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Preparation of highly purified porcine theca cells

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A novel method for purifying dispersed porcine theca cells, with less than 3% granulosa cell contamination, was developed by the repeated use of mechanical and enzymatic procedures. The steroidogenic criteria used for the identification and purity evaluation of both theca and granulosa cells were also improved. Purified theca and granulosa cells from medium-sized follicles displayed steroidogenic differences when they were cultured in the presence of 10% fetal bovine serum: (1) the theca cells synthesized oestradiol (239.1 ± 35.1 pg ml^{-1} per 2.5×10^5 cells in 40 h), but the granulosa cells did not synthesize it unless aromatizable androgens were added; (2) theca cells synthesized androstenedione (73.2 ± 14.4 ng ml^{-1} per 2.5×10^5 cells in 40 h), but granulosa cells did not; (3) FSH did not affect progesterone production in theca cells; (4) the theca cells secreted androstenedione for up to 48 h; and (5) FSH significantly stimulated progesterone production in granulosa cells during a culture for 40 h ($P < 0.05$), but not during culture for 12 h. The lack of response to FSH was used as a reliable, functional indicator of the purity of porcine theca cells. However, this criterion proved not to be useful for cells cultured for 12 h; porcine FSH had no effect on the progesterone production of theca cells co-cultured for this time with as many as 20% granulosa cells. However, after co-culturing for 40 h, this criterion resulted in the detection of only 3% granulosa cell contamination. Lack of response to FSH is a sensitive and reliable criterion for evaluating the purity of porcine theca cells, as long as FSH responsiveness of granulosa cells is fully confirmed.

Introduction

It is well known that ovarian steroidogenesis is accomplished by the co-operation of two types of somatic cell under gonadotrophic stimulation: granulosa cells and theca cells (Falk, 1959; Short, 1962; Armstrong and Perkoff, 1976; Fortune and Armstrong, 1978). Recent studies have further elucidated the fine intrafollicular events and regulatory autocrine, paracrine and endocrine mechanisms responsible for the growth and differentiation of each cell type (Gore-Langton and Armstrong, 1988). The significance of investigating pure preparations of each cell type has therefore become even more important than before. In mammals, the relatively simple culture system for isolated granulosa cells was established in the early 1960s (Bjersing, 1962; Channing, 1966, 1969; Channing and Ledwitz, 1975) and information on granulosa cell function has accumulated based on this culture system. In contrast, a dispersed theca cell culture was developed much later than the granulosa cell culture, and the information regarding theca cell function is still scanty. The main reason for this is that theca cells are embedded in connective tissue; isolating pure theca cells free from contamination by granulosa and other cells is therefore quite difficult. Although studies of dispersed mammalian theca cells have gradually become more common, only a few theca cell preparations have been established with confirmed purity.

The first successful mammalian theca cell preparation was made from the porcine ovary by Stoklosowa *et al.* (1978). Their method of cell preparation consisted of a combination of mechanical and enzymatic procedures. The subsequent identification and purity evaluation of the collected cells were made adequately using three morphological criteria. However, the collected theca cells (70% of the total), included 8% granulosa cell contamination in addition to the presence of 23% non-steroidogenic cells. Thereafter, several studies on dispersed mammalian theca cells (some of which were prepared essentially according to this method) have led to the accumulation of our knowledge of steroidogenesis in theca cells *in vitro*. Accordingly, when the identification and purity evaluation of theca cell preparations have been performed, more stress has been put on the steroidogenic rather than on the morphological differences between theca and granulosa cells. These differences can be summarized as follows: (1) porcine theca cells can synthesize C_{18} steroids, but granulosa cells cannot without the addition of extrinsic aromatizable androgens (Evans *et al.*, 1981; Tsang *et al.*, 1985, 1987); (2) porcine theca cells can synthesize C_{19} steroids, but granulosa cells cannot (Short, 1962; Bjersing and Carstensen, 1967; Younglai and Short, 1970; Fortune and Armstrong, 1978); and (3) FSH binds to granulosa cells, but not to theca cells. FSH does not therefore affect the steroidogenesis of theca cells (Nakano *et al.*, 1977; Fay and Douglas, 1987).

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In a recent study, May *et al.* (1992) used theca cell preparations that met only the second criterion mentioned above instead of strictly satisfying all three, whereas many theca cell preparations do not meet any definitive steroidogenic and morphological criteria (Evans *et al.*, 1981; Tonetta *et al.*, 1986; Morley *et al.*, 1989, 1990). In addition, meeting only the third criterion, without indicating the degree of purity, has been accepted as validation of a purified theca cell preparation (Hunter and Armstrong, 1987; Caubo *et al.*, 1989; Engelhardt *et al.*, 1991). Thus far, it has not been rigorously established whether these criteria are appropriate and necessary for the steroidogenic evaluation of theca cell preparations.

The objective of the present study was to obtain highly purified dispersed theca cells and to evaluate the steroidogenic criteria used for the definite identification of these theca cells. Theca cells were prepared from the porcine ovary so that they met all three criteria by eliminating granulosa cell contamination as thoroughly as possible through a combination of mechanical and enzymatic procedures. We report here the method of isolation of this highly purified theca cell preparation, in which the granulosa cell contamination was less than 3%. We also report that, among the three criteria, FSH unresponsiveness is the most useful steroidogenic criterion for evaluating theca cell purity, and that it is sufficiently sensitive to detect as little as 3% granulosa cell contamination under culture conditions where the FSH responsiveness of granulosa cells can be confirmed.

Materials and Methods

Cell preparation and culture conditions

Figure 1 shows a flow chart of the cell preparation procedure. Ovaries were obtained from prepubertal gilts at a local abattoir, and transported to the laboratory on ice. Nonatretic and healthy follicles (macroscopical criteria according to Moor *et al.*, 1978) of 3–6 mm in diameter were dissected out. Each follicle was placed in a Petri dish containing Dulbecco's PBS without calcium and magnesium (CMF-PBS), cut in half with a pair of fine dissection scissors, and the granulosa cells removed by gently scraping the inside of the follicle with a heat-bent Pasteur pipette. The free-floating cells in the CMF-PBS thus consisted of granulosa cells, and the remaining part of the follicles consisted of thecal sheets attached by the granulosa cells. The floating granulosa cells were enzymatically digested in a shaking water bath for 5 min at 37°C in RPMI 1640 medium (Gibco, New York) with 0.024% (w/v) Type IA collagenase and 0.001% (w/v) Type I-S hyaluronidase (Sigma Chemical Co., St Louis, MO), filtered through a 100 μm nylon mesh membrane and centrifuged at 400 ***g*** for 10 min. The pellet was washed three times with RPMI 1640 medium and recentrifuged. The cells (granulosa cells) were finally resuspended in culture medium.

It was necessary to eliminate the granulosa cells attached inside the follicles to obtain the theca cells from the thecal sheets. The most external layer of granulosa cells is sometimes attached very strongly to the basement membrane (Stoklosowa *et al.*, 1978). The residual follicles were enzymatically digested in a shaking water bath for 10 min at 37°C in RPMI 1640 containing 0.024% (w/v) collagenase and 0.001% (w/v) hyaluronidase to ensure that the granulosa cells that could not be eliminated by scraping alone were easily detachable. They were then filtrated through a 1 mm square steel-mesh basket and washed out with a vigorous stream of a large quantity of CMF-PBS using a pipette to eliminate the detached granulosa cells from the thecal sheets (Stoklosowa *et al.*, 1978). The thecal sheets were collected in a Petri dish filled with CMF-PBS, agitated and then transferred to another Petri dish filled with fresh CMF-PBS. The thecal sheets were then minced and placed on a steel mesh basket; they were then washed out with a vigorous stream of CMF-PBS until no more granulosa cells could be removed from the minced thecal sheets. The minced thecal sheets were enzymatically digested in a shaking water bath for 60 min at 37°C in RPMI 1640 containing 0.12% (w/v) collagenase and 0.005% (w/v) hyaluronidase, filtered through a 100 μm nylon mesh and centrifuged at 400 ***g*** for 10 min. The pellet was washed three times and recentrifuged in RPMI 1640

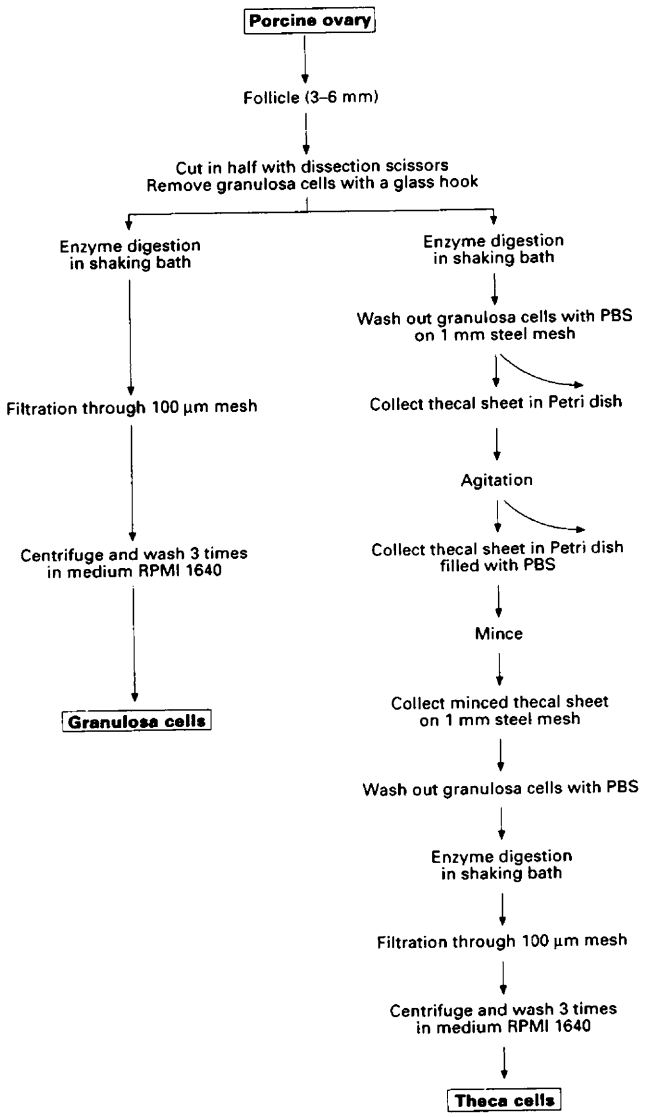

Fig. 1. Flow chart of the cell preparation procedure for porcine theca and granulosa cells.

and the purified theca cells were finally resuspended in culture medium.

Granulosa and theca cells were resuspended in RPMI 1640 medium containing 10% fetal bovine serum (Whittaker Bioproduct, Walkersville, MD). Cell viability was determined by Trypan blue exclusion (routinely 85–95%), and the number of cells was determined using a haemocytometer. In a series of 14 experiments, the average number of collected follicles was 6.4 ± 0.4 per ovary, and the average number of viable granulosa and theca cells was $41.8 \pm 5.6 \times 10^4$ and $49.2 \pm 3.6 \times 10^4$ per follicle (mean ± SEM), respectively. Both the RPMI 1640 medium and Dulbecco's CMF-PBS were supplemented with 10 000 U crystalline penicillin G potassium l^{-1} and 100 mg streptomycin sulfate l^{-1} (Meiji Seika Kaisha Ltd, Tokyo).

Cytospin smears of dispersed cells were made using a Shandon cytocentrifuge (King *et al.*, 1989) and were air dried. Some slides were stained with Oil Red O to visualize lipid droplets within steroidogenic cells (Stoklosowa *et al.*, 1978). Other slides were stained with nitro-blue tetrazolium to demonstrate the activity of 3β-hydroxysteroid dehydrogenase (3β-HSD), an enzyme that promotes progesterone synthesis in steroid-producing cells (Wattenberg, 1958). In the theca cell preparation 81 ± 7% cells stained positively for Oil Red O and 94 ± 1% stained positively in the granulosa cell preparation. In the theca cell preparation, 85 ± 7% cells stained positively for 3β-HSD while 75 ± 6% stained positively in the granulosa cell preparation (mean ± SEM of four independent experiments). 3β-HSD stained more strongly in theca cells than in granulosa cells.

Cell incubation

Aliquots of 2.5×10^5 dispersed viable cells ml^{-1} were inoculated into each well of 24-well plates (Corning; Iwaki Glass, Tokyo) in 2 ml of culture medium, in triplicate or quadruplicate. The cultures were incubated at 37°C under a humidified atmosphere of 5% CO_2 in air. Testosterone (Wakenyaku Co. Ltd, Osaka) was added to the culture media in 20 µl aliquots and dissolved in 95% ethanol to a final concentration of 0.1 µmol l^{-1}. The same volume of ethanol vehicle was added to the control media. At the end of the culture period, the media were collected and stored at − 20°C until the steroid assay was performed.

Porcine FSH

Porcine FSH used in these experiments was USDA-pFSH-I-1 (AFP-1064B), and was a generous gift from J. Bolt (USDA). Its biological FSH potency, as determined by hCG augmentation, was 82 times greater than that of NIH-FSH-S1. LH contamination as determined by radioimmunoassay (reference preparation AFP-4932B from USDA) was 0.015 ng ng^{-1} pFSH. pFSH was diluted in CMF-PBS with 1% (w/v) BSA and was stored frozen in small aliquots. They were thawed immediately before each experiment.

Steroid determination

Androstenedione was assayed after extraction with diethyl ether by radioimmunoassay, and progesterone and oestradiol were assayed without extraction by radioimmunoassay, using commercially available kits (Coat-A-Count: Nippon DPC Corporation, Tokyo). The androstenedione antiserum exhibited low crossreactivity for dehydroepiandrosterone (4.5%), oestrone (0.82%) and testosterone (0.25%), and less than 0.2% for progesterone. The antiserum against progesterone showed negligible crossreactivity (< 0.1%) for pregnenolone. The antiserum against oestradiol gave negligible crossreactivity (< 0.1%) for androstenedione, testosterone and progesterone. The sensitivities of the steroid assays were 0.01 ng ml^{-1} for androstenedione, 0.05 ng ml^{-1} for progesterone and 8 pg ml^{-1} for oestradiol. The interassay and intra-assay coefficients of variance for all the above steroid assays in the spent media were < 10%.

Statistical analyses

Statistical analyses were performed by analysis of variance. Whenever significance was observed, Scheffe's *F*-test was used for multiple comparisons. Comparisons between the two groups were performed by a paired *t* test. Data were expressed as the means ± SEM from several independent experiments performed in triplicate. In the experiments that studied the time course of androstenedione secretion by theca cells and that detected the rate of granulosa cell contamination by progesterone secretion in response to FSH, the values were the means ± SD of a representative experiment from several experiments performed in triplicate or quadruplicate. Differences were considered to be statistically different at $P < 0.05$.

Results

Oestradiol secretion by theca and granulosa cells

Theca cells alone and granulosa cells alone were inoculated at a concentration of 2.5×10^5 cells ml^{-1} culture media with 10% fetal bovine serum to ascertain the amount of oestradiol secreted by the theca and granulosa cells. The cells were cultured in the presence or absence of the substrate androgen testosterone (0.1 µmol l^{-1}), and were incubated for 40 h. Although the theca cells alone secreted 239.1 ± 35.1 pg oestradiol ml^{-1}, the granulosa cells alone did not secrete any detectable amount of oestradiol. In the presence of 0.1 µmol testosterone l^{-1}, the theca cells and the granulosa cells secreted 350.6 ± 35.2 and 739.9 ± 61.3 pg of oestradiol ml^{-1}, respectively.

Androstenedione secretion by theca and granulosa cells

In the same manner, the amounts of androstenedione secreted by the theca and granulosa cells cultured for 12 h and 40 h were examined. The granulosa cells alone secreted no detectable amount of androstenedione after 12 h and 40 h. However, the theca cells secreted 32.3 ± 5.5 and 73.2 ± 14.4 ng androstenedione ml^{-1}, respectively, during culture for 12 h and 40 h. Moreover, pFSH (5 ng ml^{-1}) had no significant effect on androstenedione secretion by theca cells cultured for 40 h (77.3 ± 14.5 ng ml^{-1}).

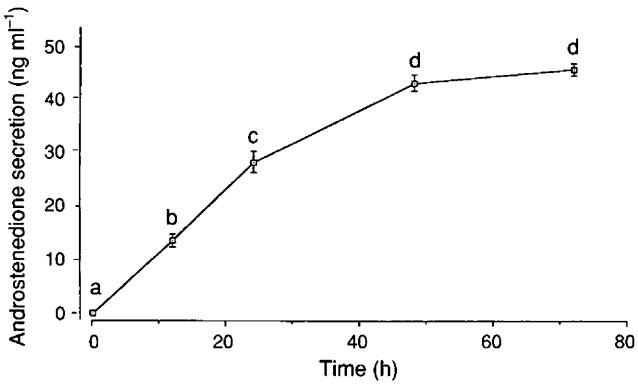

Fig. 2. Time course of androstenedione accumulation by porcine theca cells. Cells were cultured in medium containing 10% fetal bovine serum. The media were collected at the indicated times (12–72 h) after inoculation. Values represent the mean ± SD of quadruplicate cultures from a representative experiment. Values with different superscripts are significantly different ($P < 0.05$).

Time course of androstenedione secretion by theca cells

Figure 2 shows the time course of androstenedione production by the theca cells over 72 h. The production of steroid increased significantly ($P < 0.05$) until 48 h of culture; thereafter, the rate of production declined up to 72 h. Thus, all subsequent incubations were carried out for 40 h.

Progesterone secretion by theca cells and granulosa cells in response to FSH

There were no significant differences between the amounts of progesterone secreted by the granulosa cells cultured for 12 h in the absence and presence (5 ng ml^{-1} and 10 ng ml^{-1}) of pFSH. However, after being cultured for 40 h, the amount of progesterone secreted increased by a factor of about 60, 180 and 190, respectively, compared with the corresponding culture conditions for 12 h. The differences in progesterone secretion after culture for 40 h were significant ($P < 0.05$). The amount of both basal and pFSH-stimulated progesterone secretion by granulosa cells therefore increased markedly from 12 h to 40 h after inoculation (Table 1).

Similarly, there were no significant differences among the amounts of progesterone secreted by the theca cells cultured for 12 h in the absence and presence (5 ng ml^{-1} and 10 ng ml^{-1}) of pFSH. However, when cultured for 40 h, these concentrations increased only by a factor of about 3, 3 and 4, respectively, compared with values obtained after 12 h. There were no significant differences between culture periods of 12 h and 40 h, except that cells cultured in the presence of 10 ng FSH ml^{-1} for 40 h produced more progesterone than did other cells cultured for 40 h in the absence of FSH or in the presence of 5 ng FSH ml^{-1} ($P < 0.05$).

Steroid secretion in co-cultures of 80% theca cells and 20% granulosa cells

The above results show that when granulosa cells are cultured for 12 h they secrete very little progesterone and their response to FSH is not detectable after this time. We therefore examined whether theca cells that were artificially contaminated with granulosa cells and were cultured for 12 h could respond to FSH by increasing progesterone production. Progesterone secretion by theca cells contaminated with as many as 20% granulosa cells did not respond significantly to the addition of 1–10 ng FSH ml^{-1} (Table 2).

The basal oestradiol and androstenedione secretions during a culture period of 12 h were then examined (Table 2). The amount of oestradiol secreted by the co-culture was about 12.4 times and 4.8 times greater than the secretions of the theca cells alone and the granulosa cells alone in the presence of 0.1 μmol testosterone l^{-1}, respectively. The amount of androstenedione secreted by the co-culture was 36% less than that secreted by theca cells alone.

Detection of granulosa cell contamination by progesterone secretion in response to FSH

As shown above, progesterone secretion by the granulosa cells in response to FSH, in addition to the marked increase in their basal rate of secretion, became significant only when the cells were cultured for 40 h. We therefore examined whether granulosa cell contamination in the theca cell preparation could be detected by the progesterone secretion in response to FSH during this period. Theca cells were co-cultured with 0–20% granulosa cells for 40 h, and their basal and FSH-stimulated progesterone secretions were assayed (Fig. 3). There was no response to FSH in the culture of pure theca cells alone, but a response to FSH could be detected in the presence of only 3% granulosa cells ($P < 0.05$).

Table 1. Progesterone secretion by porcine theca and granulosa cells

	Culture period (h)	Without pFSH	With pFSH (5 ng ml^{-1})	With pFSH (10 ng ml^{-1})
Granulosa cells	12	0.43 ± 0.10^a	0.77 ± 0.22^a	1.10 ± 0.38^a
	40	25.03 ± 3.57^b	137.26 ± 21.36^c	214.08 ± 12.91^d
Theca cells	12	1.02 ± 0.58^a	1.16 ± 0.82^a	1.50 ± 0.03^a
	40	3.18 ± 0.33^b	3.54 ± 0.29^b	6.14 ± 1.13^c

Theca and granulosa cells were cultured in the conditions shown at a density of 2.5×10^5.
Data are presented as the mean ± SEM (pg ml^{-1}) of cumulative data from 4–6 independent experiments performed in triplicate.
Values with different superscripts within a row are significantly different ($P < 0.05$).

Table 2. Steroid secretion by porcine theca cells, granulosa cells and by a co-culture of 80% theca cells and 20% granulosa cells

Steroid	Theca cells alone	Granulosa cells alone	Co-culture of 80% theca cells and 20% granulosa cells			
			Control	pFSH (1 ng ml^{-1})	pFSH (5 ng ml^{-1})	pFSH (10 ng ml^{-1})
Progesterone (ng ml^{-1})	1.02 ± 0.58	0.43 ± 0.10	1.20 ± 0.28[a]	1.12 ± 0.35[a]	1.46 ± 0.34[a]	1.75 ± 0.43[a]
Oestradiol (pg ml^{-1})	148.7 ± 20.6	Not detected (385.2 ± 43.3*)	1848.6 ± 244.8	ND	ND	ND
Androstenedione (ng ml^{-1})	32.3 ± 5.5	Not detected	20.7 ± 1.4	ND	ND	ND

Theca cells alone, granulosa cells alone, and a co-culture of 80% theca cells and 20% granulosa cells were cultured for 12 h at a density of 2.5×10^5 cells ml^{-1} in the absence (control) or presence of porcine FSH (1, 5 and 10 ng ml^{-1}).
Data are presented as the mean ± SEM of cumulative data from four independent experiments performed in triplicate.
Differences between the control and experimental treatment values for progesterone production in co-culture were assessed by an analysis of variance, followed by Scheffe's *F*-test for multiple comparisons.
[a]Values with different superscripts within a row are significantly different ($P < 0.05$); there were no differences in progesterone production within any group.
*Value obtained when cells were cultured in the presence of 0.1 μmol testosterone l^{-1}.
ND: not determined.

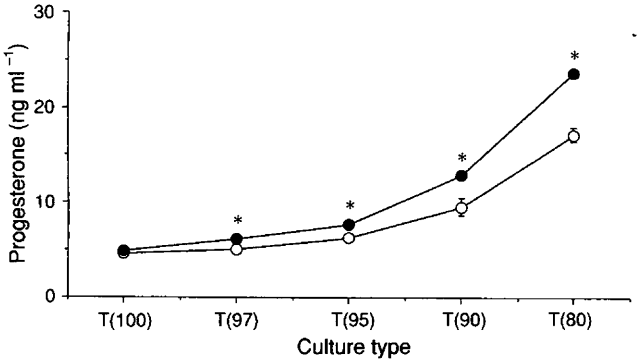

Fig. 3. Progesterone secretion by porcine theca and granulosa cells in response to porcine FSH (pFSH). Cells were inoculated into 24-well plates (2.5×10^5 cells ml^{-1}; 2 ml medium per well), and cultured in the absence (○) or presence (●) of pFSH (5 ng ml^{-1}) for 40 h. T(100): theca cells alone; T(97): 97% theca cells and 3% granulosa cells; T(95): 95% theca and 5% granulosa cells; T(90): 90% theca and 10% granulosa cells; T(80): 80% theca and 20% granulosa cells. Data are the means ± SD of triplicate cultures from a typical experiment. If no SD bar is shown, the SD was less than the size of the symbol. Values with an asterisk are significantly different ($P < 0.05$) from the respective values in the absence of pFSH. Note that the presence of 5 ng pFSH ml^{-1} had no significant effect on the amount of progesterone secretion in the culture of theca cells alone.

Discussion

The aims of the present study were to obtain a pure preparation of dispersed porcine theca cells, and to define better criteria for the identification and purity evaluation of collected theca cells. In preparing the cells, the procedure of Stoklosowa *et al.* (1978) was used with some modifications. For identification of the theca cells, the following three steroidogenic criteria were adopted and we examined whether these criteria were adequate and reliable: (1) porcine theca cells can synthesize C_{18} steroids, but granulosa cells cannot without the addition of aromatizable androgens; (2) porcine theca cells can synthesize C_{19} steroids, but granulosa cells cannot; and (3) FSH does not bind to theca cells but does bind to granulosa cells, indicating that FSH does not affect steroidogenesis in theca cells.

In the preliminary stage of our theca cell preparation, thecal sheets were obtained by carefully scraping the granulosa cells from the follicular walls, and then examining them under a compound microscope and a stereomicroscope by haematoxylin–eosin staining in frozen sections, as for most previous reports (Stoklosowa *et al.*, 1978, 1982; Tsang *et al.*, 1982). Although the thecal sheets contained no granulosa cells, as reported by Tsang *et al.* (1982, 1985), the theca cells obtained by mincing and enzymatically dispersing the thecal sheets did not satisfy the above three steroidogenic criteria (data not shown). This prompted us to remove the granulosa cells from the thecal sheets as thoroughly as possible, essentially according to the procedure of Stoklosowa *et al.* (1978). This procedure also appeared to indicate the superiority of steroidogenic criteria over standard morphological criteria, if they are chosen properly.

Theca cells secreted oestradiol, but granulosa cells did not; however, granulosa cells had an active aromatase system and could secrete a large amount of oestradiol in the presence of extrinsically added androgen. These results are consistent with previous reports (Evans *et al.*, 1981; Stoklosowa *et al.*, 1982; Tsang *et al.*, 1987). The theca cells secreted a large amount of androstenedione over 40 h, but the granulosa cells did not secrete this steroid at all. This finding is consistent with previous reports (Evans *et al.*, 1981; Tsang *et al.*, 1982, 1987) and also with the finding that C-17,18-lyase is present only in theca cells and not in granulosa cells (Rodgers *et al.*, 1986, 1987). However, it has been reported that some granulosa cell preparations secrete small amounts of not only oestradiol without extrinsic aromatizable androgen (Evans *et al.*, 1981; Stoklosowa *et al.*, 1982; Tsang *et al.*, 1987) but also androgens (Evans *et al.*, 1981; Stoklosowa *et al.*, 1982; Tsang *et al.*, 1982). These differences are probably not due to a low assay sensitivity to oestradiol and androstenedione in the present study, but rather to some contaminating theca cells in the granulosa cell preparations.

Thus, the theca and granulosa cells in the present preparation satisfied the first two criteria mentioned above very well. However, can these two criteria alone adequately validate the purity of theca cells? Although May *et al.* (1992) adopted only the second criterion as proof of the identity and purity of the theca cells, even a contamination to some degree with granulosa cells must result in androstenedione and oestradiol secretion, as theca cells alone secreted both steroids but granulosa cells alone did not secrete either. Hence, theca cells with as much as 20% granulosa cell contamination secrete androstenedione and oestradiol, satisfying both the above criteria. The demonstration of oestradiol and androstenedione secretion (criteria 1 and 2 above) does not preclude the absence of granulosa cells; it can only be interpreted as the presence of theca cells.

The third criterion is therefore needed as a reliable, functional indicator of the purity of porcine theca cells (Hunter and Armstrong, 1987; Caubo *et al.*, 1989; Engelhardt *et al.*, 1991). This criterion deals with the fact that although FSH has no steroidogenic effect on theca cells, it does have a stimulatory effect on steroidogenesis in granulosa cells. To our knowledge, there have been no reports on theca cell preparations that showed the steroidogenic effect of FSH on granulosa cells clearly.

On the basis of the report that granulosa cells, which had their culture medium changed every second day, exhibited a great potential to secrete progesterone from day 2 to the end of an 8-day culture (Stoklosowa *et al.*, 1982), we examined basal and FSH-stimulated progesterone secretion by granulosa and theca cells cultured for 12 and 40 h. Both basal and FSH-stimulated progesterone secretion by granulosa cells increased significantly in a culture period of 40 h compared with 12 h. However, although basal secretion by the theca cells increased slightly but significantly from a 12 h to a 40 h culture period, there was no effect of FSH on theca cells, except at pharmacologically high doses (10 ng ml^{-1}). In this regard, it must be stressed that the effect of FSH on progesterone production by granulosa cells was not evident until 40 h in culture. This means that the lack of response to FSH of the theca cell preparation must be confirmed under culture conditions where the FSH responsiveness of the granulosa cells is evident.

As shown in the present study, sufficient time in culture may be one of the more important conditions. Evans *et al.* (1981) reported that granulosa cells responded to FSH by increasing progesterone production when cultured for 24 h, but that they also responded to LH. It is important to note that the theca cells used for culture in this study by Evans *et al.* were not dispersed cells, but solid tissue. However, Tsang *et al.* (1985, 1987) did not find any effect of FSH on granulosa cells, which may be due to differences in culture conditions and follicular state. In an earlier report, Tsang *et al.* (1985) studied granulosa cells from prepubertal gilts treated with pregnant mares' serum gonadotrophin (PMSG) and hCG that had been cultured for only 6 h, which is probably too short a period for the effect of FSH to appear. In a later report (Tsang *et al.*, 1987), a longer culture time of 24 h was used. This may have been sufficient, but the granulosa cells were collected from the preovulatory follicles of prepubertal gilts 72 h after PMSG treatment. The lack of an FSH response in this instance may be due to the follicular state, since FSH-sensitive adenylate cyclase activity in granulosa cells decreases as the follicle is enlarged (Nakano *et al.*, 1977; Lee, 1978; Lindsey and Channing, 1979). For example, in the present study, even a theca cell preparation that had been mixed with 20% granulosa cells and that had satisfied all three criteria could be mistaken as being composed purely of theca cells when cultured for 12 h without confirming the responsiveness to FSH in the granulosa cells. FSH-stimulated progesterone production by granulosa cells is therefore a very important factor in evaluating the granulosa cell contamination of a theca cell preparation, since out of the three criteria outlined above only this characteristic is specific to and indicative of the presence of granulosa cells. In addition, the response of granulosa cells to FSH is so sensitive that even 3% granulosa cell contamination was detectable when the cells were cultured for 40 h in the presence of 5 ng pFSH ml^{-1}. Accordingly, FSH unresponsiveness (the third criterion) is the most important and reliable indicator of theca cell purity under culture conditions in which the response of granulosa cells to FSH can be confirmed.

It may be argued that a longer treatment of a higher enzymatic concentration in the theca cell preparation than in the granulosa cell preparation inhibits the response of the contaminating granulosa cells to FSH in the present theca cell preparation. However, even after the collected granulosa cells were enzymatically treated in the same manner as the theca cells, they still responded significantly to FSH ($P < 0.05$) (data not shown) with a cell viability of 70–85%.

To satisfy the third criterion, the theca cells must be maintained for at least 40 h *in vitro*, because detection of the effect of FSH on granulosa cells requires 40 h of culture. In most studies, by contrast, the cultures were maintained for a relatively short time because androstenedione production did not continue for more than 6 h (Tsang *et al.*, 1985), 14 h (Hunter and Armstrong, 1987; Engelhardt *et al.*, 1991), or at most, 48 h (Morley *et al.*, 1989).

It has recently been shown that pure FSH can activate paracrine signalling in granulosa cells, which affects androgen production in thecal and interstitial cells (Smyth *et al.*, 1993). Thus, the lack of a significant effect of FSH on androstenedione secretion by theca cells may further confirm the absence of granulosa cell contamination in the theca cell preparation studied here.

Another interesting result was the magnitude of oestradiol secretion in the co-culture of both cell types. A co-culture of 80% theca cells with 20% granulosa cells resulted in the secretion of a large amount of oestradiol (1848.6 ± 244.8 pg ml^{-1} for a 12 h culture). The amount secreted was about 12.4 and 4.8 times higher than that recorded for either theca cells alone or granulosa cells alone, respectively, cultured in the presence of 0.1 μmol testosterone l^{-1} for 12 h. Since the aromatase activity is higher in granulosa cells than in theca cells (Gore-Langton and Armstrong, 1988), the granulosa cells probably used the aromatizable androgen supplied by the theca cells to synthesize oestradiol synergistically in the co-culture system. Thus, if there is some granulosa cell contamination in the thecal preparation, the collected cells will produce more oestradiol than is produced by pure theca cells.

In summary, we have developed a method for collecting highly purified dispersed porcine theca cells with less than

3% granulosa cell contamination by improving the conventional enzymatic and mechanical methods to eliminate the granulosa cells thoroughly. The capacity of theca cells for steroidogenesis (especially for androstenedione) is high and is maintained for 48 h of culture. The criteria developed for the identification and purity evaluation of porcine theca cells are as follows: (1) porcine theca cells can synthesize C_{18} steroids, but granulosa cells cannot without the addition of exogenous aromatizable androgen; (2) porcine theca cells can synthesize C_{19} steroids, but granulosa cells cannot, and (3) FSH does not affect the steroidogenesis of theca cells. However, this is only valid under culture conditions in which the FSH responsiveness of granulosa cells can be confirmed.

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