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Expression and Regulation of FGF Receptors in Mouse Granulosa Cells

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Abstract: Signals from fibroblast growth factors (FGFs) play a critical role in regulating the development of ovarian granulosa cells; however, the expression and regulation of FGF receptors are not well understood. The present study was conducted to assess the expression kinetics of transcripts encoding FGF receptors (FGFRs) during granulosa cell development in mice. In addition, the effects of oocytes on the levels of *Fgfr* transcripts were examined, as well as the expression of *FGFR* mRNA expression in porcine granulosa cells. All four types of *Fgfr* transcripts were detected in cumulus and mural granulosa cells with a tendency toward higher expression levels in mural granulosa cells. Interestingly, the expressions of *Fgfr1* and *Fgfr2* mRNA in cumulus cells were suppressed by co-culture with oocytes, whereas their expression in mural granulosa cells was promoted. Porcine cumulus cells expressed higher levels of *FGFR1* and *FGFR2* mRNA than mural granulosa cells. The results show that FGF receptor expression in granulosa cells, at least at the mRNA level, is dynamically regulated during follicular development, and that oocytes participate in the regulation of differential expression between cumulus and mural granulosa cells. Moreover, the regulation of transcripts encoding FGF receptors in granulosa cells may differ between mice and pigs.

Key words: oocyte, FGF, Granulosa cells, Follicle, Mouse

Introduction

Development of ovarian follicles is controlled by various factors including gonadotropins, steroids, and growth factors. Among the many growth factors produced within follicles, the requirement of the transforming growth

factor- β (TGF- β) superfamily proteins for normal female fertility is well recognized. For example, female mice deficient in growth differentiation factor 9 (*Gdf9*) and/or bone morphogenetic protein 15 (*Bmp15*), two members of the TGF- β superfamily secreted by oocytes, exhibit reduced fertility, due at least in part to defective function of somatic granulosa cells [1–3]. Therefore, TGF- β superfamily proteins play a critical role in regulating the development and function of granulosa cells.

Proteins belonging to the fibroblast growth factor (FGF) family are also produced within follicles. For example, production of FGF8 by oocytes has been reported in mice and cows [4, 5]. Oocyte-derived FGF8 interacts with BMP15 to promote the expression of genes encoding glycolytic enzymes in cultured mouse cumulus cells [6]. Expression of FGF10 and FGF7 has been reported in bovine follicles [5, 7, 8]. FGF10 suppresses estradiol production in cultured bovine granulosa cells, but has no effect on their proliferation [9]. Therefore, FGFs, sometimes acting in accordance with TGF- β superfamily proteins, appear to be another critical regulator of granulosa cell development and function. In fact, several mouse lines with mutations in genes encoding FGF receptors exhibit an infertile or sub-fertile phenotype [10–13]. However, the developmental kinetics of the expression and regulation of FGF receptors in granulosa cells are not understood.

Through their production of paracrine factors, oocytes play an active role in coordinating differential gene expression between two granulosa cell sub-populations: cumulus cells, which are located near the oocytes, and mural granulosa cells, which are located far from oocytes within follicles. For example, oocytes suppress the expression of transcripts encoding luteinizing hormone receptor (*Lhcgr*) in cumulus cells [14]. Because of this oocyte effect, mural granulosa cells express significantly higher levels of *Lhcgr* mRNA than cumulus cells *in vivo*. In contrast, *Slc38a3* transcripts that encode an amino

Table 1. Primer Sets Used for RT-PCR

Gene	Accession no.	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Amplicon size (bp)
<i>Fgfr1</i>	NM_010206	CCACTCTGCGCTGGTTGAA'	AGGCACCACAGAATCCATTATGA	110
<i>Fgfr2</i>	NM_010207	GTGGTTAAAAAACGGGAAGGAGTT	ACGGGACCACACTTTCCATAATAA	101
<i>Fgfr3</i>	NM_008010	TCAGAGGCTGCAAGTGCTAA	ACTGAAGTGGCACAGCACAC	94
<i>Fgfr4</i>	NM_008011	ACTGGCTCAAGGATGGACAG	TCCGAGGGTACCACACTTTC	106
<i>Rpl19</i>	NM_009078	CCGCTGCGGAAAAAGAAG'	CAGCCCATCCTTGATCAGCTT	103

acid transporter, and transcripts encoding enzymes required for glycolysis and cholesterol biosynthesis, are promoted by oocytes in cumulus cells, and thus the levels of these transcripts are greater in cumulus cells than mural granulosa cells [3, 15, 16]. Therefore, it is likely that the transcripts differentially expressed between cumulus cells and mural granulosa cells are affected by the factors produced by oocytes [17, 18].

To understand the ovarian FGF system in more detail, the steady-state levels of transcripts encoding FGF receptor in cumulus and mural granulosa cells, and the effect of oocytes on these transcript levels were examined. In addition, the FGF receptor expression was also examined in porcine cumulus and mural granulosa cells.

Materials and Methods

Mice

C57Bl/6 x DBA/2 F1 (BDF1) mice were purchased from Sankyo Labo Service (Tokyo, Japan) or bred in the laboratory of the investigators at the University of Tokyo. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Tokyo.

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

COCs, mural granulosa cells, and oocytes were isolated from 3-week-old female mice with or without prior (44–48 h earlier) equine chorionic gonadotropin (eCG) (Asuka Seiyaku, Japan) treatment, as reported previously [15]. Cumulus cells were obtained by pipetting the COCs with a narrow-bore pipette. Cumulus and mural granulosa cells isolated without eCG-treatment were enriched in those from the late-secondary and early-antral follicles, whereas these cells isolated with eCG-treatment were enriched in those from the well-developed antral follicles. COCs were also isolated from ovaries of mice at 3 h and 6 h after human chorionic gonadotropin (hCG) treatment that was preceded by eCG treatment. Ovulated COCs were isolated from the oviducts of mice

14 h after the superovulation treatment. Some of the cells were immediately applied to a real-time PCR analysis to assess the expression levels of the transcripts encoding FGF receptors by real-time PCR (see below). Oocytes were microsurgically removed from the COCs to produce OOX cumulus cells as reported previously [19].

To analyze the effect of oocytes on the expression of *Fgfr* mRNAs, OOX cumulus cells or mural granulosa cells were cultured in the presence or absence of fully-grown germinal vesicle-stage oocytes (2 oocytes/ μ l) for 20 h. This concentration of oocytes was sufficient for full suppression of *Lhcgr* expression in mural granulosa cells *in vitro* in a previous study [14]. The culture medium used was bicarbonate buffered MEM alpha (Invitrogen) supplemented with 75 μ g/ml penicillin G, 50 μ g/ml streptomycin sulfate, and 3 mg/ml bovine serum albumin (Sigma-Aldrich). The medium was also supplemented with 10 μ M of a phosphodiesterase inhibitor, milrinone (Sigma-Aldrich), to maintain oocytes at the germinal vesicle stage. Milrinone was added to all cultures, irrespective of the presence or absence of oocytes. Cultures were performed in drops under mineral oil, and maintained at 37°C in an atmosphere of 5% O₂, 5% CO₂, and 90% N₂.

Isolation of porcine granulosa cells

Porcine COCs, cumulus cells, and mural granulosa cells were collected from antral follicles (2–5 mm in diameter) obtained from a commercial slaughterhouse, as reported previously [20].

Total RNA extraction from granulosa cells, reverse transcription, and real-time PCR

Total RNAs were isolated from COCs, cumulus and mural granulosa cells using an RNeasy Micro kit (Qiagen) according to the manufacturer's protocol. The extracted RNA was then reverse-transcribed using a QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol.

Steady-state levels of *Fgfr* transcripts were determined by real-time PCR using Thunderbird™ SYBR® qPCR Mix (TOYOBO) with a StepOnePlus™ Real-Time PCR Sys-

tem (Applied Biosystems). The PCR primer sets used are summarized in Table 1. The PCR primer sets used for the porcine FGF receptors were reported previously [21]. The results are presented as the expression levels relative to the transcript amount of a standard sample after normalization to the expression levels of a housekeeping transcript, *Rp19*, by the $2^{-\Delta\Delta Ct}$ method [22]. Melting curve analyses were performed in order to avoid false-positive signals at the end of the reaction. The reactions were run in duplicate and the PCR products were applied to agarose gel electrophoresis to confirm the sizes.

Statistical analysis

All experiments were repeated at least three times. The Tukey test or a standard *t*-test was used for multiple or paired comparisons, respectively, using the program Microsoft Excel (Microsoft) with add-in Excel-statistics software (Social Survey Research Information Co, Ltd.). A *P* value <0.05 was considered statistically significant.

Results

Kinetics of *Fgfr* mRNA expression in granulosa cells during follicle development

The expression levels of *Fgfr* mRNA in the cumulus and mural granulosa cells of ovaries before and after eCG treatment were examined using real-time PCR (Fig. 1). All *Fgfr* mRNAs were detected in both cumulus and mural granulosa cells. The expression levels of transcripts encoding FGFR1 tended to be lower in cumulus cells than in mural granulosa cells but the differences were not statistically significant. The levels of *Fgfr2* mRNA were significantly lower in cumulus cells than in mural granulosa cells before and after eCG treatment. Moreover, the *Fgfr2* mRNA levels in mural granulosa cells were significantly increased by eCG treatment. *Fgfr3* transcripts were detectable in both cumulus and mural granulosa cells before and after eCG treatment, but the levels detected were unstable, probably because of the relatively low *Fgfr3* mRNA expression levels. *Fgfr4* transcript levels were lower before eCG treatment in both cumulus and mural granulosa cells. After eCG treatment, the *Fgfr4* transcript levels increased in both cumulus and mural granulosa cells.

The expression levels of *Fgfr* mRNA in COCs during the ovulation period were examined using real-time PCR (Fig. 2). The levels of these *Fgfr* transcripts in COCs did not change significantly during the hCG-induced ovulation period or in ovulated COCs. Therefore, *Fgfr* mRNA expression was dynamically regulated during the development of follicles.

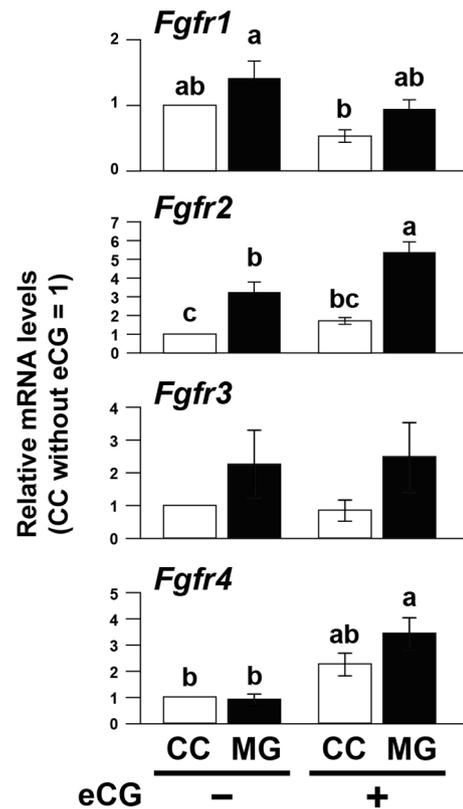


Fig. 1 Expression of transcripts encoding FGF receptors in cumulus and mural granulosa cells with or without eCG-priming. White and black bars indicate cumulus cells (CC) and mural granulosa cells (MG), respectively. Values with different letters (a, b, and c) are significantly different ($P < 0.05$). n=4.

Effects of oocytes on the expression of *Fgfr* transcripts

Since it appeared likely that the transcripts differentially expressed between cumulus and mural granulosa cells were regulated by oocytes, we tested this possibility by assessing *Fgfr* mRNA levels in cumulus cells and mural granulosa cells after co-culturing in the presence or absence of oocytes. The cumulus and mural granulosa cells were isolated from the eCG-primed mice.

As shown in Fig. 3A, the expression levels of both *Fgfr1* and *Fgfr2* mRNA were significantly lower in cumulus cells co-cultured with oocytes than in cumulus cells cultured alone. This suggests that factors secreted by oocytes suppress the expression of *Fgfr1* and *Fgfr2* mRNA in cumulus cells. We also measured the levels of *Fgfr3* and *Fgfr4*; however, these transcripts were barely detectable (data not shown), suggesting that some factors present in the follicle are required for maintaining the expression

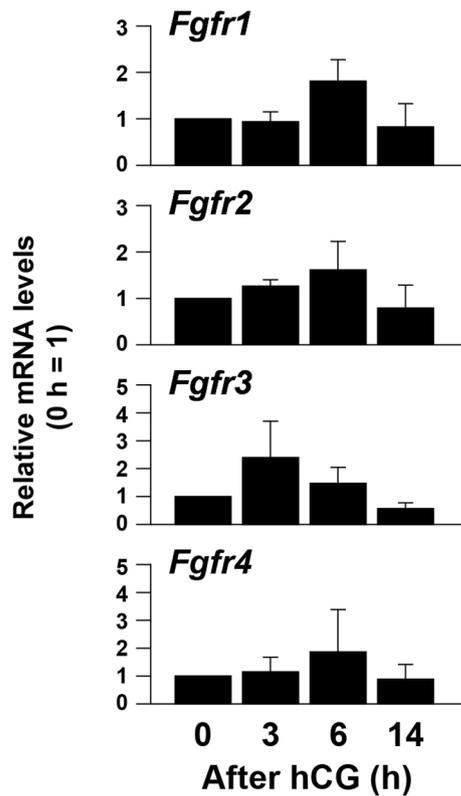


Fig. 2 Expression of transcripts encoding FGF receptors in COCs during the ovulation period. No significant differences were detected among time points. n=3.

of these transcripts *in vitro*. In contrast, mural granulosa cells co-cultured with oocytes exhibited elevated levels of *Fgfr1* and *Fgfr2* mRNA compared with those cultured without oocytes, suggesting that oocytes promote *Fgfr1* and *Fgfr2* expression in mural granulosa cells. Therefore, the effects of oocytes on the regulation of *Fgfr1* and *Fgfr2* mRNA expression differed between cumulus and mural granulosa cells: while oocytes promoted *Fgfr1* and *Fgfr2* expression in mural granulosa cells, whereas they suppressed their expressions in cumulus cells.

Fgfr transcript levels in porcine CCs and MGs

To assess the diversity of the FGF system in mammalian ovaries, the expression levels of *FGFR1* and *FGFR2* were examined in porcine cumulus and mural granulosa cells. We used the PCR primer sets for specific variants of porcine *FGFR1* and *FGFR2* reported previously (see Discussion) [21], to detect porcine *FGFR* transcripts. In contrast to the expression patterns of *Fgfr1* and *Fgfr2* mRNA in mouse cumulus and mural granulosa cells, porcine cumulus cells expressed significantly higher levels of *FGFR1* and *FGFR2* than porcine mural granulosa cells (Fig. 4).

Discussion

Although there is evidence that FGF signals are a critical regulator of follicular development in mammals, the

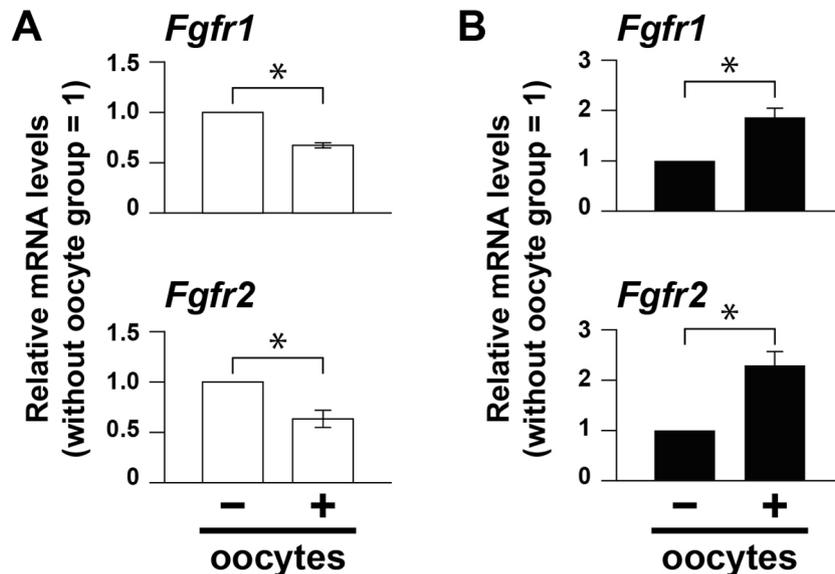


Fig. 3 Effect of oocytes on expression levels of transcripts encoding *FGFR1* and *FGFR2* in (A) cumulus cells (n=3), and (B) mural granulosa cells (n=5).

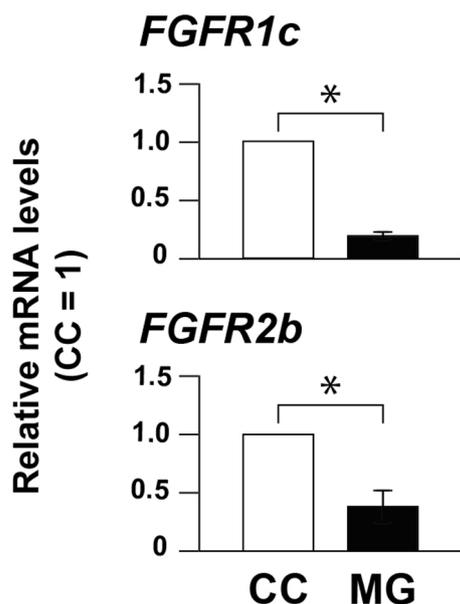


Fig. 4 Comparison of expression levels of transcripts encoding FGFR1c and FGFR2b in porcine cumulus (white bars), and mural granulosa cells (black bars). * $P < 0.05$. $n=4$.

expression profiles of FGF receptors are not well understood in mice. Therefore, this study was conducted to assess the expression kinetics and regulation of transcripts encoding *Fgfr* mRNA expression in cumulus cells and mural granulosa cells. The results show that transcripts encoding FGF receptors were detected in both cumulus and mural granulosa cells in mice, and their expression levels were dynamically regulated during follicular development. Interestingly, in mice, mural granulosa cells expressed higher levels of *Fgfr1* and *Fgfr2* transcripts than cumulus cells, and the differential expression of *Fgfr1* and *Fgfr2* between cumulus and mural granulosa cells appears to be regulated by oocytes. While the presence of oocytes suppressed *Fgfr1* and *Fgfr2* mRNA expressions in cumulus cells, their expression in mural granulosa cells was promoted. The differential expression of *Fgfrs* may elicit a differential response of cumulus and mural granulosa cells to FGF signals. Therefore, oocytes might regulate how cumulus and mural granulosa cells respond to FGF signals by controlling the expression of *Fgfr* mRNA in cumulus and mural granulosa cells.

FGF receptors undergo complex alternative splicing that results in multiple proteins with different ligand preferences. For example, *in vitro* studies using human recombinant proteins have shown that FGF8 subfamily ligands (i.e., FGF8, FGF10, and FGF22) preferentially

activate the FGFR3c and FGFR2c splicing variants and, to a lesser extent, FGFR1c [23, 24]. In the present study, we used PCR primer sets that amplify common intercellular kinase domains of mouse FGF receptors, and we detected all the splicing variants of FGFRs. It is important to assess which FGFR variants are expressed in which cell types and stages of follicular development. Since multiple FGF ligands seem to be produced within ovaries, it is possible that FGF signaling may be controlled by the levels of expression and types of splicing variants of the FGF receptors.

Mouse oocytes play a critical role in regulating transcript levels in cumulus cells and mural granulosa cells. For example, the expression of transcripts encoding enzymes involved in glycolysis and the cholesterol biosynthetic pathway are promoted by oocyte-derived factors in cumulus cells [3, 6, 15]. On the other hand, the expressions of transcripts encoding the LH receptor or SPRY2, one of the regulator of receptors tyrosine kinase signals such as FGFRs, are suppressed by oocytes [14, 25]. Interestingly, the present results show that while oocytes suppressed the expression of transcripts encoding FGFR1 and FGFR2 in cumulus cells, their expression in mural granulosa cells was promoted. Therefore, cumulus and mural granulosa cells responded differentially to oocytes in regard to the regulation of FGFRs. The reverse has been observed for regulation of a transcript encoding an amino acid transporter, SLC38A3 [16, 26]. While oocytes promoted *Slc38a3* expression in cumulus cells, they suppressed *Slc38a3* expression in mural granulosa cells. The underlying mechanism for this differential response between cumulus and mural granulosa cells have not yet been determined.

The expression levels of transcripts encoding *Fgfr1* and *Fgfr2* were significantly higher in mural granulosa cells than those in cumulus cells in mice. In pigs, on the other hand, cumulus cells expressed significantly higher levels of FGFRs than mural granulosa cells. The PCR primer sets used to detect transcripts encoding porcine FGFRs were designed to amplify specific variants of these FGFRs (i.e., FGFR1c and FGFR2b). Although further studies will be needed to examine the detailed expression profiles of each alternative splicing variant in the cumulus and mural granulosa cells of mice and pigs, it is possible that the expression of transcripts encoding FGF receptors is differentially regulated among mammalian species. In fact, broad diversity has been observed in the regulation of cumulus cell function by oocytes. For example, while the expansion of cumulus cells requires factors secreted from oocytes in mice, porcine and bovine cumulus cells do not require oocytes [27–29]. Simi-

larly, whereas oocytes promote the expression of transcripts encoding glycolytic enzymes in murine cumulus cells, bovine oocytes have less effect on glycolytic activity in bovine cumulus cells [30, 31]. Alternatively, the differences in the stages of follicular development used in the present study may account for the differential expression of transcripts encoding FGFRs between mice and pigs.

In summary, the dynamic regulation of FGF receptor mRNA expression during granulosa cell development suggest the importance of FGF signals in the regulation of the development and function of granulosa cells. In addition, oocytes appear to play a critical role in determining the expression of FGFR transcripts in granulosa cells, at least in mice. Further studies assessing the physiological role and the necessity of FGF signals are required to understand the entire FGF system in mammalian ovaries.

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