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Effect in dedicator of cytokinesis 6 (DOCK6) on steroid production in theca cells of follicular cysts

Chiaki Murayama, Eiki Yamasaki, Akio Miyamoto, Takashi Shimizu*

Graduate School of Animal and Food Hygiene, Obihiro University of Agriculture and Veterinary Medicine, Inada-machi, Obihiro, Hokkaido 080-8555, Japan

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ABSTRACT

Ovarian follicular cysts are one of the most common causes of reproductive failure in mammals. A comparative gene expression approach may aid in elucidating the causes of ovarian cyst disease. In the present study, the differential display technique was used to identify mRNA sequences that accumulate preferentially in theca cells of bovine cystic follicles. Dedicator of cytokinesis 6 (Dock6) expression was observed in the theca cells of cystic follicles. Small interfering RNA (siRNA) knockdown of *Dock6* increased progesterone (P4) production and StAR expression in theca cells of high-estrogen follicular cysts, but did not affect androstenedione (A4) production. We propose that Dock6 may be a marker associated with the development of follicular cysts. Additionally, *Dock6* may be involved in the development of cystic follicles by suppressing P4 production rather than increasing A4 production in theca cells.

1. Introduction

Ovarian follicular cysts in cattle are a major cause of infertility. In dairy cows, ovarian follicular cysts are defined as large follicular structures (25 mm in diameter or greater) that are present for 10 days in the absence of a corpus luteum [1,2]. Several studies have demonstrated that the development of ovarian follicular cysts is associated with an endocrine imbalance in the hypothalamic-pituitary-ovarian axis [2–5]. At the ovarian level, molecular changes in the growing follicle may contribute to anovulation and cystic formation. In particular, alterations in follicular steroidogenesis and luteinizing hormone (LH) receptor expression have been reported in bovine follicular cysts than in dominant follicles [6,7]. However, investigating cellular and molecular changes prior to cyst formation remains difficult.

Several studies have reported the differential gene expression during follicular development in cattle [8–11]. In addition, Grado-Ahuir et al. [12] reported that differences in the expression of gene sequence elements between cystic and non-cystic estrogenic follicles are modest in magnitude, particularly for the overwhelming majority of the differentially expressed genes, that are downregulated in the estrogenic cysts. However, although the majority of differentially expressed genes in between healthy and cystic follicles have been reported, molecules inducing cystic follicles have been not identified.

In the present study, to improve our knowledge regarding the genes expressed during cyst formation, the differential display technique was used to identify mRNA sequences that accumulate preferentially in bovine cystic follicles. Although granulosa cells have been used to analyze gene expression using microarrays, we focused on the theca cells that produce androstenedione (A4) which is necessary for estrogen production in granulosa cells. Here, we report specific genes expressed in the theca cells of cystic follicles.

2. Materials and methods

2.1. Collection of follicles

Bovine ovaries were collected at a local slaughterhouse and placed in ice-cold phosphate-buffered saline (PBS). The follicle diameters were determined from the weight of the follicular fluid as previously described by Murasawa et al. [13]. Follicles were classified into the following three categories based on the calculated follicle diameters, concentrations of estradiol (E2) and the ratio of E2 to progesterone (P4) (E/P) in the follicle fluid: preovulatory follicle (POF, diameters ≧ 8.5 mm, E/P > 1), high-E2 cystic follicle (HECF, diameters ≧ 25 mm, E/P ≧ 1) and low-E2 cystic follicle

* Corresponding author. Fax: +81 155 49 5459.
E-mail address: shimizut@obihiro.ac.jp (T. Shimizu).

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(LECF, diameters ≧ 25 mm, E/P ≦ 1). The characteristics of POFs, HECFs and LECFs are shown in Table 1. Theca cells were isolated from the several follicles using previously described methods [14,15].

2.2. Screening of differentially expressed genes using annealing control primer (ACP) system

Total RNA from theca cells of several follicles was extracted with TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and frozen at −80 °C. cDNA synthesis and PCR were performed following the procedures recommended by the manufacturer using an annealing control primer system (Seegene, Seoul, Korea). PCR products were analyzed by agarose gel electrophoresis.

2.3. DNA sequencing

PCR products were cloned into pCR 4-TOPO using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA). After the plasmids were purified by using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany), insets sizes were confirmed by *EcoRI* restriction enzyme digestion followed by agarose gel electrophoresis (1%). Sequencing of cloned DNA was performed at Hokkaido System Science Co., Ltd. (Hokkaido, Japan) by using the ABI PRISM 3130 Genetic Analyzer with the BigDye Terminator v3.1 kit (Applied Biosystems, Foster City, CA, USA) and T7 and T3 primers. The obtained sequences were compared to sequences in a public database by using BLAST at the NCBI web site (http://blast.ncbi.nlm.nih.gov) to identify the PCR products.

2.4. Bovine theca cell culture

Bovine ovaries were collected at a local slaughterhouse and placed in ice-cold PBS. Theca cells were isolated from the POFs, HECFs and LECFs using a previous method [14]. Theca cells were suspended in culture medium including 1 ml of Dulbecco's modified Eagle's/F12 medium (DMEM/F12; Invitrogen, New York, NY, USA) containing 100 μg/ml streptomycin, 100 μg/ml kanamycin and 5% fetal bovine serum (FBS; Biowest, Rue de la Caille, Muaille, France) in 24-well culture plates (NuncTM: Nalge Nunc International, New York, NY, USA) at 1×10^5 cells per well, and cultured for 24 h at 37 °C in 5% CO_2, 95% air. The wells were then washed twice with PBS to remove unattached cells.

LH stimulation of cystic follicle theca cells was examined for short-term cultures; the culture medium was replaced with 1% FCS DMEM/F12 supplemented with or without LH (bovine LH, USDA-bLH-B6, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Bertheda, MD, USA, 2.5 ng/ml) and the culture were continued for 48 h.

2.5. RNA extraction, reverse transcription (RT), and quantitative PCR

Total RNA from cultured theca cells were extracted with TRIZOL® regent (Invitrogen) following the method provided by the manufacturer and frozen at −80 °C. Before the reverse transcription (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 1st Strand cDNA Synthesis Kit (Invitrogen) 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 *StAR*, *CYP17* and β-actin were quantified by real-time PCR using the iQ5Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using a commercial kit (QuantiTectTM SYBR® Green PCR: Qiagne GmbH, Hilden, Germany). The primers were designed using Primer-3 software based on bovine sequences and are as follows: *Dock6* Forward: 5′-GGG ATC TGG TTG AGT TTC CA-3′, Reverse: 5′-TCT CCT ATG GGC AAT ATC C-3′, *P450scc* Forward: 5′-CTG CAA ATG GTC CCA CTT CT-3′, Reverse: 5′-CAC CTG GTT GGG TCA AAC TT-3′, *3β-HSD* Forward: 5′-CCT TGT ACA CTT GTG CCC TGA G-3′, Reverse: 5′-ACC TTG CAG TGA TTG GTC AGG A-3′, *LHR* Forward: 5′-AGG AAA ATG CAC GCC TGG AG-3′, Reverse: 5′-GTG GCA TCC AGG AGG TTG GT-3′, *StAR* Forward: 5′-GTG GAT TTT GCC AAT CAC CT-3′, Reverse: 5′-TTA TTG AAA ACG TGC CAC CA-3′, *GAPDH* Forward: 5′-CTC TCA AGG GCA TTC TAG GC -3′, Reverse: 5′-TGA GAA AGT GGT CGT TGA GG -3′. The amplification program consisted of 15 min for activation at 95 °C followed by 50 cycles of PCR (94 °C for 15 s, 58 °C for 30 s, and 72 °C for 20 s). The values were normalized using *GAPDH* as the internal standard.

2.6. DOCK6 siRNA transfection

The D-Nucleofector™ (Lonza Ltd. Visp, Switzerland) and Amaxa™ P3 Primary Cell 4D- Nucleofector™ X Kit (Lonza Ltd. Switzerland) were used for intracellular expression of small interfering RNA (siRNA). Briefly, the target sequence for dedicator of cytokinesis 6 (Dock6) siRNA was 5′-ggucauugguggccagauu-3′, and scramble siRNA was 5′-uugugcgucguaaugcgga-3′. Theca cells were suspended in culture medium in 2 ml of Dulbecco's modified Eagle's/F12 medium (DMEM/F12; Invitrogen, New York, USA) containing 100 μg/ml streptomycin, 100 μg/ml kanamycin and 5% fetal bovine serum (FBS; Biowest, Rue de la Caille, Muaille, France) in 6-well culture plates (NuncTM: Nalge Nunc International, New York, USA) at 5 × 105 cells per well, and cultured for 24 h at 37 °C in 5% CO_2, 95% air. Wells were then washed twice with PBS to remove unattached cells. The theca cells were placed into 0.2% trypsin buffer, and the dissociation reaction was performed for 5 min at 37 °C. Next, 6×10^5 theca cells and 0.2 mg/100 ml of the siRNA was transfected using 4D- NucleohectorTM. All procedures were performed according to the manufacturer's protocols. siRNA transfected theca cells were suspended in 0.5 ml of culture medium in 48-well culture plates (NuncTM: Nalge Nunc International, New York, USA) at 0.5×10^5 cells per well for 24 h. The culture medium was exchanged with 0 or 2.5 ng/ml LH in 1% FCS DMEM/F12 after 24 h and the cultures were continued for 48 h.

2.7. Hormone assay

The assays for P4 using culture medium, collected at each timepoint, were performed by enzyme immunoassay (EIA) after diethyl ether extraction [14]. The standard curve ranged from 50 to 50,000 pg/ml. The intra- and interassay coefficients of variation (CVs) were 7.2% and 4.3%, respectively. The EIA for androstenedione (A4) using culture medium from each 48-h timepoint was identical to the EIA for P4, as previously described [14]. The standard curve ranged from 7.8 to 8000 pg/ml. The intra- and interassay coefficients of variation averaged 8.2% and 5.5%, respectively.

Table 1
Concentrations of estradiol (E2), progesterone (P4) in follicular fluid of preovulatory follicle (POF), high-E2 cystic follicles (HECF) and low-E2 cystic follicles (LECF).

	POF	HECF	LECF
E2 conc.	221.30 ng/ml	433.74 ng/ml	8.49 ng/ml
P4 conc.	58.71 ng/ml	22.59 ng/ml	208.31 ng/ml
Follicular diameter	14.9 mm	26.2 mm	27.1 mm

Table 2
Genes identified by gene fishing PCR.

Genes	Accession no.	Follicle categories
Bos taurus nuclear receptor subfamily 5, group A, member 2, transcript variant 2 (NR5A2)	XM_002694275.1	LECF
Bos taurus brain ribonuclease (BRB)	NM_173891.2	LECF
Bos taurus 6.8 kDa mitochondrial proteolipid (MP68)	NM_001113315.1	POF, HECF, LECF
Bos taurus dedicator of cytokinesis 6 (DOCK 6)	NM_001192166.1	HECF, LECF
Bos taurus InCenP homolog family member (icp-1)-like (INCENP)	XM_002707799.1	POF
Bos taurus breast carcinoma amplified sequence 4 (BCAS4)	NM_001076474.1	POF, HECF, LECF
Bos taurus ubiquitin-like with PHD and ring finger domains 1 (UHRF1)	NM_001103098.1	POF, HECF, LECF
Bos taurus C1 domain-containing phosphatase and tensin-like protein splice-like (TENC1)	XM_002687243.1	POF, HECF, LECF
Bos taurus ribosomal protein S2 (RPS2)	NM_001033613.1	POF, HECF, LECF
Bos taurus amyloid beta (A4) precursor-like protein 2 (APLP2)	XM_002699235.1	POF, HECF, LECF
Bos taurus transcription factor 21	BC105574.1	POF, HECF, LECF

POF: preovulatory follicle, HECF: High-E2 cystic follicle, and LECF: Low-E2 cystic follicle.

2.8. Data analysis

All data are presented as means ± S.E.M. The levels of Dock6 in the theca cells of POFs, HECFs and LECFs, and changes in several factors after siRNA treatment were tested for significant differences using ANOVA, followed by the Tukey–Kramer test for multiple comparisons test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Identification of the differentially genes expressed s in theca cells of cystic follicles

Genes expressed in theca cells of POFs, HECFs and LECFs are shown in Table 2. Dedicator of cytokinesis 6 (Dock6) was observed in the theca cells of cystic follicles such as HECFs and LECFs. For confirmation of this result, we analyzed its expression in the theca cells of POFs, HECFs and LECFs (Fig. 1). *Dock6* expression in HECF and LECF theca cells was higher than in POF theca cells, consistent with the results of the differential display analysis.

3.2. Effect of Dock6 on P4 and A4 production, and steroidogenesis-related genes in theca cells of HECF

We examined whether Dock6 is associated with the steroid production in HECF theca cells. siRNA knockdown of *Dock6* increased P4 production and StAR expression in LH-stimulated theca cells of HECFs, whereas siRNA treatment did not affect A4 production (Fig. 2A, B and Fig. 3A).

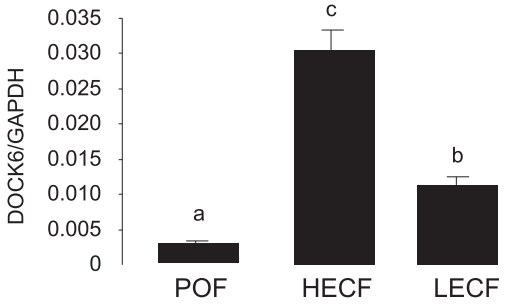

Fig. 1. Expression of *Dock6* mRNA in theca cells of preovulatory follicle (POF, n = 4), high-E2 cystic follicles (HECF, n = 4), low-E2 cystic follicles (LECF, n = 3). The data are expressed as means ± S.E.M. Different superscripts denote significantly different values ($P < 0.05$).

4. Discussion

Our data demonstrated that 11 genes were expressed in theca cells of POFs, HECFs and LECFs based on differential display. In particular, Dock6 was expressed in HECF and LECF theca cells.

Pulsatile secretion of LH is associated with cystic formation of follicles that are not able to ovulate by LH surges from the pituitary. Low LH treatment (2.5 ng/ml in the present study) which mimicked the pulsatile secretion of LH stimulated *Dock6* expression in theca cells of HECFs (data not shown). Thus, since *Dock6* may be involved in the development of HECFs under pulsatile secretion of LH, we

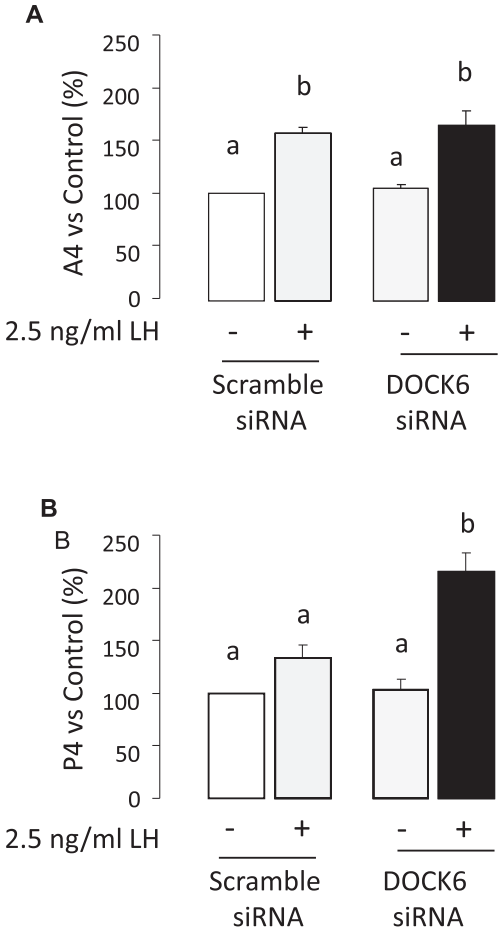

Fig. 2. Effect of DOCK6 on the production of androstenedione (A4) (A) and progesterone (P4) (B) in LH-stimulated theca cells of HECFs. The data are expressed as means ± S.E.M of four experiments with triplicate determination of each. Different superscripts denote significantly different values ($P < 0.05$).

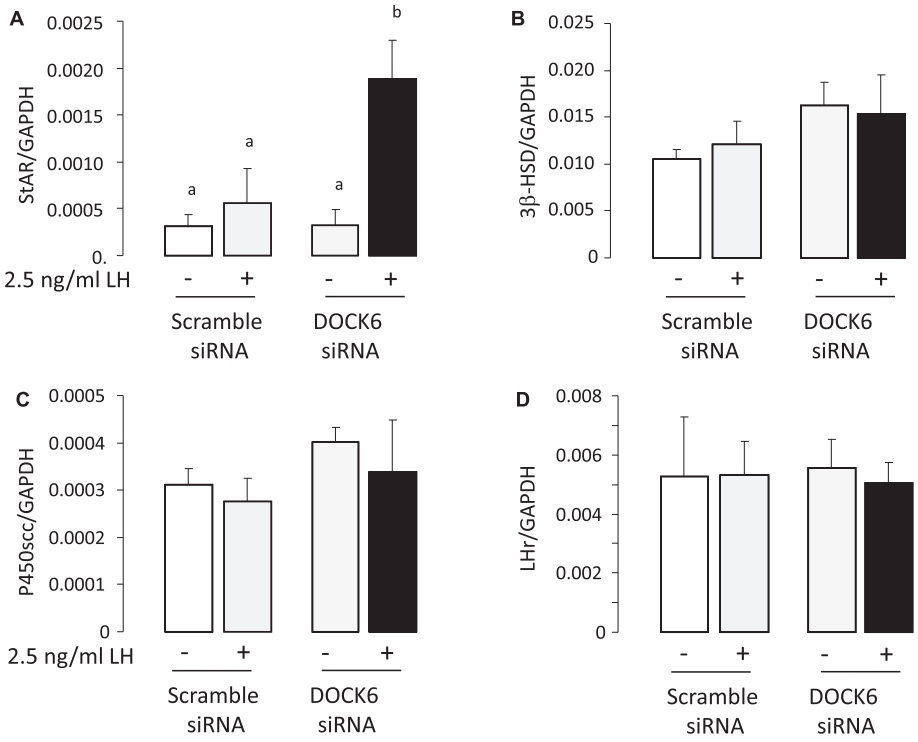

Fig. 3. Effect of DOCK6 on the expression of *StAR* (A), *3β-HSD* (B), *P450scc* (C) and *LHr* (D) mRNA in LH-treated theca cells of HECFs. Theca cells were collected from HECF and treated with or without low LH (2.5 ng/ml) for 48 h. The data are expressed as means ± S.E.M of four experiments with triplicate determination of each. Different superscripts denote significantly different values ($P < 0.05$).

examined its role in steroid production in theca cells of HECFs. In the present study, *Dock6* siRNA treatment induced P4 production, but not A4 production in theca cells of HECFs, suggesting that *Dock6* suppresses P4 production. Generally, A4 and pulsatile LH are important for the development of HECFs. A4 produced by theca cells is a substrate for E2 production in granulosa cells of follicles. Thus, Dock6 may be involved in the development of estrogen-active cystic follicles by suppressing P4 production in theca cells under pulsatile secretion of LH. In fact, the P4 concentration in the follicular fluid of HECF was lower than in other follicles.

Dock6 (also known as Zir1) is a large protein involved in intracellular signaling networks. It belongs to a family of proteins that share high sequence similarity with Dock180, the archetypal member of the DOCK family [16]. There is currently very little information about the cellular role of this protein. Interestingly, DOCK6 has been reported to exhibit dual guanine nucleotide exchange factors (GEFs) specificity towards the small G proteins RAC1 and CDC42 [17]. Our data indicated that knockdown of endogenous *Dock6* by siRNA stimulated the expression of the gene encoding StAR gene in LH-treated theca cells of HECFs. Therefore, our findings suggest that DOCK6 may be associated with the suppression of P4 production by regulating StAR expression via RAC1 and CDC42 in theca cells of HECFs.

In conclusion, of 11 genes detected using the differential display technique, *Dock6* is a candidate marker associated with the development of follicular cysts in the bovine ovary. In addition, Dock6 may be involved in the development of cystic follicles by suppressing P4 production rather than increasing A4 production in theca cells.

Conflict of interest

The authors declare that no conflicts of interest exist that would prejudice the impartiality of this study.

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