Methods and Techniques

Vol. 4, No. 1 (2020)
ISSN: 2532-5876

DOI: 10.13133/2532-5876/16965

# A Challenging Task—How to Successfully Separate Theca and Granulosa cells: A Mandatory Step for Investigating Ovary Steroidogenesis

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**Citation:** Fedeli, V, Monti, N, Canipari, R, 2020, "A Challenging Task—How to Successfully Separate Theca and Granulosa Cells: A Mandatory Step for Investigating Ovary Steroidogenesis", *Organisms; Journal of Biological Sciences*, vol. 4, no. 1 (2020), pp. 86-89. DOI: 10.13133/2532-5876/16965.

## The relevance of Theca/Granulosa cell studies

The reciprocal interaction in between theca (TCs) and granulosa (GCs) cells plays a pivotal role in allowing ovary to display a wide range of physiological functions, including steroidogenesis and reproduction capabilities. For instance, changes in the respective proportion among GCs and TCS have been correlated to progression or recovery from Polycystic Ovary Syndrome (Bevilacqua, et al. 2019). To investigate how these cells cooperate, it is important to understand how they work in isolation. Therefore, segregation of the two cell clusters constitutes a pre-requisite for planning functional studies on the ovary.

GCs are a homogeneous population of cells, a few differences between cumulus and mural cells notwithstanding. On the contrary, theca cells surrounding the antral follicle are a mixed population, composed of an inner (theca interna) and an outer layer of cells (theca externa) harbouring some appreciable morphological and functional features. The theca interna contains cells committed to endocrine function. The theca externa is a fibrous, connective tissue layer derived from fibroblast-like cells. In many mammalian species, theca cells associated with follicles undergoing atresia, still survive and remain in the ovarian stroma as nests of endocrine cells, known as secondary interstitial glands (Erickson, et al. 1985). Therefore, hereafter we shall refer to these isolated populations as theca/interstitial cells (TICs), given that both cell populations have been demonstrated to express high levels of enzymes involved in androstenedione synthesis, as 3β-Hydroxysteroid dehydrogenase (3β-HSDH) (Magoffin and Erickson 1988). Complete separation of these clusters is deprived of physiological utility, while a number of methodological hurdles make almost unpractical to actually separate them.

Indeed, to address such difficulties, several different approaches have been so far published, as briefly sketched in the followings:

1. Magoffin et al. isolate GCs and Theca/interstitial cells (TICs) from hypophysectomized immature rat ovaries. Four days after hypophysectomy ovaries are

collected, cut into 4-6 pieces, and digested in a collagenase-DNase solution (4 mg/ml of collagenase, 10 pg/d of DNase, 10 mg/ml of BSA in Medium 199 (Magoffin and Erickson 1988).

The cell suspension thus obtained is carefully layered on top of a linear (20-70%) or a discontinuous Percoll gradient. and centrifuged (4°C) at 400 x g for 20 min. The different cell populations are separated in different bands depending on their specific density. After measuring the positions of the cell bands, the bands are individually collected by aspiration.

After separation with a linear Percoll gradient, the purity of the cells is low (65%). For better cell purity cells were separated by a discontinuous gradient. This is important because the discontinuous gradient generates density steps which act as a thermodynamic barrier for the single cells. For this, the cells are blocked in the specific band and it is possible to isolate them with a higher level of purity (93%).

Each cell fraction is then washed with medium and resuspended in a known volume of medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin sulfate and 2 mM L-glutamine.

However, even though the cell purity is above 90%, the use of hypophysectomized rats is a "complicated" method for studying the endocrine regulation of ovarian TIC differentiation and not differentiated preovulatory TI cells.

The successful purity rates achieved by the Magoffin's method notwithstanding, this technique of cell segregation has been left aside by the next generation of studies. Likely, this method was discarded as the investigation was focused on human ovary cells, usually showing larger sizes than those provided by rodents, for which a direct mechanical approach was found equally reliable.

2. In the paper by Liu (Liu et al. 2017), cells are isolated from 6-week-old mice. In this study, GCs are obtained by puncturing the antral follicles with hypodermic needles, while the remaining tissue is digested with collagenase. With this method, GCs result in an almost pure population. However, as already highlighted (Ma and Hao 2018), this approach does not allow to obtain TICs with enough purity, i.e. TICs resulted usually contaminated in high proportion by GCs from secondary and preantral follicles. Likely, this unwarranted contamination arises as large follicles are mixed together with small follicles, in which GCs can hardly be segregated from TICs.

Such shortcomings hinder the reliability of the technique and the possibility to recruit a number of TICs with sufficient purity for planning successive function-related studies.

## Isolation of Theca/interstitial and Granulosa cells from antral follicles

To address these issues, herein we describe an improved method for the isolation of TICs and GCs from the murine ovary.

The method is in fact an in-depth revised version of one already described technique (Innocenti, et al. 2017), with some modifications.

We isolated ovaries from adult (6/8-week-old) female CD1 mice 48 h after subcutaneous injection with PMSG (Pregnant Mare Serum Gonadotropin) to stimulate folliculogenesis.

After 48 hours of PMSG treatment, the animals were killed using CO2. All animal procedures have been approved by the local ethics committee for animal research.

Ovaries were removed, placed in CMF (Calcium-Magnesium free PBS) and freed from the surrounding tissues under a stereomicroscope. To avoid contamination by small follicles, we first dissected large follicles away from the ovarian stroma, and then GCs were collected by puncturing and gently pressing these follicles with 25 gauge-needles in M2 medium supplemented with 0.3% BSA. GC suspension was then centrifuged at 1000 rpm for 8 min and resuspended in DMEM with 5% FBS, 1% Glutamine, 1% P/S and Gentamicin 0.4 mg/mL. After three washes with the same medium, GCs were cultured at a density of 1 x 106 cells in 60 mm dish.

The residual tissue (TICs) was smoothly scraped to eliminate adherent GCs and digested by incubation with a collagenase-DNase solution containing 4mg/mL collagenase IV, 10mg/mL DNase and 10mg/mL BSA in DMEM for 1 hour under agitation. At the end of this period, we blocked collagenase with medium supplemented with 5% FBS, filtered the cell suspension, washed the dispersed cells three times with DMEM with 5% FBS, 1% Glutamine, 1% P/S and Gentamicin 0.4 mg/mL. The cells were then cultured in 60 mm dishes.

## Identification of theca/interstitial and Granulosa cells

### Morphology

Isolated cells were observed at different times (48, 72 and 120 h). TICs and GCs were observed under light microscope, showing different morphological characteristics. TICs have a fibroblast-like phenotype, a fusiform or triangular shape, while GCs are polygonal or cuboidal, as previously described (Tian et al. 2015).

No morphological signs of apoptosis have been observed neither at 72 nor at 120 hours (Figure 1).

### Molecular characterization

To further investigate GCs and TICs populations, cells were lysed in lysis buffer for RNA extraction.

The two cell populations display a different molecular profile, which helps in recognizing their mutual differences. Namely, several markers differ significantly between the two cell populations, as reported in the literature (Hatzirodos et al. 2015).

We investigated the expression of FSH receptor (FSH-R), 17α-hydroxylase (CYP17A1), 3β-Hydroxysteroid dehydrogenase (3βHSD) genes by real time-PCR.

As expected, the expression of FSH-R was mainly observed in GCs, while in TICs its expression was almost undetectable. On the contrary, expression of CYP17A1 and 3βHSD, two markers of theca endocrine cells, was found mostly in TICs (Figure 2). Statistically, distribution was highly significant on average.

This selective compartmentalization of gene expression epitomizes the differences between the two cell populations. Noticeably, these specific patterns of gene expression reflect a very basic distinction in biological function and physiological properties among the two groups.

TICs are involved in the androgen synthesis and the production of androstenedione and testosterone. In turn, both these androgens are the very basic substrates necessary to GCs to synthesize 17-beta-estradiol and other estrogens. GCs respond to FSH stimulation with an increased expression of aromatase and a consequent increase of estradiol.

It is noteworthy that only a few GCs express CYP17A1 and 3βHSD, whereas only a small proportion of TICs show detectable levels of FSH-R mRNA.

The method we propose, although being similar to that described by Liu (Liu et al. 2017), presents some

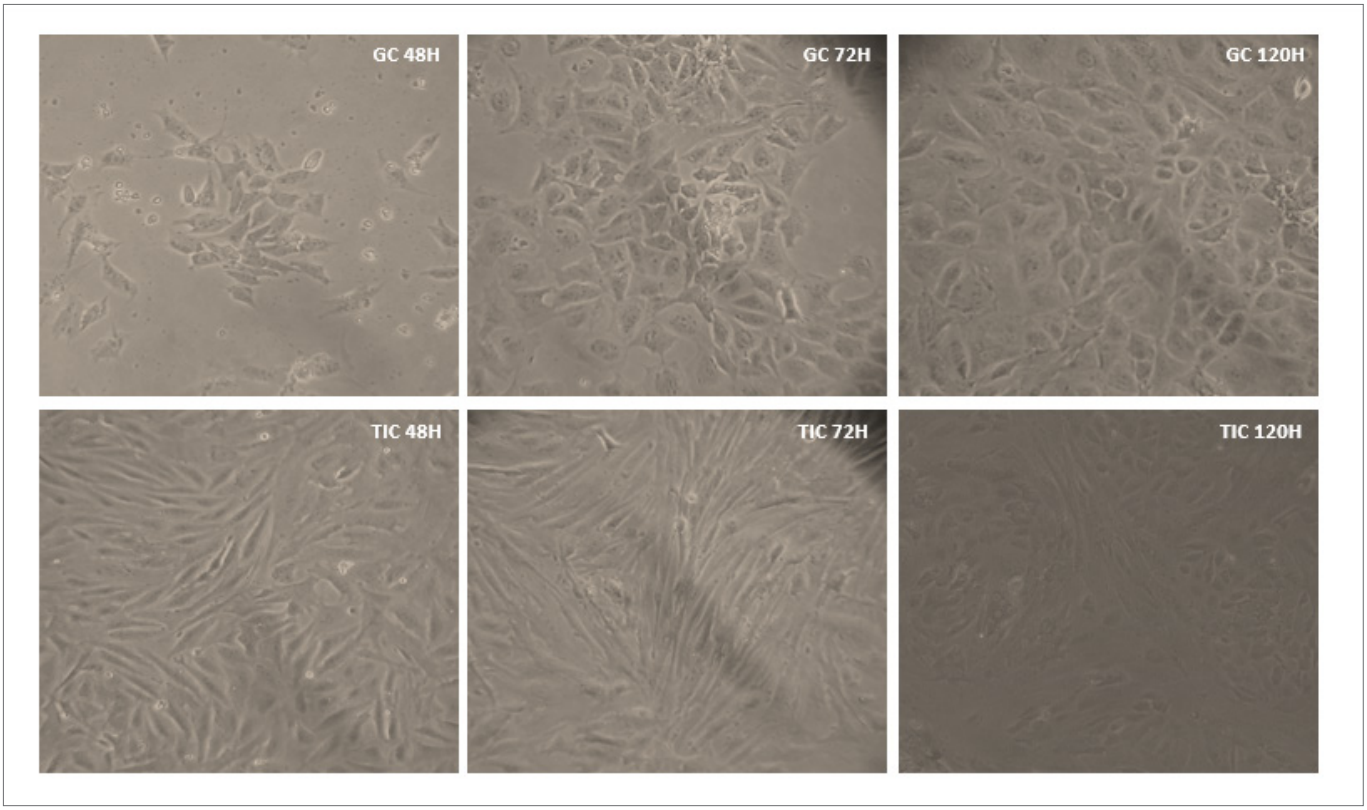


**Figure 1:** Morphological characteristics of GCs and TICs, light microscope. Magnification: 40x.

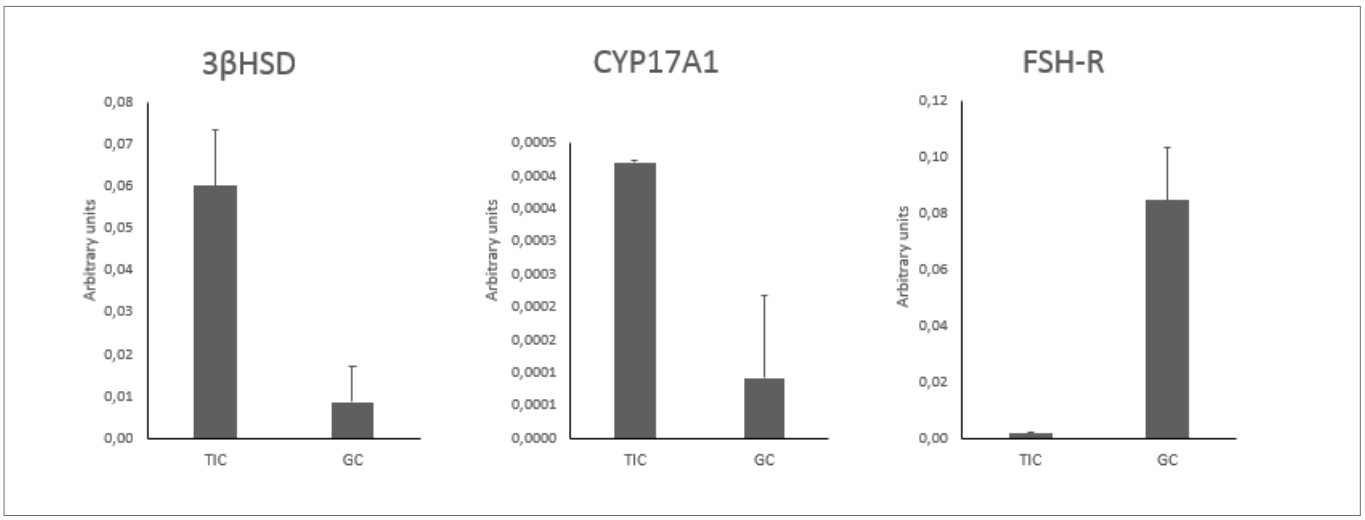


**Figure 2:** Differential genes expression in GCs and TICs. Values are normalized versus Actin expression. These differences reached statistical significance in all the studied markers. 3βHSD: P = 0.023; CYP17A1: P = 0.049; FSH-R: p = 0.020. Results are the mean ± s.e.m. of 3 independent experiments.

critical differences. To avoid the mix between GCs and TICs, mostly depending on the compresence of small follicles, we isolated preantral follicles with a 25 gauge-needles, under the stereomicroscope, and then we discarded them. This approach helps in eliminating granulosa cells from preantral follicles that otherwise might pollute theca cells. Consequently, we were able to recover both TICs and GCs with a high degree of purity.

Further studies are warranted to confirm the suitability of the proposed method and to improve isolation techniques for TCs and GCs.

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