Video Article

A Tissue Culture Model of Estrogen-producing Primary Bovine Granulosa Cells

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Abstract

Ovarian granulosa cells (GC) are the major source of estradiol synthesis. Induced by the preovulatory luteinizing hormone (LH) surge, cells of the theca and, in particular, of the granulosa cell layer profoundly change their morphological, physiological, and molecular characteristics and form the progesterone-producing corpus luteum that is responsible for maintaining pregnancy. Cell culture models are essential tools to study the underlying regulatory mechanisms involved in the folliculo-luteal transformation. The presented protocol focuses on the isolation procedure and cryopreservation of bovine GC from small- to medium-sized follicles (< 6 mm). With this technique, a nearly pure population of GC can be obtained. The cryopreservation procedure greatly facilitates time management of the cell culture work independent of a direct primary tissue (ovaries) supply. This protocol describes a serum-free cell culture model that mimics the estradiol-active status of bovine GC. Important conditions that are essential for a successful steroid-active cell culture are discussed throughout the protocol. It is demonstrated that increasing the plating density of the cells induces a specific response as indicated by an altered gene expression profile and hormone production. Furthermore, this model provides a basis for further studies on GC differentiation and other applications.

Video Link

The video component of this article can be found at https://www.jove.com/video/58208/

Introduction

Successful ovulation and luteinization depend on finely tuned and well-orchestrated molecular alterations in different somatic follicular cell types. Since details of these developmental processes are not yet fully understood, further clarification is required. *In vivo* approaches are elaborate and costly and, in particular, fail to address specific molecular mechanisms that occur during folliculogenesis. Therefore, reductionistic *in vitro* models are needed in addition, to provide insight into cellular and molecular details. Different studies describe the culture of whole follicles in the context of *in vitro* fertilization techniques[1,2,3]. Because researchers are interested in mechanisms of differentiation, many studies focus on follicular GC. These cells, directly associated with the oocyte, are the major sources of estrogen production and, thus, play an essential role throughout folliculogenesis and luteinization[4].

Immortalized cell lines of GC have been developed from different species. Most of them, however, do not show a sufficient steroid hormone production[5]. So far, only one cell line of bovine GC has been established[6], but this line lost its steroidogenic activity after several passages[7]. Therefore, since steroidogenesis and, in particular, estradiol production is an essential feature of GC functionality, it is advisable to study these aspects in primary cell culture models. In previous studies, it was demonstrated that a considerable estradiol production can only be observed under serum-free culture conditions[8,9]. Further on, the supplementation of a precursor of estradiol synthesis is another prerequisite, as GC fail to express the necessary enzyme that can convert progesterone to androstenedione[10]. Additionally, the synergistic effect of FSH and IGF-1 supplementation *in vitro* revealed an optimized activity of aromatase, the key enzyme of estradiol synthesis[11]. In the present protocol, other important factors that have a substantial impact on the GC culture model are also described. In particular, the cell plating density has tremendous effects on the outcome of the experiment[12]. Furthermore, a cryopreservation technique of bovine GC that does not significantly interfere with GC physiology in culture could be established. This technique helps to improve the organization of cell culture work and to optimize the preferred plating density.

Protocol

NOTE: Bovine ovaries were obtained from a commercial slaughterhouse. The collection of abattoir byproducts does not require an ethical approval according to the German law.

1. Working Conditions and Preparations

1. To guarantee sterility, perform all media and tissue preparation as well as all culture work in a specialized cell culture lab using a laminar flow bench.
2. Prepare 1x phosphate buffered saline (PBS, pH 7.4) supplemented with 100 IU penicillin, 0.1 mg/mL streptomycin and 0.5 µg/µL amphotericin for the transport of ovaries.
   NOTE: The maximal duration between receiving the ovaries and isolating the GC is 2 h in this set-up.

2. Isolation of Bovine Granulosa Cells

1. **Wash the ovaries several times in 1x PBS (with 100 IU penicillin, 0.1 mg/mL streptomycin, and 0.5 µg/µL amphotericin) to remove any blood from the surface before starting the isolation procedure. Use a beaker, place the ovaries inside, fill it with 1x PBS, and discard the PBS again.**
   1. Repeat this washing step 3–4x, until the ovaries are cleaned from any remaining blood.
2. Wipe one ovary using a lab wipe soaked with 70% alcohol, to minimize possible contaminations.
3. With a 3 mL syringe and an 18 G needle, aspirate the GC by puncturing small- to medium-sized follicles (<6 mm, measured with a ruler), and pool the follicular fluid in one 50 mL centrifuge tube.
   NOTE: To moisten the syringe, take a little amount of 1x PBS (with 100 IU penicillin, 0.1 mg/mL streptomycin, and 0.5 µg/µL amphotericin). This helps to collect small amounts of follicular fluid and prevents cells from sticking inside the syringe.
4. After puncturing several follicles, rinse the syringe and needle with 1x PBS for intermediate cleaning.

3. Cryopreservation of Cells

1. **Use trypan blue staining and count the cells under a hemocytometer.**
   1. To count the number of viable cells, prepare a tube with 1.5 µL of a 0.25% trypan blue solution.
      NOTE: The living cells will remain unstained because trypan blue can only access the cell barrier of dead cells, which then appear blue.
   2. Add 15 µL of the granulosa cell suspension to the trypan blue solution and mix them gently.
   3. Place the mixture in both chambers of a hemocytometer. Count the number of living (*e.g.*, unstained) cells in one big square per chamber.
      NOTE: Although some blood cells can be seen, they can be distinguished by their very small size.
   4. Calculate the number of living cells with the mean of both chamber squares according to the general guidelines; the proportion of living cells can vary between ovaries but should exceed 60%[13].
2. **Take a sample from the freshly isolated granulosa cell pool as a reference for further analysis.**
   1. Depending on the cell count of the isolated GC, set aside an appropriate number of cells as reference samples for RNA, DNA, or protein preparation.
      NOTE: For RNA isolation and the subsequent evaluation of the gene expression, 1 x 10^5 cells are sufficient, whereas other subsequent analyses might require different, generally higher cell numbers.
   2. Place one or several aliquots of the reference cells in a fresh tube and centrifuge it for 1 min at room temperature and maximum speed (12,000 x *g*) to obtain a cell pellet. Discard the supernatant.
   3. Immediately shock-freeze the samples in liquid nitrogen. Store the sample at -80 °C.
3. **Perform the cryopreservation as follows.**
   1. Prepare the freezing medium using 80% fetal calf serum (FCS) and 20% dimethyl sulfoxide (DMSO).
   2. In order to gently pellet the GC, centrifuge the GC for 3 min at room temperature and 500 x *g*.
   3. Remove the supernatant carefully and gently resuspend the cells in 100% FCS (*e.g.*, 1 mL) until no more cell clumps are visible.
   4. Add 1 volume (*e.g.*, 1 mL) of the prepared freezing medium to the cell suspension and transfer the mixture to a cryogenic vial.
      NOTE: The final concentration of the cryo-protecting media will be 90% FCS and 10% DMSO.
   5. Place the cryogenic vial in a specialized (commercially available) freezing container that prevents rapid freezing. The cooling rate of the samples should not exceed -1 °C/min. Transfer the freezing container into a -80 °C freezer for at least 4 h.
   6. For longer storage, place the cryogenic vials in ultra-low temperature containers (*e.g.*, liquid phase nitrogen containers).
      NOTE: The protocol can be stopped here, as the cryopreservation allows longer storage.

4. Preparation of Media and Culture Plates for the Cell Culture

1. **Coat cell culture plates with 0.02% collagen R.**
   NOTE: Collagen R is known to be essential for an improved attachment of GC in culture.
   1. Prepare a 0.02% collagen R solution with purified water suitable for cell culture.
   2. Add 150 µL per well in a 24-well plate (= 2 cm^2/well) and ensure that the whole surface of the culture well is covered with the collagen R solution.
   3. Let the solution dry with the lid open for a few hours or overnight in the laminar flow hood.
   4. When the culture plates are completely dry, irradiate them with UV light for 10–15 min to minimize contamination.
   5. If necessary, store the plates at 4 °C for up to 2 months.

2. Prepare media and supplements for culture work under a laminar flow hood. For a successful recovery of the GC, they have to be processed immediately after thawing.
3. **Prepare the following media.**
    1. Supplement α-Minimal Essential Medium (α-MEM) with 2 mM L-glutamine, 0.084% sodium bicarbonate, 0.1% bovine serum albumin (BSA), 20 mM HEPES, 4 ng/mL sodium selenite, 5 µg/mL transferrin, 10 ng/mL insulin and 1 mM non-essential amino acids.
    2. Additionally, add 100 IU penicillin and 0.1 mg/mL streptomycin to the media.
    NOTE: The antibiotics, once warmed, are stable for approximately 48 h. Therefore, aliquot the desired amount of media for the culture set-up and planned media exchange. Pre-warm only the media aliquots to 37 °C in a water bath. The prepared media can be stored at 4 °C for no longer than 3 months.
    3. To induce steroid activity in cultured bovine GC, supplement the α-MEM additionally with 20 ng/mL follicle-stimulating hormone (FSH), 50 ng/mL R^3 IGF-1, and 2 µM androstenedione.
    NOTE: Always supplement these components shortly before the onset of a culture or media exchange. Avoid repeated freeze-and-thaw cycles for FSH and IGF-1 stock solutions, as this might compromise the steroid activity.

5. Cell Culture Work

1. Thaw the cells quickly in a water bath at 37 °C for 3–4 min and transfer the cell suspension into 37 °C pre-warmed α-MEM (without hormone supplement). For centrifugation, a final volume of 10 mL is recommended.
2. Centrifuge the GC for 3 min at room temperature and at 500 x *g*. Discard the supernatant. Add the pre-warmed and supplemented α-MEM and resuspend the cells carefully.
3. Seed the GC at a density of 1 x 10^5 or 1 x 10^6 cells per well in a final volume of 500 µL. Include technical replicates of at least three culture wells per condition.
4. Incubate the cell culture in an incubator at 37 °C with 5% CO_2 for 8 d.
5. Perform a media exchange every other day. Replace only two-thirds of the culture media to reduce stress and to ease the adaptation of the cells to the fresh media.
NOTE: The GC form a typical fibroblast-like phenotype after a few days in culture and tend to cluster together. In the high cell-density culture, larger clusters are found more frequently as compared to the normal density GC culture (**Figure 1**, left *vs.* right panel).

6. Subsequent Analysis of Cultured Granulosa Cells

1. To perform steroid hormone analysis, collect the media and freeze it at -20 °C. Use appropriate methods to analyze the estradiol and progesterone concentration in the spent media [*e.g.,* radioimmunoassay (RIA)][14].
2. For the subsequent analysis of different target molecules (RNA, DNA, proteins, *etc.*), lyse the cells directly in the culture dish with an appropriate lysing agent as recommended by the supplier.
NOTE: For most isolation procedures, it is possible to stop the protocol here, as the lysed cells can be stored at -20 °C for up to 1 week. For longer storage, the cells should be kept at -80 °C.
3. To determine the transcript abundance, synthesize cDNA and measure specific transcripts by quantitative real-time polymerase chain reaction (qPCR) techniques[15]. A statistical evaluation should include at least three replications of different cell cultures, originated from different cell preparations (biological replicates).

Representative Results

GC plated at 1 x 10^5 cells/well display a typical fibroblast-like appearance as they form a cell extension comparable to fibroblasts and tend to build clusters (**Figure 1a** and **1c**). Increasing the plating density by 10-fold to 1 x 10^6 cells/well did not change the morphology but more cell clusters can be observed (**Figure 1b** and **1d**, arrows). As already shown in a previous publication, the collagen coating of the culture dishes largely improved the attachment of the cells[13].

Initial cryopreservation did not considerably change the physiological characteristics of cells in culture as compared to those directly cultured from freshly isolated samples. This is shown in **Figure 2** as the transcript abundance (measured by qPCR) of several marker genes did not differ when comparing cultured cells derived from either frozen or freshly isolated pools.

Figure 3 shows representative results of the steroid hormone analysis (measured by RIA) in bovine GC cultured at normal *vs.* high density. The estradiol concentration is significantly decreased in the high-density culture compared to the normal-density culture, whereas the progesterone production tended to be higher.

A comparative analysis of several genes (measured by qPCR) in high- *vs.* normal-density cultures revealed a significant effect (**Figure 4**). *CYP19A1*, encoding the key enzyme of estradiol biosynthesis aromatase, as well as the gonadotropin receptor *FSHR*, were significantly down-regulated. In contrast, the genes *RGS2* and *VNN2* showed a significant up-regulation. These results clearly suggest that specific processes of cellular differentiation are induced by increasing the cell plating density.

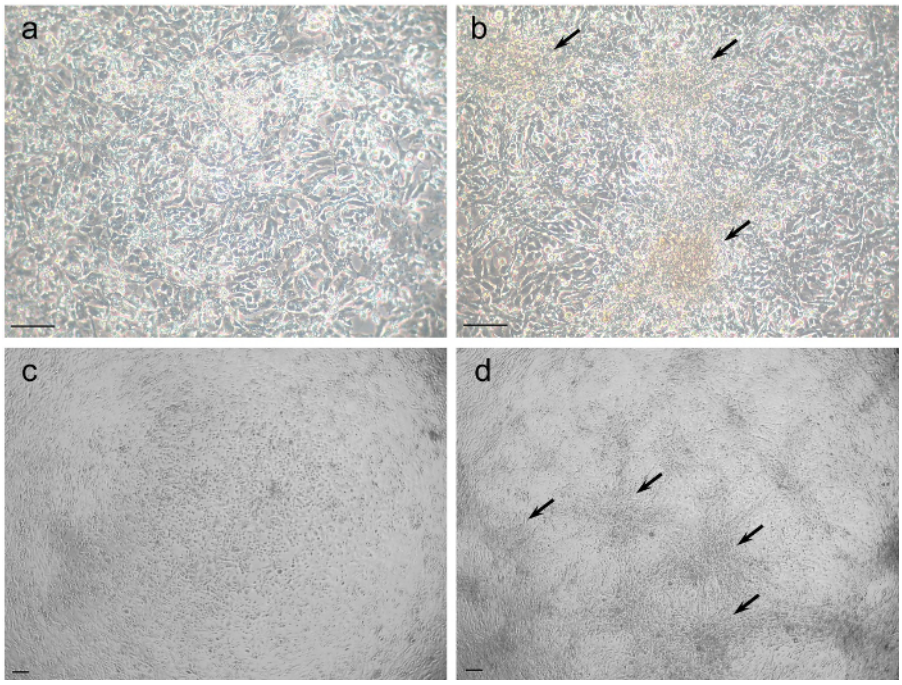

Figure 1: Cultured bovine granulosa cells at normal (left panels) and high cell density (right panels). (**a** and **c**) Cells cultured at a density of 1 x 10^5 cells/well displayed a typical fibroblast-like phenotype. (**b** and **d**) GC cultured at a high plating density (1 x 10^6 cells/well) tended to form large cell clusters (arrows) more frequently as compared to cells under a normal density (left panel). Scale bars = 100 µm. Please click here to view a larger version of this figure.

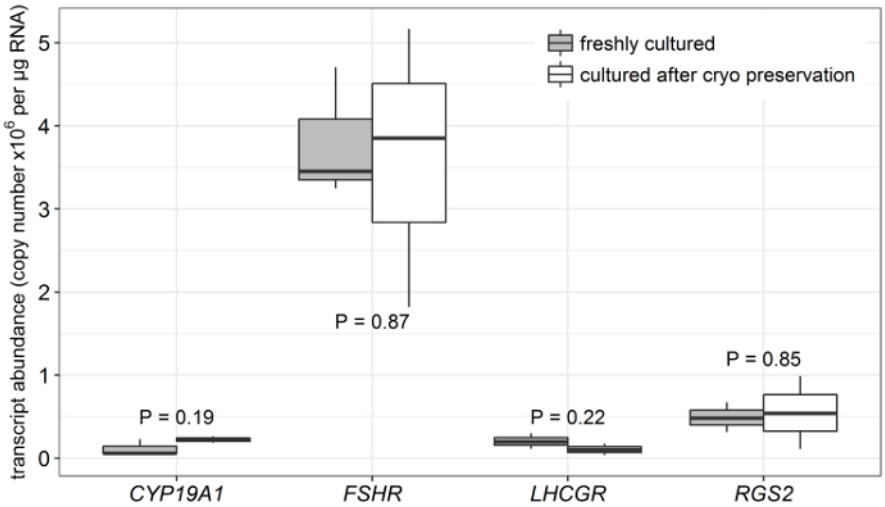

Figure 2: Comparison of cells cultured directly after isolation and after cryopreservation. The cells were cultured at a density of 1 x 10^5 cells per well. The gene expression of several marker transcripts was evaluated and revealed no difference between cultured cells with or without an initial cryopreservation. The boxplot shows the median of *n* = 3. Two-tailed Student's *t*-test, the *p*-values are included. This figure is reproduced from Baufeld and Vanselow[16]. Please click here to view a larger version of this figure.

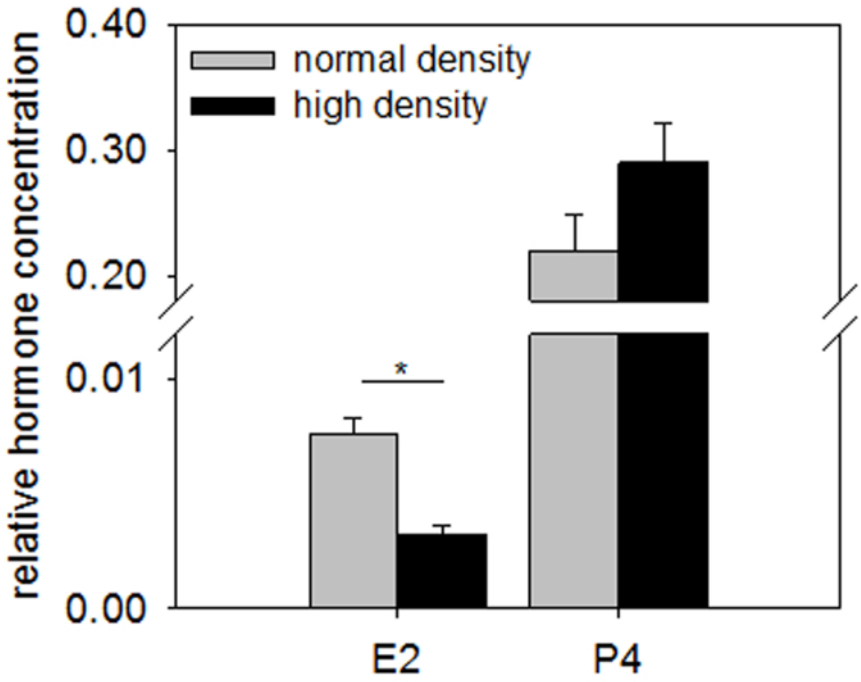

Figure 3: Steroid hormone concentration in granulosa cells cultured at different plating densities. The estradiol (E2) concentration decreased significantly when GC were cultured at a high cell density, whereas the progesterone (P4) concentration only tended to be higher. The hormone concentration was corrected for the DNA content to normalize for different cell numbers. The mean and SEM of $n = 3$ are shown. $p > 0.05$, two-tailed Student's *t*-test. This figure is reproduced from Baufeld *et al.*[15]. Please click here to view a larger version of this figure.

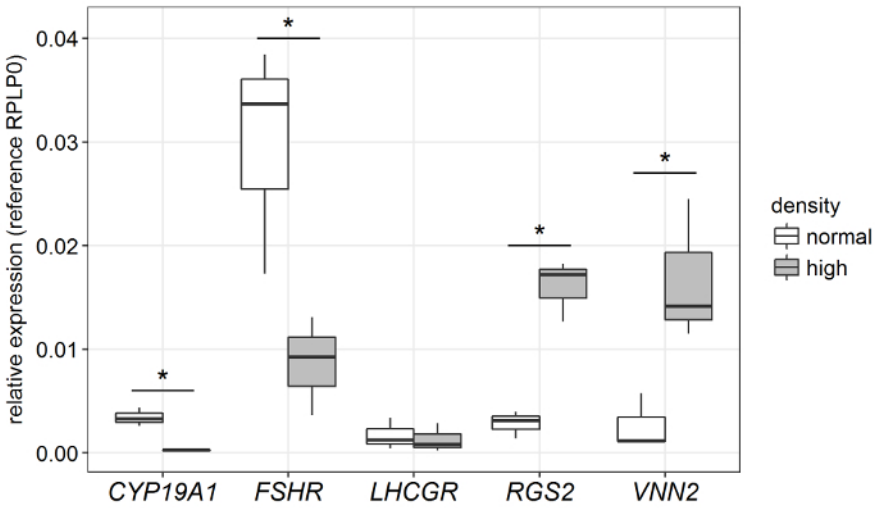

Figure 4: Abundance of functional key transcripts in granulosa cells cultured at a normal *vs.* a high plating density. Bovine GC cultured under serum-free conditions for 8 d revealed a specific regulation of several selected marker genes. The boxplot displays the median of $n = 3$ individual replicates. $p < 0.05$, two-tailed Student's *t*-test. Please click here to view a larger version of this figure.

Discussion

The presented cell culture model provides a tool to analyze granulosa cell differentiation *in vitro*. Several studies showed that a serum-free cultivation is a prerequisite to maintaining steroid activity in cultured bovine GC or GC of other species[8,9]. Additionally, coating the culture dish with components of the extracellular matrix (*e.g.*, collagen R)[13], improved the attachment of the cells significantly. Another important feature is the prolonged culture period. Recently, it has been demonstrated that a long-term culture is necessary to obtain sufficient steroidogenic activity and a balanced expression of granulosa cell identity markers[17]. It appears that GC require the time to recover from the physical stress during the isolation procedure.

The media supplements FSH, IGF-1, and androstenedione are known to induce aromatase activity in cultured GC. Especially, the supplementation with androstenedione is absolutely necessary, as the GC need a precursor for estradiol synthesis. This has been published

previously[11,18] and, therefore, was not further investigated during the present study. However, an adaptation of FSH, IGF-1, and androstenedione concentrations might be necessary for other experimental set-ups.

The cryopreservation technique described here can help to improve the organization of tissue culture experiments by making them more independent from the varying supply with ovaries. According to previous testing, cryopreservation does not affect the GC phenotype or steroid production in culture. Also, the abundance of marker transcripts in cultured cells did not reveal significant differences comparing samples prepared from freshly isolated cells with those previously subjected to cryopreservation[16].

A crucial parameter for the present GC culture model is the cell plating density. As shown by the **Representative Results**, increasing the plating density induced remarkable changes of physiological and molecular characteristics. Several genes are regulated in a specific manner, resembling the changes that are induced by LH stimulation *in vivo*[4,19]. The fact that an increasing cell density can drive differentiation-like processes in cultured bovine GC has to be meticulously considered in this GC *in vitro* model to avoid conflicting results between replicates. Therefore, contradictory results with other studies might be ascribed to different cell densities and should be examined more closely.

The culture model described here revealed to be non-responsive to LH, as the transcripts of the receptor *LHCGR* are close to the detection limit. Hence, a simulation of the LH surge alike the *in vivo* situation failed to induce differentiation[13]. Nonetheless, this model provides a helpful tool to study estradiol-active GC in primary culture, in particular as no functional bovine GC lines exist at present.

Different treatment protocols can be tested in the present GC culture model that help to unravel regulatory mechanisms of steroid production or GC differentiation. Further, single factors that are involved in developmental processes can be separately analyzed. Therefore, this culture model provides a basis for many different applications.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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