Advances in In Vitro Culture of Poultry Follicular Granulosa Cells: Postprint

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Abstract

Granulosa cells in the poultry ovary play important roles in follicular development and atresia, including signal transduction, nutrient supply, and ion homeostasis. The proliferation and differentiation of follicular granulosa cells are themselves coordinately regulated by reproductive hormones and cytokines, and their underlying mechanisms represent an active area of current research. In vitro culture of poultry follicular granulosa cells can serve as an ideal cellular model for studying reproductive physiological regulation. This article provides a brief overview of the current research status on in vitro culture of poultry follicular granulosa cells and the roles of granulosa cells in follicular growth, development, and atresia, and summarizes the establishment methods for primary granulosa cell in vitro culture models.

Full Text

Research Progress on in vitro Culture Methods of Poultry Follicular Granulosa Cells

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Abstract

Granulosa cells in the poultry ovary play crucial roles in follicular development and atresia, including signal transduction, nutrient supply, and ion balance regulation. The proliferation and differentiation of follicular granulosa cells are themselves regulated by a combination of reproductive hormones and cytokines, and the underlying mechanisms represent a current research hotspot. In vitro culture of poultry follicular granulosa cells provides an ideal cellular model for studying reproductive physiological regulation. This review briefly summarizes the current research status of in vitro culture of poultry follicular granulosa cells and the roles of granulosa cells in follicular growth and atresia, and outlines the methods for establishing primary granulosa cell culture models.

Keywords: poultry; follicular granulosa cells; in vitro culture

Introduction

The ovary is the primary reproductive organ in female animals, and granulosa cells within follicles play an extremely important role in maintaining ovarian reproductive function. The follicular wall in poultry is structurally composed of the perivitelline layer, granulosa cell layer, theca interna, theca externa, and peripheral connective tissue and germinal epithelium (Figure 1 [Figure 1: see original paper]). The main functions of the follicular wall are to provide nutrients for oocyte growth and to synthesize and secrete steroid hormones, thereby regulating follicular development, growth, and ovulation [1]. Research indicates that egg-laying performance in poultry largely depends on follicular development status [1]. Follicular granulosa cells participate in follicular regulation by synthesizing hormones and growth factors (such as progesterone, stem cell factor, and epidermal growth factor), including primordial follicle recruitment, growing follicle development, dominant follicle selection, and mature follicle ovulation [2]. Studies have shown that granulosa cell apoptosis is the fundamental cause of follicular atresia, and since the number of atretic follicles directly affects egg production rate [2], granulosa cell development determines follicular growth and the ultimate laying performance of poultry. Theoretically, establishing a primary granulosa cell culture model is an important approach for exploring the endocrine function and regulatory mechanisms of the poultry ovary and follicular development mechanisms. This not only helps to study the role of granulosa cells during follicular atresia but also provides important guidance for developing measures to improve avian reproductive capacity in production.

Figure 1. Schematic diagram of poultry mature follicle structure [1]. (A) The whole mature follicle; (B) Enlarged view of the circled area in A; (C) Enlarged view of the circled area in B. 1: Primary oocyte; 2: Vitelline membrane and zona radiata; 3: Enveloping layer of the vitelline membrane; 4: Stratum granulosum; 5: Basal lamina; 6: Theca folliculi interna; 7: Theca folliculi externa; 8: Connective tissue layer; 9: Reproductive epithelium layer; 10: Follicle band; 11: Follicle stalk; 12: Artery; 13: Vein; 14: Granulosa cells.

1. Role of Granulosa Cells in Follicular Development and Growth

The transition from primordial to growing follicles in poultry is hormone-independent and is initiated through the binding of stem cell factor secreted by granulosa cells to c-Kit receptors in oocytes, thereby recruiting primordial follicles and promoting oocyte growth [2]. Cytokines such as the insulin-like growth factor family, epidermal growth factor family, and transforming growth factor-β superfamily can promote granulosa cell proliferation and progesterone secretion, participate in maintaining functional coupling between oocytes and granulosa cells, and consequently facilitate oocyte development and maturation [3]. Oocytes uptake small molecular metabolites from surrounding granulosa cells via gap junctions and regulate intracellular pH through ion exchange with granulosa cells. Oocytes and granulosa cells mutually influence each other through gap junction proteins; granulosa cells can transmit information among themselves via gap junctions to promote oocyte maturation and development, while oocytes can also secrete cytokines that feedback to promote granulosa cell proliferation and differentiation. To improve the in vitro maturation environment for oocytes, follicular layer cells such as granulosa cells are initially added for co-culture [4]. Fukui et al. [5] found that co-culture of granulosa cells with oocytes in vitro could promote oocyte maturation. Thus, the interaction between granulosa cells and oocytes determines the developmental fate of follicles [6].

Follicular atresia is a normal reproductive physiological process referring to the degeneration and eventual elimination of follicles during development prior to ovulation. In poultry, the number of atretic follicles directly affects egg production rate. Studies have identified granulosa cell apoptosis during follicular atresia in chickens, rats, pigs, cattle, and sheep; when more than 10% of granulosa cells undergo apoptosis in a developing follicle, it indicates that the follicle has initiated or is undergoing atresia, and once a follicle enters atresia, it cannot return to normal developmental trajectory [7]. Research on mammalian granulosa cells cultured in vitro has found that progesterone inhibits follicular granulosa cell apoptosis, and in birds, progesterone is primarily produced by follicular granulosa cells [2]; therefore, avian granulosa cells regulate the occurrence of follicular atresia. Numerous studies have used different hormones or drugs to induce in vitro cultured granulosa cells and investigated their effects on follicular atresia based on granulosa cell apoptosis status. For instance, Li [8] found that follicle-stimulating hormone could significantly inhibit granulosa cell apoptosis in experiments with buffalo follicular granulosa cells cultured in vitro.

In summary, follicular granulosa cells constitute the largest cell population in mature follicles, surrounding and nourishing oocytes, and play crucial regulatory roles during follicular maturation in poultry. Studying the biological regulation of granulosa cells is therefore of great significance. In vitro culture of granulosa cells represents an excellent experimental approach for such research; however,

since no established granulosa cell line currently exists, primary cells can only be obtained through isolation from follicles.

2.1. Extraction and Isolation of Granulosa Cells

The primary objective of establishing primary granulosa cell culture is to obtain sufficient quantities of viable follicular granulosa cells. Based on domestic and international literature, methods for isolating the granulosa cell layer can be broadly categorized into two types, with the choice of method depending on follicle size.

Method 1: For large follicles in poultry, the method first published by Gilbert et al. [9] for separating avian follicular granulosa cells is generally employed. Specifically, after euthanizing the animal, the ovary is aseptically extracted. Follicles are grasped with forceps, rapidly incised, inverted in a culture dish containing liquid medium, and the yolk is washed away to separate the composite membrane from the yolk. The obtained composite membrane consists of the perivitelline layer, granulosa cell layer, and basal lamina.

Method 2: For small yellow follicles, the isolation of the granulosa cell layer is modified from the method of Gilbert et al. [9]. After yolk release, the composite membrane is inverted in sterile physiological saline, agitated to allow the granulosa cell layer to separate and settle, and the precipitate is collected as the granulosa cell layer and dispersed granulosa cells. Current isolation methods for avian follicular granulosa cells do not yield pure granulosa cells, as they contain yolk and follicular theca cells. In Method 1, the granulosa cell layer is relatively loose and often mixed with yolk, though yolk can be reduced through cell washing, whereas Method 2 may affect cell viability due to agitation of the composite membrane.

After separating the granulosa cell layer, further extraction is required to obtain granulosa cells, which can be achieved through mechanical dissociation or enzymatic digestion [10]. Mechanical dissociation: The obtained granulosa cell layer is placed in pre-cooled phosphate-buffered saline (PBS), minced with ophthalmic scissors, and filtered to obtain granulosa cells [11]. Enzymatic digestion: The granulosa cell layer is digested in culture medium containing collagenase, and after termination and centrifugation, the precipitate consists of granulosa cells. Asem et al. [10] summarized that mechanically dissociated granulosa cells cultured in Earle' s Balanced Salt Solution (EBSS) secreted significantly less progesterone in response to hormone stimulation compared to enzymatically digested granulosa cells cultured in M199 medium. In recent years, domestic and international reports have typically employed a combination of mechanical and enzymatic digestion methods, where after mechanical isolation of the granulosa cell layer, it is directly minced into fragments (without adding buffer solution) and then digested with culture medium containing collagenase type II, followed by filtration after digestion termination. After digestion, erythrocyte lysis is recommended; as shown in Figure 1, the isolated composite membrane layer

contains veins, and the extracted granulosa cells will also contain blood cells, which affect cell morphology observation. However, erythrocyte lysis may affect cell viability, and therefore can be selected based on experimental objectives.

2.2. Culture Methods for Granulosa Cells

Granulosa cell culture typically involves supplementation of culture medium with serum (primarily fetal bovine serum, FBS) to provide essential nutrients, binding proteins, and factors promoting cell adhesion and spreading, thereby protecting cells from mechanical damage. Depending on experimental requirements, FBS supplementation in avian granulosa cell culture medium generally ranges from 0% to 10%; some experiments add 0.1% bovine serum albumin (BSA) to serum-free or low-serum medium [6, 12–18]. However, Chiang et al. [19] found that BSA may alter granulosa cell physiological responses; for example, BSA addition can rapidly increase voltage-activated calcium currents in granulosa cells. Therefore, BSA supplementation is not recommended when studying granulosa cell function. Picton et al. [20] demonstrated that serum-free medium could maintain normal differentiation of porcine follicular granulosa cells cultured in vitro. Although omitting serum reduces interference with experimental treatments, it affects cell growth to some extent (as serum contains growth-promoting factors). To balance cell growth and experimental treatment, researchers often culture cells in medium containing 5% serum, observe cell adhesion, then replace the original medium with serum-free medium and continue culture for 24–48 h before experimental treatment, thereby reducing interference from unknown substances in serum [21]. The in vitro research model for chicken granulosa cells established by Professor Zhang Caiqiao' s laboratory at Zhejiang University involves culturing small yellow follicle granulosa cells in low-concentration (0.5%) FBS for 16 h before switching to serum-free medium [6].

Temperature is an essential requirement for maintaining cell growth, and optimal culture temperature varies among cell types. Generally, cell viability increases with temperature until reaching the upper tolerance limit, beyond which cell viability becomes inhibited and cellular function is compromised [22]. The in vitro culture temperature for granulosa cells differs among animal species. Human and porcine granulosa cells are cultured at 37 °C [23-24], rat and mouse granulosa cells are generally cultured at 37 °C, while bovine and ovine granulosa cells require 38.0–38.5 °C. Poultry have higher body temperatures than mammals, thus it is inferred that culturing poultry follicular granulosa cells at 38.5–41.0 °C may yield higher proliferation rates than at 37 °C. For example, Wen et al. [14] cultured goose F1–F3 follicle granulosa cells at 38.5 °C; Rangel et al. [23] chose 41 °C for culturing chicken F1 follicle granulosa cells, although many researchers still opt for 37 °C.

In addition to appropriate temperature, cell growth depends on nutrient supply. Regarding culture medium selection, mammalian granulosa cell culture media vary among species. Dulbecco' s Modified Eagle Medium (DMEM) works well

for rat or mouse granulosa cells, while M199 medium yields higher survival rates for ovine granulosa cells [21]. Asem et al. [10] compared different media and found that chicken follicular granulosa cells secreted the most progesterone when cultured in M199 or F12 medium [7], whereas Wen et al. [14] used DMEM/F12 medium for culturing goose follicular granulosa cells in vitro [14]. Most avian granulosa cell studies have selected M199 medium [21].

2.3. Identification of Granulosa Cells

After obtaining follicular granulosa cells, cell viability must first be assessed using trypan blue staining [14], generally requiring cell viability above 90%. Cell proliferation rate can also be measured using the thiazolyl blue tetrazolium bromide (MTT) assay [25] or the cell counting kit-8 (CCK-8) assay [14] to observe cell proliferation. In the MTT assay, errors increase due to the influence of different lysing agents' solubilization capacity and their background signals on optical density (OD) measurements, as well as the tendency to lose some cells during manipulation. In contrast, the CCK-8 reagent results in minimal cell loss and smaller errors during actual operation [26].

Isolated granulosa cells can be identified through immunohistochemistry for follicle-stimulating hormone receptor (FSHR) or anti-Müllerian hormone (AMH). Granulosa cells are the only cells in ovarian tissue that express FSHR, and numerous studies have selected FSHR immunohistochemical staining for identifying follicular granulosa cells. AMH, a member of the transforming growth factor-β superfamily, is primarily expressed in granulosa cells of growing follicles but not in atretic follicle granulosa cells or theca cells; therefore, some studies have also chosen AMH immunohistochemical staining for granulosa cell identification [27].

Research on granulosa cell culture began in the early 1970s. Through continuous improvement and refinement, in vitro culture methods for human and mammalian granulosa cells have become an important tool for studying reproductive physiology and endocrinology, and have gradually been applied to research on poultry granulosa cells and follicular development regulation. Compared with animal experiments, in vitro culture of follicular granulosa cells offers advantages of lower cost, shorter duration, and easier reproducibility, providing distinct benefits for studying the production and regulation of follicular cell steroid hormones. In vitro culture of granulosa cells can avoid interference from other cytokines, facilitating deeper investigation into the physiological regulatory mechanisms of granulosa cells. Follicular granulosa cell culture can be used not only to analyze the physiological activity of hormones such as FSH, but also to explore the effects of certain factors or drugs on the ovary.

However, granulosa cells isolated from poultry follicles have not been assessed for purity using FSHR immunohistochemistry, nor have purification methods been investigated. Consequently, granulosa cells obtained through primary cell isolation may contain oocytes, theca interna cells, yolk, and other substances.

The isolated and cultured granulosa cells still face many challenges, such as whether they can maintain their viability, proliferative capacity, and characteristic phenotype over extended periods. Studies have found that long-term cultured primary granulosa cells gradually assume a fibroblast-like morphology, with most cells beginning to show degenerative responses around day 7 of culture [28]. Therefore, optimizing granulosa cell purification methods, extending the growth duration of granulosa cells during primary culture, and preventing degenerative responses are important research directions for granulosa cell culture methods. Achieving these goals will undoubtedly provide reliable methods for more in-depth studies on the effects of gonadotropins, steroid hormones, and polypeptide growth factors on granulosa cell function.

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