

# Involvement of Ad4BP/SF-1, DAX-1, and COUP-TFII transcription factor on steroid production and luteinization in ovarian theca cells

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**Abstract** To examine the essential mechanisms of steroid production in ovarian theca cells, we analyzed the expression of genes associated with steroid production using simple culture system with serum medium. In addition, we examined the involvement of DAX-1, COUP-TFII, and Ad4BP/SF-1 transcription factors on the steroid production in theca cells. Theca cells begin to display an elongated or fibroblastic aspect within 24 h of culture. Over the next 48 h, they metamorphosed from the fibroblastic to the epitheloid phenotype. The number of theca cells increased during culture period. Androstenedione and progesterone production per cell decreased at 48–96 h compared with 0–48 h of culture. Steroidogenic acute regulatory protein (StAR) and CYP 17 genes expression decreased at 48 h compared with 0 h of culture, and afterward maintained a low level. In contrast, expression of  $3\beta$ -HSD and P450<sub>scc</sub> mRNAs increased at 48 h compared with 0 h of culture. Protein expression of Ad4BP/SF-1 maintained a constant level during culture. COUP-TFII protein expression showed a peak level at 24 h of culture period. DAX-1 protein expression began to increase at 48 h of culture. Our data suggested that the inhibition in CYP 17 and StAR genes by DAX-1 transcription factor may be associated with the decrease in androstenedione and progesterone production by theca cells during in vitro culture. Such an essential pathway for steroid production might indicate the importance of theca cell function in bovine ovary.

**Keywords** Androstenedione · CYP 17 · DAX-1 · Luteinization · Progesterone · Theca cell

## Introduction

The ovarian follicle develops through several distinct stages such as primordial, antral, and Graafian follicles throughout the estrous cycle [1–3]. The ovarian follicle is composed of the oocyte, granulosa cells, and theca cell layers. Of these cells, thecal cell contributes to the ovarian function by producing steroid hormone.

The first step in the biosynthesis of steroid hormones is the conversion of cholesterol into pregnenolone. Steroidogenic acute regulatory protein (StAR) plays a key role in the intra-mitochondrial movement of cholesterol [4]. Pregnenolone is converted into dehydroepiandrosterone by cytochrome P450  $17\alpha$ -hydroxylase/C17, C20-lyase (CYP 17), and afterward converted by  $3\beta$ -hydroxysteroid dehydrogenase ( $3\beta$ -HSD) into androstenedione [5, 6]. Pregnenolone is metabolized either via the  $\Delta^4$ -pathway to progesterone or by  $3\beta$ -HSD [5].

The genes expression associated with steroid hormones synthesis is regulated by transcription factors. Of many transcription factors, dosage-sensitive sex reversal adrenal hypoplasia congenital critical region in the X chromosome gene 1 (DAX-1) [7], chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) [8, 9], and adrenal 4 binding protein/steroidogenic factor-1 (Ad4BP/SF-1) [10, 11] are associated with the steroidogenesis. Expression of P450<sub>scc</sub> and StAR genes that mainly involve in steroid production are controlled by the Ad4BP/SF-1 transcription factor that binds to specific sites in the promoter regions of these genes in bovine luteal cells [12, 13]. DAX-1 and COUP-TFII have been reported to function as transcriptional suppressors of Ad4BP/SF-1, which regulate

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expression of the steroidogenic gene in the adrenal gland [14]. However, the association of DAX-1 and COUP-TFII on steroid production by bovine theca cells is still unknown.

It would be valuable, in terms of a better understanding of the molecular mechanism of steroid hormone production in theca cells, to investigate the expression of genes associated with progesterone and androstenedione synthesis during *in vitro* culture. Therefore, using simple culture system (serum medium), we investigated the morphological changes, the mRNA expression of StAR, cytochrome P450 side-chain cleavage (P450<sub>scc</sub>), 3 $\beta$ -HSD, and CYP 17, and the protein expression of DAX-1, Ad4BP/SF-1, and COUP-TFII transcription factors in theca cells.

## Materials and methods

### Bovine theca cell culture

Bovine ovaries were collected <20 min after slaughter at a local abattoir and placed in ice-cold phosphate-buffered saline (PBS; Sigma Chemical Co., St. Louis, MO, USA). Theca cells were isolated from the ovaries using the following methods [15]. Briefly, healthy developing follicles were assessed according to [16] for a vascularized pink theca externa and amber follicular fluid without debris. Large follicles (>10 mm in diameter) were selected and follicular fluid was aspirated using a syringe with a 22-gauge needle. Follicles were opened by making a small incision on the surface. Granulosa cells were removed by gentle scraping with a medicine spoon under a stereomicroscope. We checked the complete removal of granulosa cells under stereomicroscope. The thecal layer was placed into PBS containing 2 mg collagenase (452 U/mg, type1, Sigma), 1 mg hyaluronidase (391 U/mg, type VIII, Sigma), 1 mg protease (4.5 U/g, Sigma), 0.4% (v/v) bovine serum albumin, and the dissociation reaction was performed for 40 min at 37°C. Centrifugal separation was carried out by 350  $\times$  g. Then, Tris-HCl Buffer (pH 8.0) was put into the tube for 1 min at 37°C. Dispersed cells were washed twice with PBS. Theca cells were seeded at a moderate number cells per well (6-well culture plate) in 3 ml of Dulbecco's modified Eagle's/F12 medium (DMEM/F12; Sigma Chemical Co.) containing amphotericin B (10  $\mu$ l/ml), gentamicin (5  $\mu$ l/ml), and 5% fetal calf serum (FCS; Biowest, Rue de la Caille, Muaille, France) as a preincubation for 48 h. After preincubation, theca cells were re-seeded at a density of 1  $\times$  10<sup>5</sup> cells per well (12-well culture plate) in 2 ml of DMEM/F12 containing amphotericin B (10  $\mu$ l/ml), gentamicin (5  $\mu$ l/ml) and 5% FCS. The cells were cultured for 0–120 h at 37°C in a 5% CO<sub>2</sub> atmosphere. The medium was changed every 48 h. To examine the proliferation of

theca cells, the number of theca cells was counted by using hemocytometer every 24 h.

### RNA extraction, reverse transcription (RT), and Quantitative PCR

Total RNA (500 ng) from cultured theca cell was extracted with TRIZOL<sup>®</sup> reagent (Life Technologies, Inc.) following the method provided by the manufacturer and frozen at –80°C. Before the RT reaction, samples treated with DNase using a commercial kit (SV Total RNA Isolation System: Promega Co., Madison, WI, USA). Single-strand cDNA was reverse transcribed from total RNA using a first-strand cDNA synthesis kit for RT-PCR (Roche Diagnostics Co., Indianapolis, IN, USA) with random primers. The RT conditions consisted of 10 min of annealing at 25°C, 50 min of cDNA synthesis at 42°C, and 15 min of inactivation at 70°C.

The mRNA expression of 17 $\alpha$ -hydroxylase/C17-20 lyase (CYP 17), 3 $\beta$ -HSD, P450<sub>scc</sub>, StAR, Ad4BP/SF-1, DAX-1, COUP-TFII, LDLR, SR-B1, and  $\beta$ -actin were quantified by real-time PCR by Light Cycler (Roche Diagnostics Co., Indianapolis, IN, USA) using a commercial kit (QuantiTect<sup>™</sup> SYBR<sup>®</sup> Green PCR: QIAGEN GmbH, Hilden, Germany). The primers were designed using Primer-3 software based on bovine sequences (Table 1). The amplification program consisted of 15 min for activation at 95°C followed by 40 cycles of PCR (94°C for 15 s, 58°C for 30 s, and 72°C for 20 s). For quantification of the target genes, a series of standards were constructed by amplifying a fragment of DNA (450–550 bp) that contained the target sequence for real-time PCR (100–200 bp). The values were normalized using  $\beta$ -actin as the internal standard.

### Western blot analysis

A sample of cultured theca cells was extracted with lysis buffer (20 mM Tris-HCl pH 7.0, 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% TritonX-100, 1% Protease inhibitor Cocktail, 1% Phosphatase inhibitor Cocktail 1, and 1% Phosphatase inhibitor Cocktail 2). The resulting total cellular protein was heated to 95°C for 5 min, electrophoresed in a 12% SDS-PAGE gel, and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with PBS buffer containing 0.05% Tween 20 (Sigma Chemical Co.) and 5% nonfat dry milk (Wako, Osaka, Japan) for 1 h at room temperature and incubated overnight at 4°C with anti-mouse-SF-1 rabbit antibody PA1-800 (Affinity Bioreagents, Inc., Golden, USA. 1:1,000 dilution), anti-rabbit-DAX-1 human antibody (Santa Cruz Biotechnology, Inc., CA, USA. 1:1000 dilution), anti-goat-COUP-TFII human antibody (Santa Cruz Biotechnology,

**Table 1** Primer pairs used for detection of mRNAs

Gene	Primer sequence	Size (bps)	GeneBank accession/Emsembl
CYP 17	Forward: 5'-TGG ATC GTG GCC TAC CTC CT-3' Reverse: 5'-AGG TCG CCA ATG CTG GAG TC-3'	215	M12547
P450scc	Forward: 5'-CTG CAA ATG GTC CCA CTT CT-3' Reverse: 5'-CAC CTG GTT GGG TCA AAC TT-3'	209	K01230
3 $\beta$ -HSD	Forward: 5'-TCC ACA CCA GCA CCA TAG AA-3' Reverse: 5'-AAG GTG CCA CCA TTT TTC AG-3'	118	X17614
StAR	Forward: 5'-GTG GAT TTT GCC AAT CAC CT-3' Reverse: 5'-TTA TTG AAA ACG TGC CAC CA-3'	203	NM174189
SF-1	Forward: 5'-TGT GTG GGG ACA AGG TGT-3' Reverse: 5'-GCT TGC GCT GAG TCT TGT-3'	147	D13569
DAX-1	Forward: 5'-CCT GCA GTG CGT GAA GTA-3' Reverse: 5'-AGG GTG TTG GCA CTG ATG-3'	153	AF421373
COUP-TFII	Forward: 5'-GCC TCA AAA AGT GCC TCA A-3' Reverse: 5'-GGC CAG TTC GCA AAT GTT-3'	245	Q9TTR2
LDLR	Forward: 5'-ACA ACC CCG TGT ACC AGA AG-3' Reverse: 5'-AGG GTC AGG GGA GAA AGT GT-3'	195	K01830
SR-B1	Forward: 5'-GTG TCC TTC CTG GAG TAC CG-3' Reverse: 5'-GAA CAC GGT GAA GAG GCC AG-3'	336	AF019384
$\beta$ -actin	Forward: 5'-CCA AGG CCA ACC GTG AGA AGA T-3' Reverse: 5'-CCA CGT TCC GTG AGG ATC TTC A-3'	256	AY141970

Inc., 1:200 dilution), or anti- $\beta$ -actin mouse monoclonal clone AC-15 antibody (Sigma Chemical Co., 1:5,000 dilution). After washing three times (5 min each) with PBS buffer with 0.05% Tween 20 and treated with HRP-conjugated anti-mouse (Rockland Immunochemicals, Inc., USA., 1:10,000 dilution), anti-rabbit (GE Healthcare, 1:5,000 dilution) or anti-goat IgG antibodies (Rockland, 1:5,000 dilution) for 1 h at room temperature. Immunoactive bands were detected using ECL Western Blotting Detection Reagents (Amersham Biosciences, UK). Densitometric analysis was performed by using a Polaroid 667 image (Nippon Polaroid KK, Tokyo, Japan).

#### Hormone assay

The assays for progesterone using culture medium from each 48 h timepoint were performed by enzyme immunoassay (EIA) after diethyl ether extraction [17]. The standard curve ranged from 50 to 50,000 pg/ml. The intra- and interassay coefficients of variation (CVs) were 7.5% and 4.3%, respectively.

The EIA for androstenedione using culture medium from each 48 h timepoint was identical to the EIA for progesterone, as previously described [18]. Basically, standards or samples were incubated with 100  $\mu$ l polyclonal antibody (raised in a rabbit against A-3-CMO-BSA; Cosmo Bio Co., Tokyo, Japan, 1:750,000) solution and

100  $\mu$ l A-3-CMO-horseradish peroxidase (Cosmo Bio Co., 1:500,000) for 24 h at 4°C. The standard curve ranged from 7.8 to 8,000 pg/ml. The intra- and interassay CVs averaged 8.9% and 4.8%, respectively.

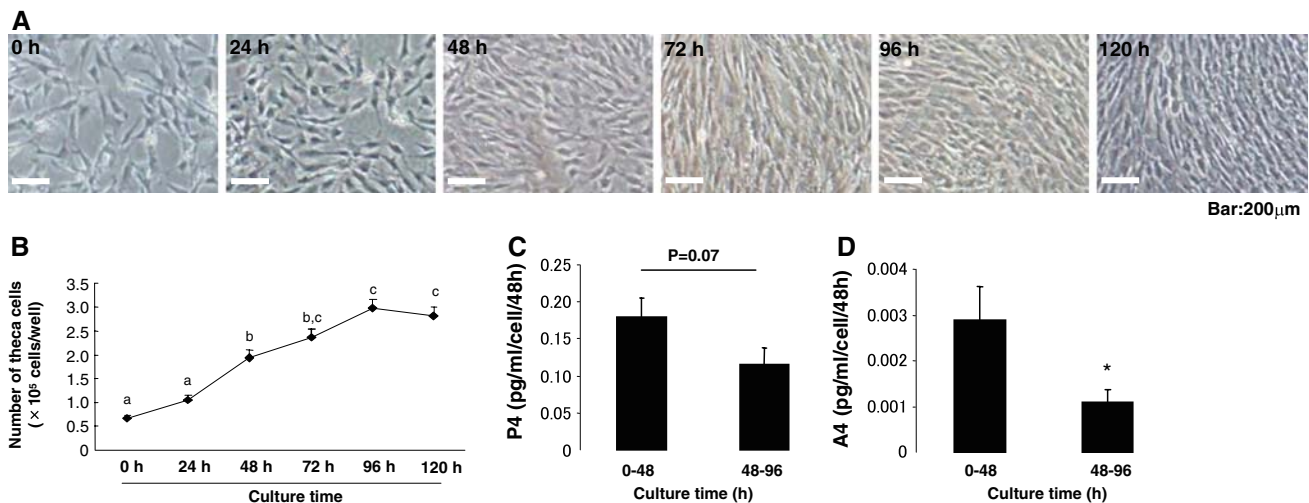
#### Data analysis

All data are presented as mean  $\pm$  SEM. The levels of several factors in the theca cells at different culture timepoints were tested for significant differences using ANOVA, followed by the Tukey-Kramer test as a multiple comparison test. The relationship between androstenedione and progesterone per cell was analyzed by Pearson's correlation coefficient test. Differences were considered significant at  $P < 0.05$ .

## Results

Cell proliferation, morphological changes, androstenedione, and progesterone

Theca cells attached to the substrate and begin to display an elongated or fibroblastic aspect within 24 h of culture (Fig. 1a). Over the next 48 h, they metamorphosed from the fibroblastic to the epitheloid phenotype (Fig. 1a). The number of theca cells increased during culture period



**Fig. 1** Morphological observation (a), cell proliferation (b), and progesterone (c), and androstenedione (d) per cell by theca cells cultured in serum medium. The data are expressed as mean  $\pm$  SEM of four separate experiments with triplicate determinations at each

(Fig. 1b). Androstenedione ( $P < 0.05$ ) and progesterone production ( $P = 0.07$ ) per cell decreased at 48–96 h compared with 0–48 h of culture (Fig. 1c and d).

Expression of genes associated with androstenedione and progesterone production

A significant difference was not observed in the expression of LDLR or SR-B1 mRNAs during luteinization (Fig. 2a and b). Steroidogenic acute regulatory protein expression decreased at 48 h compared with 0 h of culture, and afterward maintained a low level (Fig. 2c). Expression of  $3\beta$ -HSD and P450<sub>scc</sub> mRNAs increased at 48 h compared with 0 h of culture (Fig. 2d and e). Expression of CYP 17 decreased at 48 h compared with 0 h of culture, and decreased further at 96 h (Fig. 2f).

mRNA and protein expression of transcription factors

Expression of Ad4BP/SF-1, DAX-1, and COUP-TFII mRNAs increased at 96 h compared with 0 h of culture (Fig. 3a–c). Protein expression of Ad4BP/SF-1 maintained a constant level during culture (Fig. 4a). COUP-TFII protein expression showed a peak level at 24 h throughout the culture period (Fig. 4c). DAX-1 protein expression began to increase at 48 h of culture (Fig. 4b).

## Discussion

In the present study, theca cells cultured in serum medium displayed the structural and functional changes during in vitro culture. Our study demonstrated that theca cells

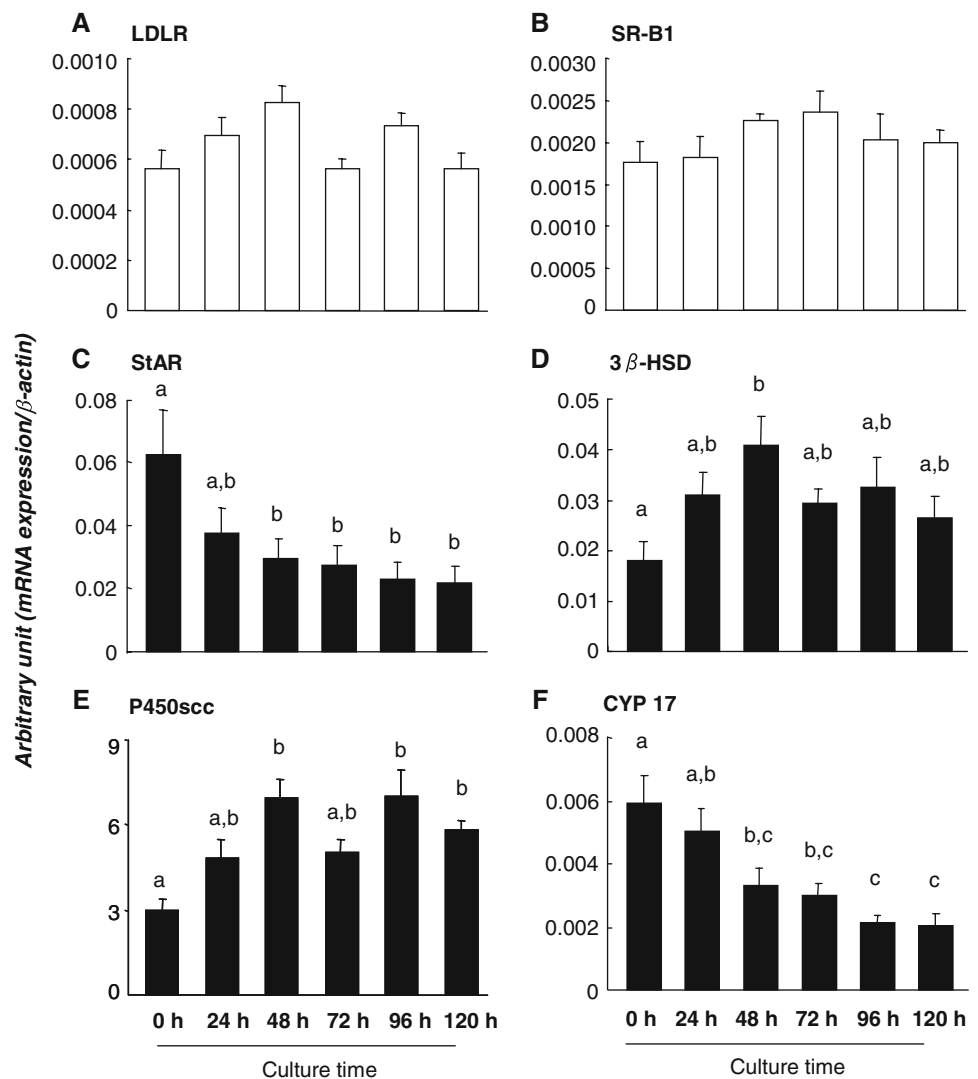
begin to display an elongated or fibroblastic aspect within 24 h of culture, and over the next 48 h, they metamorphosed from the fibroblastic to epitheloid phenotype. This morphological change is known as in vitro luteinization. In addition, we observed the cell proliferation during culture period. These observations suggested that serum may induce the morphological change and proliferation of theca cells.

In steroid production, our present study indicated that androstenedione and progesterone production per cell decreased at 48–96 h compared with 0–48 h of culture. These data demonstrated that androstenedione and progesterone production per cell decreased during theca cell luteinization induced by serum. In vivo, thecal cell luteinization occurs after ovulation induced by LH surge. A previous study using bovine thecal cells indicated that after 3 days of culture, androgen production declined drastically and was not maintained by LH treatment [19]. On the other hand, progesterone production remained relatively constant by LH treatment throughout the 8-day culture period [19]. Our present study and other suggested that LH may affect a switch from the major pathway for androgen production to progesterone production [5].

Since progesterone and androstenedione production per cell did change during in vitro culture, we examined the expression of genes associated with these hormones. The SR-B1 content by granulosa cells in in vitro culture is directly correlated with the acquisition of cholesterol [20], which is a substrate for progesterone synthesis. In the present study, the mRNA expression levels of LDLR and SR-B1 in theca cells did not change throughout the culture period. This result suggested that the theca cells may take in constant amount of cholesterol during in vitro culture. StAR imports cholesterol into mitochondria, and is

culture timepoints. Different superscripts denote significantly different values ( $P < 0.05$ ). An asterisk denotes a significant difference at  $P < 0.05$

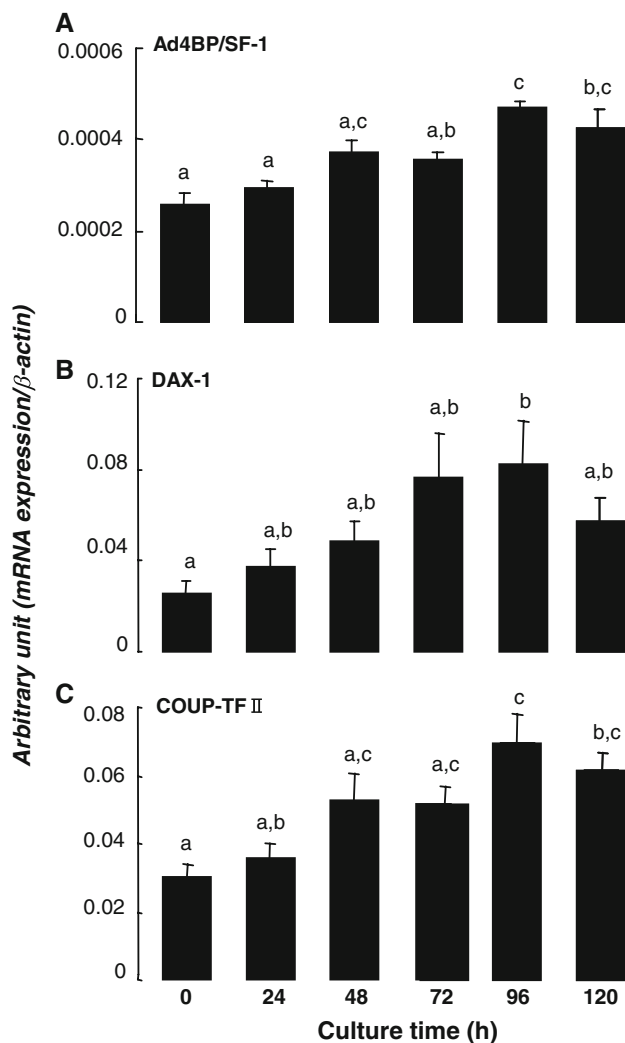
**Fig. 2** Expression of LDLR (a), SR-B1 (b), StAR (c),  $3\beta$ -HSD (d), P450scc (e), and CYP 17 (f) genes in theca cells. The data are expressed as mean  $\pm$  SEM of four separate experiments with triplicate determinations at each culture timepoints. Different superscripts denote significantly different values ( $P < 0.05$ )



essential for steroidogenesis [21]. P450scc is acquired within 7 h of the ovulatory stimulus in the rat CL [22], and P450scc expression increases during luteinization of porcine granulosa cells in *in vitro* culture [23].  $3\beta$ -HSD catalyzes the formation and/or degradation of  $5\alpha$ -androstanes and  $5\alpha$ -pregnanes, such as dihydrotestosterone (DHT) and dihydroprogesterone (DHP) [24, 25]. In the present study, StAR expression in theca cells decreased at 48 h compared with 0 h of culture, and afterward maintained a low level. On the other hand, the expression of  $3\beta$ -HSD and P450scc mRNAs increased at 48 h compared with 0 h of culture. These data suggested that decrease in progesterone production per cell was induced by the suppression of StAR gene expression. Therefore, our study suggested that StAR may be an essential factor for progesterone production in thecal cell luteinization. The CYP 17 enzyme represents a single protein that catalyzes two biochemical reactions, the 17  $\alpha$ -hydroxylation of progesterone or pregnenolone and the subsequent cleavage of the C17–20

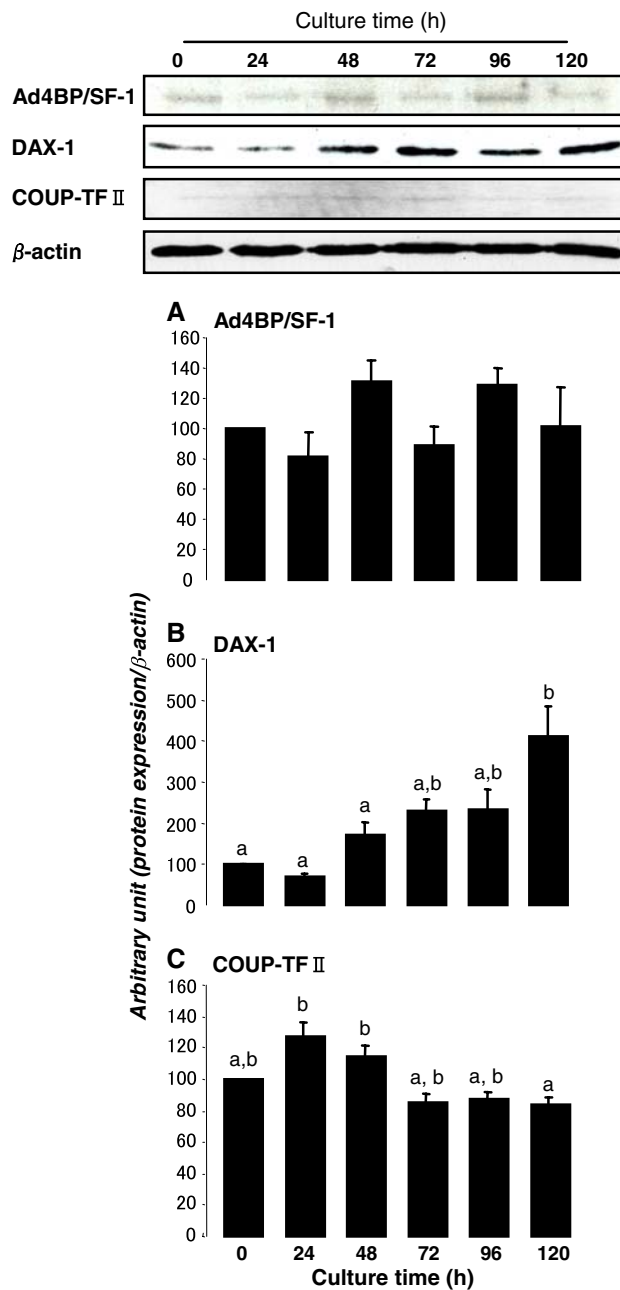
bond to produce androstenedione or dehydroepiandrosterone. In the present study, CYP 17 expression in the theca cells decreased at 48 h, and afterward decreased further at 96 h of culture. This result suggested that the decrease in CYP 17 expression may provoke the decrease in androstenedione during the luteinization of theca cell.

Changes in expression of genes associated with steroid production are regulated at the level of transcription factors. Recent studies have demonstrated the possible involvement of Ad4BP/SF-1, DAX-1, and COUP-TFII on steroidogenic enzyme levels in the ovaries of human and cow [26, 27]. Our data indicated that the expression of Ad4BP/SF-1, DAX-1, and COUP-TFII mRNAs in the theca cells increased at 96 h compared with 0 h of culture. In addition, protein expression of Ad4BP/SF-1 and COUP-TFII in the theca cells was at a constant level during the entire culture period. In contrast, protein expression of DAX-1 in the theca cells showed a tendency to increase at 48 h of culture, and significantly increased at 120 h of



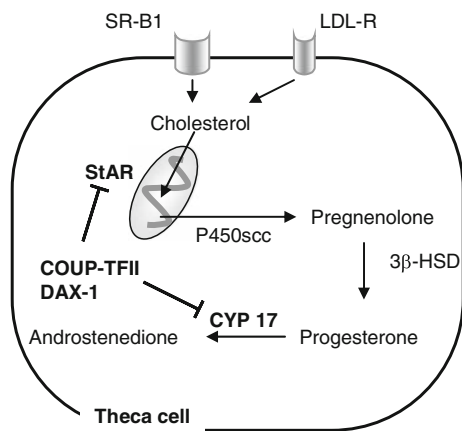
**Fig. 3** Expression of Ad4BP/SF-1 (a), DAX-1 (b), and COUP-TFII (c) genes in theca cells. The data are expressed as mean  $\pm$  SEM of four separate experiments with triplicate determinations in each experiment at each culture time. Different superscripts denote significantly different values ( $P < 0.05$ )

culture. The bovine CYP 17 gene contains at least two cAMP-responsive sequences (CRS1 and CRS2) [28]. The CRS2 region of the CYP 17 promoter binds Ad4BP/SF-1 and COUP-TF [2]. COUP-TFII was found to inhibit Ad4BP/SF-1-mediated CYP 17 gene transcription [29]. Moreover, Ad4BP/SF-1 binding sites are required for DAX1 to maximally inhibit SF-1-stimulated human CYP 17 gene transcription [30]. Therefore, our data suggested that COUP-TFII and DAX-1 may suppress Ad4BP/SF-1-mediated CYP 17 gene expression during luteinization of theca cells. In StAR gene expression, binding sites for DAX-1 present in the promoter of StAR gene [31]. DAX-1 acts as a powerful transcriptional repressor of StAR gene expression [31]. Therefore, our data suggested that increase



**Fig. 4** Expression levels of Ad4BP/SF-1 (a), DAX-1 (b), and COUP-TFII (c) proteins in theca cells. Electrophoresis images are representative of three independent experiments. The total proteins isolated from cultured theca cells were immunodetected with specific antibody, which was normalized over the expression of actin. The scanning data of the respective band intensities have been shown, where pixel value of each band was calculated by the NIH Image software (NIH, Bethesda, MD). The data are expressed as mean  $\pm$  SEM of three independent experiments at each culture timepoints, and indicated as a percentage at 0 h of culture. Different superscripts denote significantly different values ( $P < 0.05$ )

in DAX-1 protein may be associated with the suppression of StAR gene expression as well as CYP 17 gene in theca cell cultured in serum medium.



**Fig. 5** Functional characteristics of steroid production in theca cell. The theca cells may take in constant amount of cholesterol during in vitro culture. However, androstenedione and progesterone production per cell decreased in theca cell cultured in serum medium. COUP-TFII and DAX-1 transcription factors may involve in the inhibition of StAR and CYP 17 genes. Thus, transcription factor may play an important role in the control of theca cell physiology and function

In conclusion, the scheme of the mechanism of theca cell luteinization is shown in Fig. 5. Our data demonstrated that the decrease in androstenedione and progesterone production that is induced by suppressing CYP 17 and StAR may be decreased by an inhibitory effect of COUP-TFII and DAX-1 transcription factors. Thus, the present study indicated the functional and morphological fundamental changes in thecal cell.

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