1077, 15 minutes of centrifugation at 600 X g luteinized HG cells were separated from red blood cells. In the middle of the Histopaque and the medium, a thin layer of granulosa cells formed. The cells were washed three times with DME/F12 culture medium, which contained 5% foetal bovine serum, 5% horse serum, insulin (6.25 g/mL), transferrin (6.25 g/mL), selenic acid (6.25 ng/mL), BSA (1.25 mg/mL), and linoleic acid (5.35 g/mL). In addition, 20,000 cells per sq.-cm culture well were plated and allowed to confluent with Ultroser at 2% and antibiotics. Using this procedure, the attachment of cells was determined being approximately 50%.

Transfection of retroviruses

They wanted to see if they could immortalise these cells, they had been placed in primary cultures of HG cells. High-level infection tittered recombinant amphotropic as previously stated, a retrovirus was

ISSN: 2278-4632
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used. To control transcription of HPV16 E6/E7 DNA fragments (B-10) was inserted into retroviral vector pLXSN, the neomycin resistance gene was also present in the vector, which was triggered by the simian virus-40 promoter. To create recombinant viruses, previously described methods were used. In summary, transfection of plasmid DNA into the Psi-2 cell line for ecotropic packaging by precipitation of calcium phosphate, as well as viruses produced in order to infect the cells, Psi-2 cells were used. PA317 cell line for amphotropic packaging. Both cell lines are Mouse fibroblast lines derived from the NIH 3T3 cell line, PA317 clonal line-derived viruses had been used to infect HG cells, 7 days after the establishment of the primary culture. After selection in geneticin sulphate and infection with pLXSN16E6/E7. Plated infected cells in terms of cloning density in growth medium on 100-mm dishes lacking serum however containing geneticin (500 µg/mL) as the method of selection. The addition of neomycin phosphotransferase resulting in the formation of colonies of cells unaffected to the toxic effects of geneticin, which were subsequently isolated and altered into the HGL clones. With population doublings ranging from 35 to 75, the clone HGL5, which was used in the description studies, was examined. Cells reacted to steroid production similarly, at this time, however, the interval between cell divisions shortened from 48 to 96 hours (18).

Characterization

- DNA isolation and polymerase chain reaction (PCR) analysis
- CAMP measurements and steroid hormone levels
- RNA isolation and Northern analysis

4. ADENOVIRUS

Adenoviral vectors can effectively transduce all human cell types (except for blood cells absence of the adenovirus receptor). Adenoviral vectors, on the other hand, are unable to bind to the target cell's genome, leading to transient transgene expression. An oncoprotein from the adenovirus E1A 12S gene can create primary cells in culture as cell lines. It has two exons that encode it. The E1A 12S gene, which is encoded by both exons, contains four regions that are necessary for primary epithelial cell immortalization, according to extensive mutational analysis.

Although the expression of two regions is necessary to reactivate dormant cells into the cell cycle, it is unable to prolong the lifespan of these cells in culture, preventing the possibility of immortalization. The first exon encodes these regions. There is a third first-exon region that is necessary, but its purpose is still unknown. When combined with an activated Ras gene, 12S causes tumorigenic transformation, but these three regions are also necessary for this to happen. Cell proliferation, cell survival in culture, and the production of an autocrine growth factor all depend on the fourth region. The second exon encodes these functions. Either the wild-type 12S or the immortalization-competent mutants maintain the epithelial morphology and keratin expression in immortalised cells.

Cell culture

KGN cells remained grown at 37°C in a moist incubator with a CO2 content of 5% in DMEM/nutrient combination F-12 Ham supplemented containing 10% foetal bovine serum

Cell transfection

Let-7e, a mimic, NC a mimic. The logarithmic phase KGN cell line was trypsinized prior to seeding into 6-well plates. The cells developed to 30–40% confluence and rapidly transfected using Lipofectamine containing a 20 nm let-7e mimic, in addition negative controls that correspond, following an overnight incubation at 37°C with a CO2 content of 5%. The cells that were transfected were collected 48 hours after transfection for additional research (19).

Characterisation

- Quantitative real-time PCR and RNA extraction
- Western blot analysis
- Cell Counting Kit-8 assay
- Ethynyl-2-deoxyuridine assay

ISSN: 2278-4632
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- Autophagy identification
- Statistical analysis

5. LENTIVIRUS

Reverse transcriptase, an enzyme that turns viral RNA into double-stranded DNA, and integrase, an enzyme that splices viral DNA into host DNA, are both made by lentiviruses, a type of retrovirus. No one can tell the difference when viral DNA is incorporated into host DNA and divides alongside the host cell. The primary distinction between lentivirus and retrovirus is that the latter only infects dividing cells, whereas the latter can infect postmitotic cells like neurons because it can infect both dividing and non-dividing cells.

Due to their ability to actively pass through the nucleus membrane, Lentiviral vectors are capable of transducing both cells that divide and non-dividing. Lentiviral vectors, like retroviral vectors, also integrate into the genome of the host cell once they reach the nucleus. Lentiviral vectors are therefore becoming more and more common in gene transduction applications, both in vitro and in vivo. The small insertion size of lentiviral vectors is one drawback. The majority of lentiviral vectors have an insertion size limit of 5.0 kb. When insertion sizes are higher than 3.0 kb, virus titres are significantly decreased. The Lenti-SV40 titre is predicted to be low because the SV40 genome is larger than 5.0 kb.

Primary cultured human granulosa cell preparation

Small watchmaker forceps were used to remove the granulosa layers from each follicle. There was no sign of metastasis in the right ovary in the permanent section. There was no evidence of metastasis in the right ovary in the granulosa in the permanent section. In DMEM with 10% FCS, 100 IU/ml penicillin, 100 g/l streptomycin, 25 mg/l amphotericin, and L-glutamine were added to the mixture, and the granulosa layers had been resuspended. In 6-well dishes treated with collagen, the cells were seeded.

Gene transfer through lentivirus

The Gateway system was used to produce lentiviral vector plasmids through recombination. The advanced tTA segment derived from the pTet-Off A sophisticated plasmid was reassembled utilising a lentiviral vector, CSII-CMV-RfA, in the LR response to produce CSII-CMV-Tet-Off. The promoter of elongation factor 1 (EF) in CSII-EF-MCS was substituted in search of a Tet-responsive promoter (TRE-Tight) derived from pTRE-Tight, then a modified RfA fragment, in order to create a Tet-responsive lentivirus vector. Human cyclinD1, hTERT cDNA recombination and human mutant CDK4 (CDK4R24C) resulted in the development of CSII-TRE-Tight-CyclinD1, CSII-TRE-Tight-CDK4R24C, CSII-TRE-Tight-16E6E7, CSII-TRE-Tight-16E6, and CSII-TRE-Tight-16E6 (20).

Characterization

- Quantitative RT-PCR
- Oestradiol and progesterone levels in culture medium are measured.
- Bromodeoxyuridine (BrdU) assay and cell count
- BMP effects and Western blot analysis

METHODS TO CHARACTERISE

Radioimmunoassay

For the radio immunochemical assessment, flu microspheres coated with anti-rabbit antibodies were used along with the scintillation proximity assay. First, the concentrations of the flu microspheres and antibodies were optimised using the previously mentioned antibody properties. Microscopy confirmed the successful incorporation of the SV40 T-antigen with immunofluorescence using the mouse antiSV40 T-antigen monoclonal antibody PAb 1604 and goat anti-mouse IgG antibody that was FITC-labelled. Prior to being fixed by 100 % methanol at - 20°C for 30 minutes and permeabilized with acetone/methanol (1: 1) at - 20°C for 5 minutes, cells were cultured on glass coverslips for 1-2 days. The cells were washed in PBS and then incubated with 5 percent normal goat serum for 45 minutes at room temperature. After that, mouse anti-SV40 T-antigen Mab (1:20) was added. PBS was used to wash the cells and then exposed to FITC-labelled goat anti-mouse IgG antibody (1:100) for 1 hour at

ISSN: 2278-4632
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room temperature prior to mounting on glass slides with gelvatol. The primary antibody was not present in the negative controls (15).

Immunofluorescence staining.

for immunofluorescence seeded hTERT-GGCs and GGCs into 6-well plates, 30 minutes of fixation with 4% PFA, and permeabilized using 1% Triton X-100 for 5 minutes. Primary antibodies were used to treat cells against TERT for an overnight incubation at 4°C following an hour of PBS containing 1% bovine serum albumin was used for blocking. The nuclei were stained with DAPI (4,6-diamidino-2-phenylindole), and cells were observed using laser scanning confocal microscopy following three PBS washes and an hour of incubation at room temperature of Alexa-labelled donkey anti-rabbit IgG at a 1:500 dilution combination (3).

RT PCR

Positive controls included human ovarian tissue and FCs, while negative controls included chondrocytes. At passage 10 or 60, ovarian tissue, FCs, GCs, and chondrocytes were all used to extract total RNA. RNA was isolated from the homogenised solution after ovarian tissue samples measuring 5x5x5 mm were promptly placed in a sterile culture tube with (1.5ml) for 15 minutes, 30°C for 10 minutes, 42°C for 60 minutes, 99°C for 5 minutes, and 5°C for 5 minutes made up the RT temperature protocol. The following thermocycling parameters were used for the PCR reaction: 95°C for 3 minutes, 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and 2934 cycles, followed by 10 minutes at 72°C. The amplified products were observed by separation on 1.2 percent agarose gels and ethidium bromide staining.

Western blot analysis

Granulosa cells from PCOS and healthy hGCs, was isolated from follicular fluid, protein in its entirety was recovered. The protein content was known by using Bradford assay 20 g of each sample Protein was extracted using SDS-PAGE before being blotted onto PVDF membranes. The membranes were treated overnight at 4 C with anti-FSHR, anti-aromatase, anti-BMP15, anti-GDF-9, anti-AMH, anti-hTERT, and anti-GAPDH antibodies following blocking containing 5% BSA in a Tris buffered saline solution containing Tween 20. The appropriate primary antibody and either mouse, rabbit, or goat secondary antibodies conjugated to horseradish peroxidase after which they were incubated with the membranes at room temperature for 60 minutes (HRP). The membranes were dyed after being washed twice with TBST for 19.5 and 39.5 minutes (1).

Monitoring the levels of oestradiol, progesterone, and anti-Mullerian hormone (AMH)

By cultivating hGCs and determining concentrations of oestradiol and AMH in the cell culture media, aromatase activity was identified. 5×10^{5} hGCs were propagated in 6-well culture plate with fresh media female human serum supplemented, 48 hours later the culture medium from PCOS and normal hGCs were collected. After that, Elecsys and Cobas Immunoassay Analysers performed an electrochemiluminescence immunoassay to measure the levels of AMH. An immunoassay system was also utilised to measure the amounts of oestradiol and progesterone using chemiluminescence (1).

Cell Counting Kit-8 assay

According to the manufacturer's instructions, the Cell Counting Kit-8 (CCK-8) was used to determine the viability of the KGN cell line. In 96-well plates transfected KGN cells were collected and seeded at a density of 2×10^{3} cells/well. The cells were then cultured for 2 hours at 37 C with 5% CO2 in 10 l CCK-8 solutions in each well. A microplate reader was used to measure the absorbance at 450 nm for the first five days, at daily intervals (21).

Histological staining of the cells

Cells were 90-95 percent confluent and allowed to adhere before being fixed in 4 percent formaldehyde for 25 minutes at 4 °C (approx. 72 h). For morphological investigation, conventional haematoxylin and eosin (HE) staining was employed. The morphology of dyed AVG-16 cells was examined utilising a light microscope (21)

Colony formation assay.

At passage 8 cells were planted at a density of 100 single cells per plate for colony formation experiments. Cells were stained with crystal violet after 20 days of incubation, and the number of clones was determined visually. Images of a large colony produced from a single cell were taken using

an inverted light microscope (x40 magnification) on days 1, 2, 3, 6, 12, and 15 to ascertain the efficacy of colony formation, three replicated experiments were carried out. The mean SD was used to calculate efficiency (16).

Analysis using flow cytometry

Cells were taken out for flow cytometric examination at passages 10 and 30, the cells were treated with 2.5 µg/test of Human BD Fc Block TM for 10 minutes at 4° C prior to labelling with fluorescence-conjugated antibodies. Phycoerythrin-conjugated CD13 (1:200 dilution), CD29 (1:200 dilution), CD31 (554061; 1:200 dilution), CD34 (1:200 dilution), CD45 (1:200 dilution), CD49f (1:200 dilution), CD90 (1:200 dilution), CD105 (1:200 dilution), and CD166 (1:200 dilution) were treated with the cells were examined with a flow cytometer after being washed three times in PBS. Data from flow cytometry were analysed using CXP software (16).

Conclusion

I want to conclude that developing a PCOS cell line from granulosa cells using an immortalization technique is possible using various viral transfectors. Immortalization of granulosa cells has already been accomplished in various organisms such as pigs, mice, and humans using various vector systems. Various techniques are used by scientists to characterise immortal granulosa cells.

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