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Original Article

Expression and regulation of estrogen receptor 2 and its coregulators in mouse granulosa cells

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Abstract. The cooperative effects of estrogen and oocyte-derived paracrine factors (ODPFs) play critical roles in the normal development of ovarian follicles; however, the mechanism underlying this cooperation has not been well studied. The present study aimed to determine whether ODPFs affect estrogen signaling by regulating the expression of estrogen receptor (ESR) and its coregulators in mouse granulosa cells. Some transcripts encoding ESR coregulators were differentially expressed between cumulus and mural granulosa cells (MGCs). The transcript levels of ESR coregulators, including nuclear receptor corepressor 1 and activator 2, in cumulus cells were significantly suppressed by ODPFs; however, they increased when cumulus cell-oocyte complexes were treated with the transforming growth factor beta receptor I inhibitor, SB431542. Moreover, MGCs exhibited significantly higher ESR2 protein and transcript levels than those in cumulus cells. ODPFs promoted *Esr2* expression in cumulus cells but had no effect on that in MGCs. Overall, regulation of the expression of ESR2 and its coregulators in cumulus cells by oocytes seems to be one of the mechanisms underlying estrogen-oocyte cooperation in well-developed antral follicles in mice.

Key words: Cumulus cells, Estrogen receptor 2, Oocytes

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Cumulus cells are a subpopulation of specialized ovarian granulosa cells that support oocyte development. Therefore, normal development and function of cumulus cells are critical for the production of functional oocytes and normal female fertility. The development and function of cumulus cells are governed by multiple intra- and extra-follicular signals, and oocytes play pivotal roles in coordinating these signals [1, 2]. For example, in cumulus cells, oocytes suppress luteinizing hormone (LH) signaling by suppressing LH receptor expression [3], but stimulate epidermal growth factor (EGF) signaling by promoting the expression of EGF receptors in mice [4]. Moreover, oocytes facilitate fibroblast growth factor (FGF) signaling in cumulus cells by suppressing the expression of the FGF receptor antagonist *Spry2* [5]. Furthermore, they control the expression of transcriptional regulators, such as transcription factors and their coregulators, in granulosa cells to determine the cumulus cell lineage [6]. This suggests that oocytes regulate the expression of signal receptors and key transcription factors, as well as their coregulators, to coordinate intra- and extra-follicular signals in cumulus cells.

In addition to oocytes, estrogen is important for the normal development of ovarian follicles. Mice deficient in estrogen receptor 2 (ESR2; also known as estrogen receptor beta), a predominant estrogen receptor expressed by granulosa cells, exhibit female subfertility due to the attenuated responsiveness of granulosa cells to gonadotropins [7, 8], reduced estrogen production [9, 10], and ovulation failure, partly due to the impaired development of cumulus cells [9–12]. Additionally, estrogen participates in natriuretic peptide type C-mediated maintenance of oocyte meiotic arrest by helping to maintain the expression of natriuretic peptide receptor 2 by cumulus cells [13, 14]. Therefore, estrogen signaling is a critical regulator of cumulus cell development and female fertility.

Several studies have shown that oocytes require estrogen signaling to regulate the functions of granulosa cells. This was first shown by Otsuka *et al*., who reported that signals produced from oocytes are required for the amplification of follicle stimulating hormone (FSH) signaling by estrogen in diethylstilbestrol-primed rat preantral granulosa cells [15]. Furthermore, we have previously shown that estrogen signaling is required for cumulus cells to maintain their competence to undergo cumulus expansion, and that oocytes are required for this estrogen function in mice [16]. The requirement of oocyte signaling for estrogen function seems to be partly due to the ability of oocytes to modulate the effects of estrogen on cumulus cells [17]. However, the mechanism by which oocytes control estrogen signaling in cumulus cells remains largely unknown.

Estrogen signaling in cumulus cells is primarily mediated by the nuclear receptor ESR2. Cytoplasmic ESRs bind to estrogen to form dimers and then translocate into the nucleus, where they act as transcription factors. The transcriptional activity of ESRs is regulated by multiple cofactors, including nuclear receptor coactivators (NCOAs) and corepressors (NCORs). Previously, we have shown that the expression of one of the nuclear receptor coregulators, *Nrip1*, in cumulus cells is regulated by oocyte-derived paracrine factors (ODPFs) in mice [16]. This suggests that oocytes organize ESR coregulators to regulate estrogen signaling in cumulus cells;

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however, their effects on the expression of other ESR coregulators in cumulus cells have not been studied.

To understand the mechanisms by which oocytes regulate estrogen signaling in cumulus cells, we examined their effects on the expression of transcripts encoding ESR2 and its coregulators in mouse cumulus cells.

Materials and Methods

Mice

Female BDF1 mice were either purchased from Sankyo Lab Service (Tokyo, Japan) or produced and raised in the research colony of the investigators at the University of Tokyo. All experiments were conducted using three-week-old female BDF1 mice that had been injected with equine chorionic gonadotropin (ASKA Pharmaceutical, Tokyo, Japan) 42–46 h prior to the experiment. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Tokyo.

Isolation and culture of cumulus cell-oocyte complexes (COCs), cumulus cells, mural granulosa cells (MGCs), and oocytes

COCs, MGCs, and fully grown germinal vesicle-stage oocytes were isolated and cultured as described previously [6]. Fresh cumulus cells were obtained by removing oocytes from COCs via repeated pipetting using fine-bore pipettes. Oocytectomized cumulus cells (OOXs) were produced by microsurgically removing oocytes from COCs, as described previously [18]. The culture medium used was bicarbonate buffered MEMα (Life Technologies, Carlsbad, CA, USA) containing 75 μg/ml penicillin G, 50 μg/ml streptomycin sulfate, 3 mg/ml bovine serum albumin, and 10 μM of the phosphodiesterase inhibitor milrinone (all from Sigma-Aldrich, St. Louis, MO, USA). COCs or OOXs were cultured in drops of 30 μl of culture medium in liquid paraffin for 20 h. MGCs were cultured as monolayers in 100 μl of culture medium in each well of a 96-well plate, as described previously [6]. In some experiments, FSH (100 ng/ml; R&D Systems, Minneapolis, MN, USA) or oocytes (2 oocytes/μl) were added to the culture medium. All cultures were maintained at 37ºC in 5% CO_2.

Real-time polymerase chain reaction (PCR)

Total RNA was extracted using ReliaPrep RNA Cell Miniprep System (Promega K.K., Tokyo, Japan) and reverse transcribed using RevarTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan), according to the manufacturer's protocol. Real-time PCR was performed using THUNDERBIRD qPCR Mix (Toyobo) on an ABI Step One Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR primers used are listed in Table 1. The steady-state levels of transcripts were determined via the $2^{-\Delta\Delta Ct}$ method using the housekeeping gene ribosomal protein L19 as an internal control [19]. A single product of the estimated size was identified by agarose gel electrophoresis for each set of primers, and a dissociation curve analysis was performed at the end of the amplification process to confirm the specificity of the PCR products. Each reaction was conducted in duplicate and real-time PCR experiments were repeated at least three times using biologically independent samples.

Western blot analysis

Western blot analysis was performed as previously described [20]. Anti-ERβ antibody (sc8974; Santa Cruz Biotechnology, Dallas, TX, USA) and peroxidase-conjugated goat anti-rabbit IgG (AP132P; Millipore, Burlington, MA, USA) were used as the primary and secondary antibodies, respectively. Signals were visualized using the ImmunoStar LD western blotting detection kit (Wako Pure Chemicals, Osaka, Japan) and a C-DiGit Blot Scanner and Image Studio for the C-DiGit system (LI-COR, NE, USA).

Statistical analysis

Statistical analyses were performed using a standard *t*-test in Microsoft Excel (Microsoft, Redmond, WA, USA). Statistical significance was set at $p < 0.05$. Values are presented as the mean ± standard error of the mean.

Results

Differential expression of transcripts encoding NCORs and NCOAs between cumulus cells and MGCs in vivo

Because of the difference in the distance from oocytes, transcripts regulated by ODPFs tend to be differentially expressed between cumulus cells and MGCs [21]. Therefore, we first compared the expression levels of transcripts encoding NCORs (*Ncor1* and *Ncor2*) and NCOAs (*Ncoa1-7*) between the two cell types. Efficient isolation of each cell type was confirmed by assessing the levels of known cumulus cell- and MGC-enriched transcripts, namely solute carrier family 38 member 3 (*Slc38a3*; encodes an amino acid transporter) and

Table 1. PCR primer sets used in this study

Symbol	Accession No.	Forward primer	Reverse primer	Product size (bp)
Slc38a3	NM_023805	TATCTTCGCCCCCAACATCTT	TGGGCATGATTCGGAAGTAGA	107
Lhcgr	NM_013582	GGATAGAAGCTAATGCCTTTGACAAC	TAAAAGCACCGGGTTCAATGTATAG	96
Ncor1	NM_001252313	CCCATTTCCAGCGTGTTAGT	GGAGCTTCATGTTTGCTTCC	118
Ncor2	NM_011424	TGGGTCTGAAGACCTTACCA	TCAGCTCCTCCTTGGACAGT	128
Ncoa1	NM_010881	GTCACTCCAGCCCACCAC	TATTTGCCCATGGAATCTGA	125
Ncoa2	NM_001302702	TCACTGCATTGGCTCTTCTG	CATTCCTTGCGTTTTCTGGT	122
Ncoa3	NM_008679	GTGGACTCCGAGATCGGTAA	TCGCCTAGTCCACTCATCCT	94
Ncoa4	NM_001033988	TCGCGAGAACTCTGTCTTCA	CCACTCTGTTCCAGGGATGT	139
Ncoa5	NM_144892	CGAGATCATCGAGACCCTGT	TTCCTCCGGCAATAGTCATC	122
Ncoa6	NM_019825	GACCTGTGGCATCTGGAAAT	CAGGGCTCGTCATCCTTATT	87
Ncoa7	NM_172495	AATCCTTTGCCAGTCACACC	CCCATAGACATCAGGAGACCA	143
Esr2	NM_207707	GGGTGATTTCGAAGAGTGGA	CGTGTGAGCATTCAGCATCT	182
Rpl19	NM_001159483	CCGCTGCGGGAAAAAGAAG	CAGCCCATCCTTGATCAGCTT	103

LH/choriogonadotropin receptor (*Lhcgr*; encodes the LH receptor) (Fig. 1A) [3, 22]. The expression levels of *Ncor1*, *Ncor2*, *Ncoa1*, *Ncoa2*, *Ncoa4*, *Ncoa6*, and *Ncoa7* were significantly lower in cumulus cells than those in MGCs (Fig. 1B, C), suggesting that ODPFs suppress the expression of these transcripts in nearby cumulus cells *in vivo*.

Effects of oocytes on the expression of transcripts encoding NCORs and NCOAs in cumulus cells in vitro

To directly assess the effects of oocytes on the expression levels of transcripts encoding NCORs and NCOAs in cumulus cells *in vitro*, oocytes were microsurgically removed from COCs, and the resulting oocytectomized cumulus cell complexes (OOXs) were cultured with or without oocytes (2 oocytes/μl). The relative expression levels of transcripts in cumulus cells were determined after 20 h of culture. As shown in Fig. 2, the expression levels of *Ncor1* (Fig. 2A) and *Ncoa2* (Fig. 2B) transcripts were significantly downregulated in OOXs cocultured with oocytes as compared with those in OOXs cultured without oocytes. This suggested that steady-state levels of these transcripts in cumulus cells were suppressed by oocytes *in vitro*.

Growth differentiation factor 9 (GDF9) is an ODPF that plays a critical role in cumulus cell development. The GDF9 signal in cumulus cells is mediated by transforming growth factor beta receptor I (TGFBR1), also known as activin receptor-like kinase 5. To determine whether oocyte-derived GDF9 is required for the proper regulation of coregulators in cumulus cells, COCs were treated with the TGFBR1 inhibitor SB431542, and the expression levels of these transcripts were determined. *Ncor1* and *Ncoa2* expression levels were significantly higher in COCs treated with SB431542 than in those without the treatment with SB431542 (Fig. 3A, B), suggesting that ALK5-mediated ODPF signaling, including GDF9, suppressed the expression of these transcripts *in vitro*. In addition, while oocyte coculture had no significant effects on the levels of *Ncoa1*, *Ncoa3*, *Ncoa4*, and *Ncoa6* (Fig. 2B), SB431542 treatment significantly promoted the expression of these transcripts (Fig. 3B). This indicated that GDF9 may suppress the expression of these transcripts in cumulus cells, but its suppressive effects may be masked by other ODPFs, which may promote the expression of these transcripts.

Effects of oocytes on ESR2 expression in cumulus cells and MGCs

To assess the effects of oocytes on the expression of ESR2 in cumulus cells, we first compared the mRNA and protein levels of ESR2 between cumulus cells and MGCs (Fig. 4A, B), and found that both transcript and protein expression levels were significantly higher in MGCs than those in cumulus cells. These high *Esr2* levels in MGCs compared to those in cumulus cells suggest that oocytes suppress the expression of *Esr2* in cumulus cells *in vivo*; however, *Esr2* expression in OOXs was significantly promoted by coculture with oocytes (Fig. 4C). Moreover, *Esr2* expression in COCs significantly decreased after the treatment with SB431542 (Fig. 4D). These results suggested that *Esr2* expression in cumulus cells depends on the oocyte stimulation.

It is well accepted that FSH plays a critical role in MGC development [23]. As *Esr2* expression was significantly higher in MGCs than that in cumulus cells, we hypothesized that FSH may promote *Esr2* expression in MGCs. Therefore, we examined the effect of FSH on the expression of *Esr2* in MGCs. MGCs were cultured as monolayers with or without FSH, and the levels of *Esr2* transcripts were examined. *Esr2* expression significantly decreased with culture, and this reduction was not prevented by FSH supplementation (Fig. 4E). Moreover, oocyte coculture had no significant effect on *Esr2* levels in MGCs (Fig. 4F). Therefore, it appears that *Esr2* expression in MGCs is independent of control by FSH or oocytes, but requires some factors present within the follicles.

Discussion

Although evidence has shown that oocytes play a critical role in the modulation of estrogen signaling in cumulus cells, the underlying mechanism remains unknown. Herein, we showed that transcripts

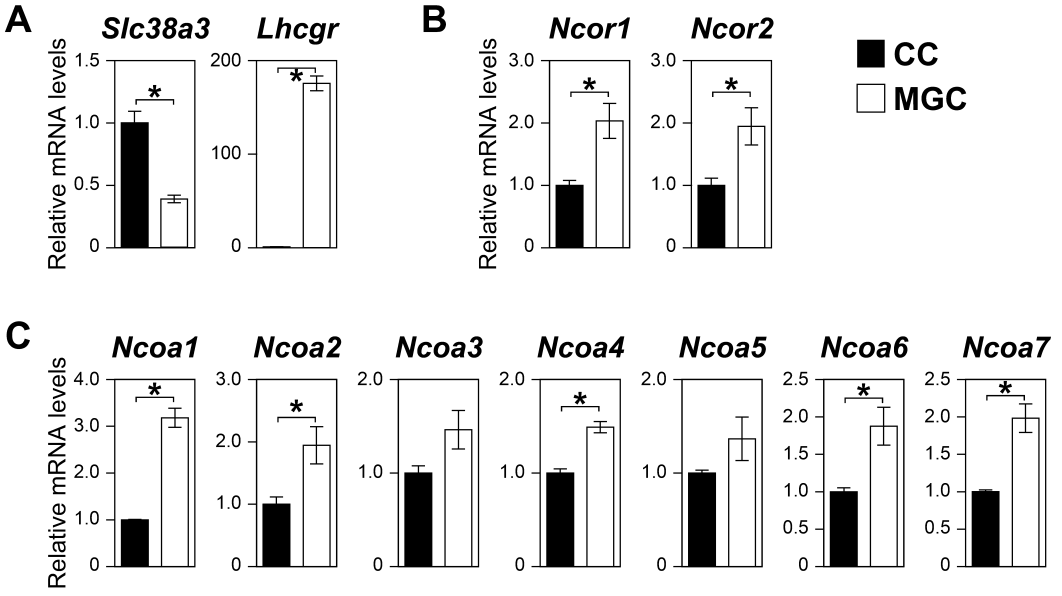

Fig. 1. Differential expression of nuclear receptor coregulators between cumulus cells and MGCs. Levels of transcripts encoding (A) SLC38a3 (cumulus cell-enriched transcript) and LHCGR (MGC-enriched transcript), (B) NCORs, and (C) NCOAs were examined using real-time PCR. Asterisks denote a significant difference ($P < 0.05$).

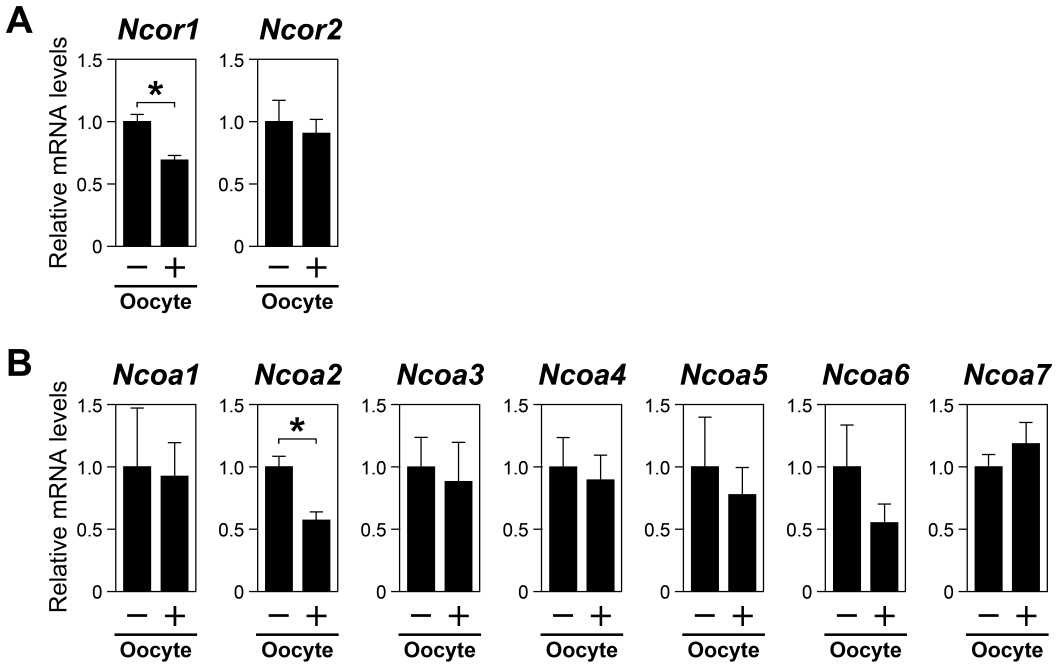

Fig. 2. Effect of oocytes on the expression levels of nuclear receptor coregulators in cumulus cells. The isolated cumulus cell complexes (OOXs) were cocultured with oocytes (2 oocytes/μl), and the levels of transcripts encoding (A) NCORs and (B) NCOAs were determined using real-time PCR. Asterisks denote a significant difference ($P < 0.05$).

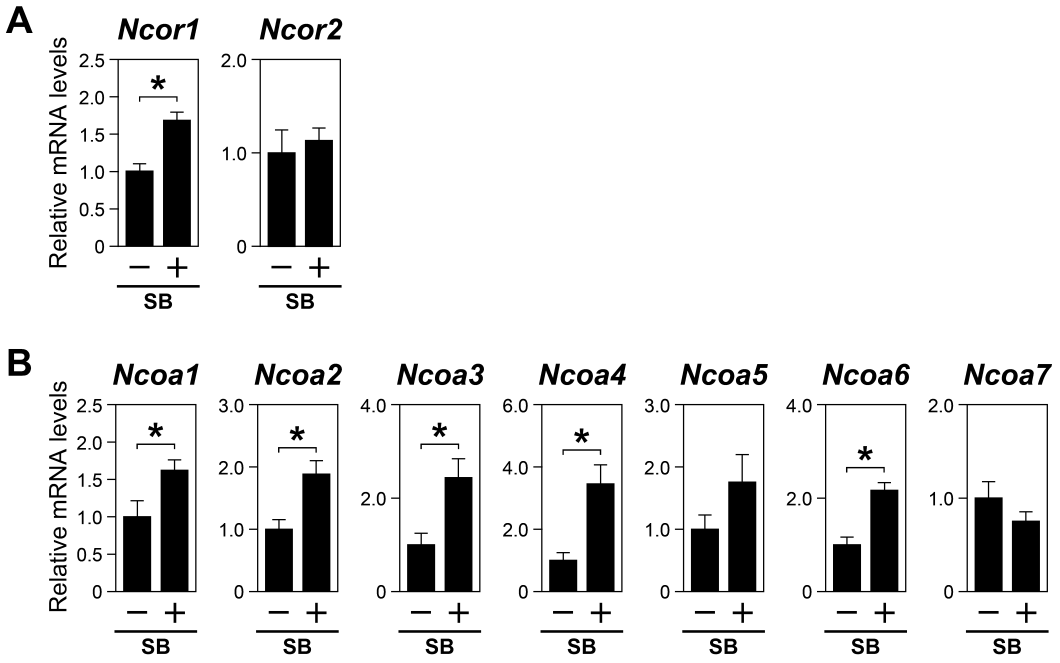

Fig. 3. Effects of SB431542 on the expression levels of nuclear receptor coregulators in cumulus cells. COCs were treated with SB431542 (SB) (10 μM), and the levels of transcripts encoding (A) NCORs and (B) NCOAs were determined using real-time PCR. Asterisks denote a significant difference ($P < 0.05$).

encoding NCORs and NCOAs were differentially expressed between cumulus cells and MGCs, and that some of these transcripts were regulated by oocyte-derived signals. Moreover, the mRNA and protein levels of ESR2 in cumulus cells increased after coculture with oocytes. These results suggest that mouse oocytes regulate estrogen signaling by modulating the expression of both ESR2 and its coregulators in cumulus cells. In this study, we did not examine the effects of other intrafollicular factors, such as FSH, on gene expression in cumulus cells. However, interactions among interfollicular factors are critical for the regulation of granulosa cell development, and therefore need to be assessed in future studies.

The requirement of estrogen signals in the development of ovarian

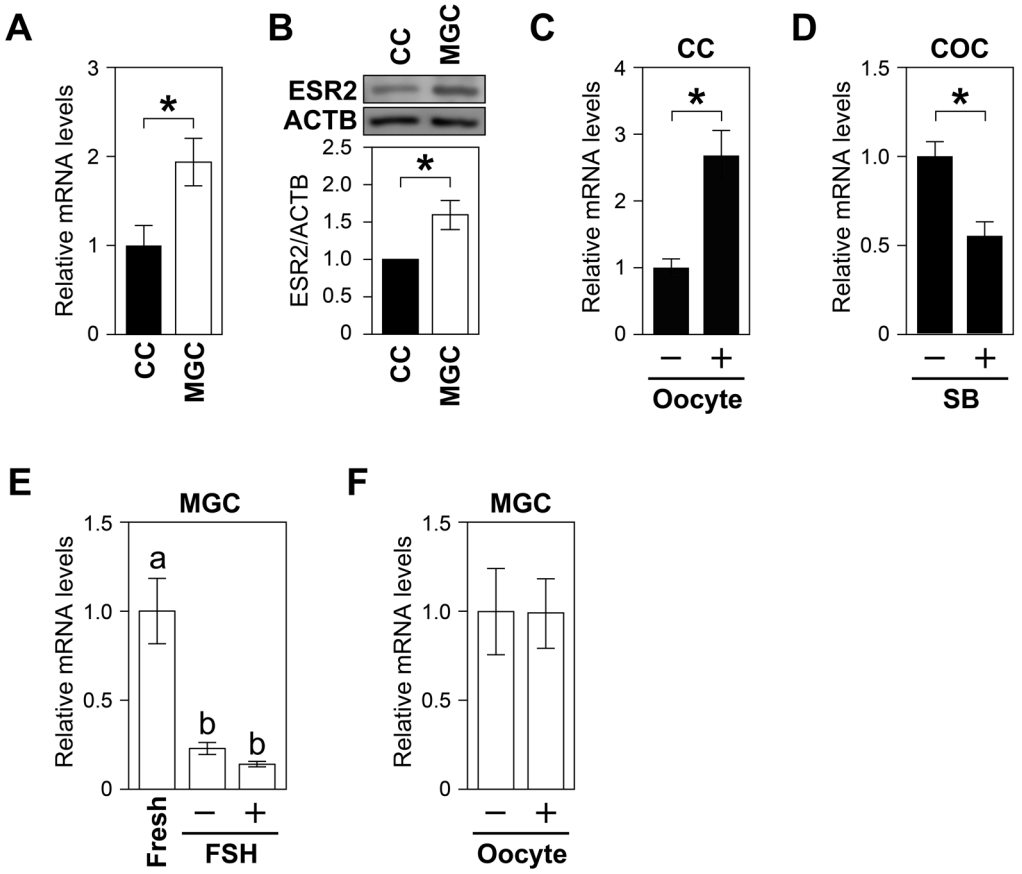

Fig. 4. Expression and regulation of ESR2 in granulosa cells. (A) mRNA and (B) protein expression levels of ESR2 in the two cell types were compared via real-time PCR and western blotting, respectively. (C) Effects of oocytes on *Esr2* expression in cumulus cells. (D) Effects of SB431542 (SB) on *Esr2* expression in COCs. (E) Effects of FSH (10 ng/ml) on *Esr2* expression in MGCs. (F) Effects of oocytes (2 oocytes/μl) on *Esr2* expression in MGCs. Asterisks or different letters (a and b) denote significant differences ($P < 0.05$).

follicles and female fertility is well documented in the phenotypes of estrogen receptor-knockout mice. Female mice deficient in estrogen receptor 1 (*Esr1*), also known as estrogen receptor alpha, are infertile because of their anovulation phenotype [24], whereas female *Esr2* knockout mice are subfertile [11]. The *Esr2*-deficient ovary contains reduced numbers of large antral follicles and corpora lutea compared to wild-type mice, while atresia of large antral follicles is increased [9]. Moreover, *Esr2*-deficient MGCs exhibit an attenuated response to FSH-induced differentiation, and cumulus cells of *Esr2*-deficient mice fail to undergo cumulus expansion [7, 10, 25]. These deficiencies in granulosa cell development in *Esr2* knockout mice probably account, at least in part, for the reduced fertility in the mutant mice. Therefore, although both ESR1 and ESR2 are indispensable for female fertility, ESR2 seems to be a critical mediator of estrogen signaling in granulosa cells. The results presented here suggest that the expression of ESR2 and its coregulators in cumulus cells is regulated by oocytes. This may provide useful insights into the mechanism by which oocytes regulate the development and function of granulosa cells as well as female fertility.

In the present study, *Ncor1* and *Ncoa2* were differentially expressed in cumulus cells and MGCs *in vivo*, and their expression in cumulus cells was suppressed by oocytes *in vitro*. Therefore, it is likely that the differential expression of these coregulators observed *in vivo* can be attributed to oocyte control. Since NCOR1 is a corepressor of nuclear receptors, whereas NCOA2 is a coactivator, it is possible that by repressing both *Ncor1*and *Ncoa2* expression, oocytes may consequently affect the quality of estrogen signaling without affecting its intensity.

NOCR1 was originally identified as a coregulator of thyroid hormone and retinoic acid receptor [26] and is now reported to be a corepressor of various other nuclear receptors, including peroxisome proliferator-activated receptor, liver X receptor, and estrogen-related receptor alpha [27–29]. Although few studies have assessed the involvement of NCOR1 in ESR2 signaling, recruitment of a corepressor complex containing NCOR1 to the *ESR1* promoter region by ESR2 has been reported in human breast cancer cell lines [30]. Transcripts encoding NCOR1 have been reported to be expressed in rat ovaries and bovine corpus luteum [31, 32]. Moreover, increased NCOR1 expression in granulosa cells has been implicated in the pathogenesis of polycystic ovary syndrome (PCOS) and clinical pregnancy failure in humans [33]. Interestingly, some variants of ODPF-encoding genes, such as *GDF9* and bone morphogenetic protein 15, have also been reported to be associated with PCOS [34, 35]. Therefore, it may be surmised that dysregulation of oocyte suppression of NCOR1 expression in cumulus cells may partly account for the pathogenesis of PCOS, however, this requires further research.

In contrast to NCOR1, the expression and function of NCOA2 in mammalian ovaries has not been well studied. NCOA2 is a member of the steroid receptor coactivator (SRC) family, which is known as a coactivator for many members of the nuclear receptor superfamily,

including those required for reproductive biology [36]. Although no direct evidence for the recruitment of NCOA2 in ESR2-dependent transcription has been reported, colocalization of ESR2 and NCOA2 has been reported in human placental syncytiotrophoblast cells [37]. Loss of the *Ncoa2* gene results in subfertile phenotypes in both sexes of mice; *Ncoa2*-deficient males are subfertile due to defects in spermatogenesis and age-dependent testicular degeneration, whereas females exhibit subfertility mainly due to placental hypoplasia [38]. In addition, *Ncoa2*-deficient mice exhibit higher energy expenditure and are resistant to diet-induced obesity [39] owing to elevated glycolysis and fatty acid degradation in the liver [40]. Therefore, it is well accepted that the SRC proteins, including NCOA2, are important regulators of cellular metabolic activities [41]. Interestingly, our previous studies have identified several metabolic pathways that are promoted by ODPFs, including glycolysis, cholesterol biosynthesis, and amino acid uptake by cumulus cells [22, 42–44]. Accordingly, the present study showed that ODPFs suppressed *Ncoa2* expression in cumulus cells. Therefore, it is possible that ODPFs regulate metabolic activities in cumulus cells by regulating *Ncoa2* expression. Further analysis is warranted to assess this possibility.

In addition to *Ncor1* and *Ncoa2*, the present analyses identified several other nuclear receptor coregulators that were also differentially expressed between cumulus cells and MGCs. Nevertheless, the mechanism by which the differential expression of these coregulators is maintained remains to be determined. The differential expression of these coregulators likely comprises differential intracellular signaling of nuclear receptors between the two granulosa cell populations, which may account for the differences in the cellular response to extracellular signals of these two cell types.

In summary, the present study provides valuable insights into the mechanism by which oocytes control estrogen signaling in cumulus cells. In addition, considering that the follicular estrogen production is also influenced by oocytes [45, 46], the interaction between oocytes and estrogen signaling seems to exist in multiple layers of signal regulation, including ligand (estrogen) production, receptor expression, and receptor coregulator expression.

Conflicts of interests: The authors declare that there are no conflicts of interests.

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