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# Effects of granulosa cells on steroidogenesis, proliferation and apoptosis of stromal cells and theca cells derived from the goat ovary☆

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ABSTRACT

The aim of this study was to investigate the effect of granulosa cells from small antral follicles on steroidogenesis, proliferation and apoptosis of goat ovarian stromal and theca cells *in vitro*. Using Transwell co-culture system, we evaluated androgen production, LH responsiveness, cell proliferation and apoptosis and some molecular expression regarding steroidogenic enzyme and apoptosis-related genes in stromal and theca cells. The results indicated that the co-culture with granulosa cells increased steroidogenesis, LH responsiveness and *bcl-2* gene expression as well as decreased apoptotic *bax* and *bad* expressions in stromal and theca cells. Thus, granulosa cells had a capacity of promoting steroidogenesis in stromal cell and LH responsiveness in cortical stromal cells, maintaining steroidogenesis in theca cells, inhibiting apoptosis of cortical stromal cells and improving anti-apoptotic abilities of stromal and theca cells.


## 1. Introduction

It is generally accepted that early follicular development, which is regulated by ovarian autocrine/paracrine regulators [1–3], is closely related to oocyte-granulosa cells-ovarian stromal cells/theca cells [4,5]. In the communication between oocyte and surrounding granulosa cells/theca cells, oocyte plays a dominant role in controlling folliculogenesis and regulating differentiation and function of surrounding granulosa cells. Oocyte is essential for follicular formation and development [6,7], because oocyte-derived factors, such as growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15), are involved in regulation of follicle formation, growth and granulocyte differentiation [8,9]. Besides oocyte itself, granulosa and theca cells also play important parts in follicular development. In the last decade, some researches on follicle cell interaction and function mainly focused on the interaction of granulosa cells with oocyte, but there were a few studies on the effect of ovarian stromal cells and theca cells on early follicular development [10].

Functional theca cell layer formation is a key physiological process for early follicular development [11]. The major functions of theca cell layer are (1) to support structure and blood containing ovarian regulators for the developing follicle, (2) to provide androgen–androstenedione and testosterone for estrogen biosynthesis of nearby granulosa cells and (3) to stimulate early follicle growth by secreting androgen [12,13]. After activating primordial follicles, primary follicles secrete some factors to recruit fibroblast-like precursor cells or stem cells from the ovarian stroma to differentiate into functional theca cells [14,15], and then form theca cell layer to play the role of autocrine/paracrine. Orisaka et al.

*Abbreviations:* SCs, ovarian cortical stromal cells; SMs, ovarian medullary stromal cells; TCs, theca cells; GCs, granulosa cells; STAR, steroidogenic acute regulatory protein; CYP11A1, Cholesterol side-chain cleavage cytochrome P450; CYP17A1, 17alpha-hydroxylase/C17, 20-lyase; HSD3B1, 3beta-hydroxysteroid dehydrogenase/isomerase; LHR, luteinizing hormone receptor; Bcl-2, B-cell lymphoma protein 2; Bax, BCL-2-associated X protein; Bad, BCL-2-associated agonist of cell death; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

☆ Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006), and were approved by the animal ethics committee of Northwest A&F University.

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investigation suggested that granulosa cells from small antral follicles mainly recruited theca cells from ovarian cortical stromal cells during early follicular development [14].

Transwell co-culture system is a 3-dimensional (3D) culture environment [16] which has been used in the study of cell migration and cancer cell invasion, and interaction between cells [17,18]. This Transwell co-culture system permits two kinds of cell populations to grow together in the same well, but prevents the direct cell-to-cell contact by a porous membrane [19]. Thus, the growth factors, cytokines and paracrine signals may be allowed to pass the porous membrane to influence the biologic activity and function of other cells [20].

In recent years, effect of granulosa cells on ovarian stromal cells to differentiate into theca cells has been studied in bovine ovary [14], but we have not found any research on the influence of granulosa cells from small antral follicles on the function of androgen production and cell proliferation and apoptosis in ovarian stromal/theca cells in the goat ovary. The objective of this study was to use the Transwell co-culture system to investigate the effects of granulosa cells on the functions of ovarian stromal cells and theca cells by measuring the change of androgen production, acquisition of LH responsiveness and cell proliferation and apoptosis in ovarian stromal cells and theca cells in the goat ovary. The findings would provide basic information for the studies on the cell interaction during follicular development.

## 2. Materials and methods

### 2.1. Isolation of SCs and SMs

According to Orisaka et al.'s research [14], we defined the cells isolated from ovarian cortex stroma as ovarian cortical stromal cells (SCs), and the cells isolated from ovarian medulla stroma as ovarian medullary stromal cells (SMs). The 20 ovaries without corpus luteum were collected from 1 to 2 years old goats at a local slaughter and dissociated according to the established laboratory standard of Northwest A&F University, China. Ovaries were transported to laboratory in a buffered salt solution within 3 h. SCs and SMs were isolated by a modified method [14]. Briefly, the tissue was first washed with 75% alcohol for 1 min, followed by three washes in D-Hank's solution to eliminate alcohol. Then, the ovarian surface epithelium was removed using forceps and the ovarian cortex and medulla was cut into about 2–3 mm thick strips. The strips containing follicles or small blood vessels were removed using an aseptic needle under stereomicroscopy (Nikon; Japan). Then, the strips were cut into small fragments and incubated in DMEM/F12 medium (GIBCO; Grand Island, NY, USA) containing 5 mg/mL collagenase (170 U/mg, type 4; GIBCO; Grand Island, NY, USA), 0.4% bovine serum albumin (BSA; GIBCO; Grand Island, NY, USA), and 0.2% glucose (pH 7.4; Sigma; St. Louis, MO, USA) for 90 min at 37 °C. The cells were dissociated by frequent agitation of the strips. After three time washing, the dispersed cells were cultured in DMEM/F12 medium with 10% fetal bovine serum (FBS; GIBCO; Grand Island, NY, USA) in 60 mm plastic dish (Corning Incorporated; Corning, NY, USA) at 37 °C under an atmosphere of 5% $CO_2$ in humidified air until use.

### 2.2. Preparation of GCs and TCs

Granulosa cells (GCs) and theca cells (TCs) from theca cells layer were isolated from antral follicles (1–4 mm diameter) by a modified methods [14,21,22] and the follicles were assumed to be in gonadotropin-independent stage and before recruitment into the further stage [1,23,24]. Briefly, the follicles with clear surface were harvested from ovaries by an aseptic needle under stereomicroscopy (Nikon; Japan). For GC collection, the antral follicles were cut into small pieces in DMEM/F12 medium and GCs were released using an aseptic needle by frequent agitation of the medium. Cell viability was estimated using trypan blue stain exclusion test. For TC isolation, antral follicles were cut into halves and the TCs were removed by a scalpel under stereomicroscopy. The remaining theca layer was cut into small fragments and TCs were dissociated by the above method. The isolated cells were washed for three times in D-Hank's solution and in DMEM/F12 medium, respectively. GCs were used immediately after collection, but TCs were cultured in DMEM/F12 medium with 10% FBS at 37 °C under the 5% $CO_2$ atmosphere and humidified air.

### 2.3. Purity of SCs, SMs and TCs

The purity of SCs, SMs and TCs was evaluated by a slightly modified method [25,26]. Briefly, 1000–3000 SCs, SMs and TCs were cultured in DMEM/F12 medium containing 10% FBS in 48-well plastic plates (Corning Incorporated; Corning, NY, USA) at 37 °C under an atmosphere of 5% $CO_2$ in humidified air for 48 h. The cells were washed with PBS for three times and fixed with 4% paraformaldehyde (Beyotime Institute of Biotechnology; Shanghai, China) solution at room temperature for 30 min. After washing and permeabilization with PBS containing 0.2% Triton X-100 (Sigma; St. Louis, MO, USA), the cells were blocked with Immunol Staining Blocking Buffer (Beyotime Institute of Biotechnology; Shanghai, China) at 4 °C for 6 h. Again after washing with PBS for three times, the cells were incubated with anti-vimentin (Rabbit; 1:100; abcom; Cambridge, MA, USA), anti-cytokeratin (Rabbit; 1:100; abcom; Cambridge, MA, USA), or anti-factor VIII (Mouse; 1:100; abcom; Cambridge, MA, USA) antibodies at 4 °C for 12 h to identify the mesenchymal, epithelial, and endothelial cells. After washing, the cells were incubated with Alexa Fluor 488/555-labeled Goat Anti-Rabbit/Mouse secondary antibody (1:500; Beyotime Institute of Biotechnology; Shanghai, China) at room temperature for 2 h. After washing with PBS, cells were incubated with DAPI Staining Solution (Beyotime Institute of Biotechnology; Shanghai, China) for 5 min, and were examined under a fluorescence microscope (Nikon; Japan).

### 2.4. Co-culture in polyester membrane Transwell-clear inserts

To examine the effect of GCs on the function of SCs, SMs and TCs, a co-culture of GCs and SCs, SMs and TCs was established in a polyester membrane Transwell-clear insert (thickness, 10 μm; growth surface area, 1.12 $cm^2$; membrane pore size, 0.4 μm; Corning Incorporated; Corning, NY, USA). About $1 \times 10^5$ viable GCs per well were cultured in Transwell-clear inserts with 0.5 mL DMEM/F12 medium containing 10% FBS at 37 °C under an atmosphere of 5% $CO_2$ in humidified air for 24 h. After the Transwell-clear inserts were transferred into 12-well plastic plates (Corning Incorporated; Corning, NY, USA), $3 \times 10^5$ SCs, SMs and TCs per well were seeded into 12-well plastic plate for culture. As controls, SCs, SMs and TCs were only cultured in the 12-well plastic plate with 1.5 mL DMEM/F12 medium. For steroid assay and Real Time quantitative PCR analyses, cells were cultured in serum-free DMEM/F12 medium supplemented with 1% Insulin-Transferrin-Selenium (GIBCO; Grand Island, NY, USA) for up to 72 h.

To determine the effect of GCs on LH responsiveness, after SCs, SMs and TCs were cultured alone or co-cultured with GCs for 24 h, they were cultured with DMEM/F12 medium with 1% Insulin-Transferrin-Selenium and LH (0, 0.01, 0.1, and 1 IU/mL; Porcine Luteinizing Hormone for Injection; Ningbo Sansheng Pharmaceutical Co., Ltd.; Ningbo, Zhejiang, China) for another 48 h [14].