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Fibroblast Growth Factors and Epidermal Growth Factor Cooperate with Oocyte-Derived Members of the TGFbeta Superfamily to Regulate *Spry2* mRNA Levels in Mouse Cumulus Cells[1]

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ABSTRACT

Mouse oocytes produce members of the transforming growth factor beta (TGFbeta) superfamily, including bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9), as well as fibroblast growth factors (FGFs). These growth factors cooperate to regulate cumulus cell function. To identify potential mechanisms involved in these interactions, the ability of fully grown oocytes to regulate expression of BMP or FGF antagonists in cumulus cells was examined. Oocytes promoted cumulus cell expression of transcripts encoding antagonists to TGFbeta superfamily members, including *Grem2*, *Htra1*, *Htra3*, and *Nog* mRNAs. In contrast, oocytes suppressed cumulus cell expression of *Spry2* mRNA, which encodes a regulator of receptor tyrosine kinase signals, such as FGF and epidermal growth factor (EGF) receptor signals. The regulation of *Spry2* mRNA levels in cumulus cells was studied further as a model for analysis of potential mechanisms for cooperativity of FGF/EGF signaling with oocyte-derived members of the TGFbeta superfamily. Oocytes suppressed basal and FGF-stimulated *Spry2* mRNA levels in cumulus cells but promoted EGF-stimulated levels. Furthermore, recombinant TGFbeta superfamily proteins, including BMP15 and GDF9, mimicked these effects of oocytes. Elevated expression of *Spry2* mRNA in cumulus and mural granulosa cells correlated with human chorionic gonadotropin-induced expression of mRNAs encoding EGF-like peptides. Therefore, oocyte-derived members of the TGFbeta superfamily suppress FGF-stimulated *Spry2* mRNA levels before the luteinizing hormone surge but promote *Spry2* mRNA levels stimulated by EGF receptor-mediated signals after the surge.

BMP15, EGF, FGF8, follicular development, GDF9, gene regulation, granulosa cells, oocytes, sprouty 2

INTRODUCTION

Mammalian oocytes play a key role in orchestrating the development and function of surrounding granulosa cells. For example, paracrine signals derived from oocytes affect proliferation, steroidogenesis, and several metabolic processes of granulosa cells and, at least in mouse, promote cumulus expansion [1–3]. Critical paracrine factors mediating the oocyte-to-granulosa cell communication include members of the transforming growth factor beta (TGFβ) superfamily, such as bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) [4–7]. *Bmp15* and/or *Gdf9* null mice exhibit reduced fertility, at least in part because of impaired functions of the granulosa cells and lower developmental competence of the oocytes [6–9]. Likewise, oocyte-secreted factors, BMP15 and GDF9, improved the developmental competence of bovine oocytes undergoing maturation in vitro [10]. Therefore, precise coordination of oocyte-derived paracrine signals in the follicles is essential for normal development of both granulosa cells and oocytes.

In addition to TGFβ superfamily members, oocytes as well as follicular somatic cells express various fibroblast growth factor (FGF) ligands and receptors in many mammalian species, including mouse [9, 11–13], rat [14–17], bovine [18–20], and human [21, 22]. In cultured granulosa cells, FGFs stimulate mitosis in rabbit, pig, guinea pig, and human [23]; alter steroidogenic activity in rat and bovine [24–28]; suppress luteinizing hormone (LH) receptor formation [24, 26]; and inhibit apoptosis in rat [15]. Furthermore, FGFs promote primordial follicle development in rat and human ovarian organ culture [17, 29, 30] and growth of bovine oocytes enclosed in granulosa cell complexes [31]. Therefore, it has become evident that FGF signals also play roles during mammalian follicular development. Interestingly, a recent study has shown that oocyte-derived FGFs, mainly FGF8, act in concert with BMP15 to induce glycolysis in cumulus cells, which are granulosa cells associated with oocytes [9]; however, the mechanisms by which these signals cooperate are unknown.

Cooperation of BMP and FGF signals is found in several tissues where both BMP and FGF signals are present. For example, during calvarial suture osteogenesis, increases in FGF signals augment BMP signals by suppressing expression of *Nog* transcript, encoding the BMP antagonist noggin [32]. Similarly, in chondrocytes, FGF signals suppress levels of *Nog*, *Grem1*, and *Chrd* mRNA encoding BMP antagonists, noggin, gremlin1, and chordin [33, 34]. Furthermore, during heart tract development, loss of FGF8 signal results in elevated expression of *Grem2* mRNA, which is correlated with disrupted BMP and TGFB signals [35]. These reports suggest that suppression of transcripts encoding BMP/FGF antagonists might be involved in the cooperation of BMP and FGF signals in cumulus cells during ovarian follicular development.

To understand further how oocytes regulate development and function of surrounding granulosa cells, we sought to identify potential mechanisms involved in the signal cross talk between BMP and FGF signals in cumulus cells. We screened granulosa cell expression of antagonists of BMP or FGF action

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and found that *Spry2* mRNA levels in cumulus cells were suppressed by oocyte-derived factors. *Spry2* mRNA encodes sprouty 2 (SPRY2) protein, which regulates signals of receptor tyrosine kinases, including FGF and epidermal growth factor (EGF) receptors [36, 37]. In mammalian cells, SPRY protein inhibits FGF receptor signals but appears to potentiate EGF receptor signals [36, 37]. Because EGF receptor signals play a critical role after the LH surge [38–41], the effects of EGF, as well as FGFs, on *Spry2* mRNA levels in cumulus cells were examined.

MATERIALS AND METHODS

Isolation and Culture of Cumulus Cell-Oocyte Complexes, Fully Grown Oocytes, Mural Granulosa Cells, and Oocytectomized Cumulus Cells

All experiments were conducted using B6SJLF1 mice maintained at The Jackson Laboratory according to the Guide for the Care and Use of Laboratory Animals (Institute for Learning and Animal Research).

Cumulus cell-oocyte complexes (COCs), fully grown oocytes, and mural granulosa cells were isolated from antral follicles of 22- to 24-day-old female mice that had been injected with 5 IU of equine chorionic gonadotropin (eCG) 44 to 48 h earlier as reported previously [42]. Oocytectomized (OOX) cumulus cells were produced by microsurgically removing oocytes, but not the zona pellucida, from the COCs [43]. In the experiments shown in Figure 4, COCs and mural granulosa cells were isolated from 22- to 24-day-old female mice injected with 5 IU of human chorionic gonadotropin (hCG) after the 48-h eCG injection as indicated in the figure legend.

The culture medium used was bicarbonate-buffered MEMα (Life Technologies, Inc.) with Earles salts, supplemented with 75 μg/ml of penicillin G, 50 μg/ml of streptomycin sulfate, 0.23 mM pyruvate, and 3 mg/ml of bovine serum albumin (Sigma-Aldrich Co.). Oocytes were maintained at the germinal vesicle stage by addition of the phosphodiesterase inhibitor milrinone (10 mM; Sigma-Aldrich) throughout the experiments. Milrinone was added to all culture medium even if no oocytes were present for experimental balance. Cultures were performed in drops of culture medium under washed mineral oil for the indicated period. All cultures were maintained at 37°C in a modular incubation chamber (Billups Rothenberg) infused with 5% O_2, 5% CO_2, and 90% N_2.

In some experiments, culture medium was supplemented with recombinant human TGFB2 (10 ng/ml; Leinco Technologies), human BMP4 (100 ng/ml; R&D Systems), human BMP6 (100 ng/ml; R&D Systems), human BMP15 (500 ng/ml), mouse GDF9 (500 ng/ml), human FGF1 (Sigma), human FGF2 (BD Biosciences), human FGF4 (R&D Systems), mouse FGF8B (Sigma), or mouse EGF (BD Biosciences) as indicated in figure legends. Recombinant BMP15 and GDF9 were produced at Baylor College of Medicine by Q. Li and M.M. Matzuk or were obtained from R&D Systems. Control buffer from purification of recombinant proteins had no effect on transcript levels in cumulus cells. We obtained identical results using either preparation of recombinant GDF9 or BMP15.

Real-Time RT-PCR

Steady-state levels of transcripts were assessed using real-time RT-PCR as reported previously [9]. The results were first normalized to the expression levels of a housekeeping gene, ribosomal protein L19 (*Rpl19*), by the $2^{-\Delta\Delta Ct}$ method [44] and then presented as the expression levels relative to the transcript amount of a control group. PCR primers used were shown in Table 1. *Lhcgr*, *Pfkp*, *Rpl19*, *Areg, Btc,* and *Ereg* primers were reported previously [9, 45]. To avoid false-positive signals, dissociation-curve analyses were performed at the end of analyses, and the PCR products were applied to agarose gel electrophoresis to confirm the sizes. Moreover, the PCR products were purified and sequenced to verify sequence identity. The reactions were conducted at least in duplicate.

In Situ Hybridization

A fragment of *Spry2* cDNA (1152 bp, GenBank accession no. NM_011897) was amplified by PCR using the primers 5′-TTCCCCGTGGTTTTAATCCA-3′ and 5′-GGCCTGTTCACTCGGTCA TAA-3′. The PCR product was cloned into pCRII–TOPO plasmid using the TOPO TA Cloning Kit (Invitrogen). The identity and orientation of the sequence were confirmed by sequencing. The plasmid DNA was digested with restriction enzyme *Xho*I (Roche Diagnostics) and used for in vitro transcription to make [^{33}P]Cytidine-5′-triphosphate labeled riboprobes for in situ hybridization as reported previously [9]. No signal was detected with a *Spry2* sense probe (see Supplemental Fig. S1 available online at www.biolreprod.org).

Statistical Analysis

All experiments were repeated at least three times. The Tukey-Kramer Honestly Significant Difference (HSD) test or a standard *t*-test were used for multiple or paired comparison, respectively, using JMP software (SAS Institute, Inc.). Relative fold-change values were $\log_{10}$ transformed to scale the data such that the normality and equal variance assumptions of the Tukey-Kramer HSD test were satisfied (see Fig. 4). A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Expression of Transcripts Encoding BMP/FGF Antagonists in Granulosa Cells and Paracrine Regulation by Oocytes

To identify transcripts encoding BMP/FGF antagonists for which the expression levels in granulosa cells are suppressed by oocytes, expression levels of mRNA encoding BMP antagonists (*Bambi*, *Grem2*, *Htra1*, *Htra3*, *Nog*, and *Twsg1*) and an FGF antagonist (*Spry2*) were compared between freshly isolated cumulus and mural granulosa cells. The rationale was as follows: Transcripts that are differentially expressed between cumulus cells, which are associated with oocytes, and mural granulosa cells, which are located far from oocytes, may be regulated in vivo by oocyte-derived factors. Because BMP signals in granulosa cells are mediated by SMAD proteins [46], levels of transcripts encoding SMAD inhibitors (*Smad6*, *Smad7*, *Tob1*, and *Tob2*) also were examined. These transcripts were selected as candidate transcripts based on their expression in ovary and cumulus cells, indicated by the publicly available GNF SymAtlas database (http://symatlas.gnf.org) and our microarray data [7], respectively. Efficient separation of cumulus and mural granulosa cells was confirmed by assessing mRNA levels of *Slc38a3* and *Pfkp*, encoding an amino acid transporter and a glycolytic enzyme (markers of cumulus cells), respectively, and *Lhcgr*, encoding LH receptor (a marker of mural granulosa cells) [2] (Fig. 1A).

Expression levels of *Nog* and *Smad7* mRNA were significantly higher in cumulus cells than in mural granulosa cells, suggesting a possibility that expression levels of these transcripts in cumulus cells are promoted by oocyte-derived factors (Fig. 1A). On the other hand, levels of *Grem2*, *Twsg1*, *Tob1*, *Tob2*, and *Spry2* mRNA were significantly higher in mural granulosa cells than in cumulus cells; thus, oocyte-secreted factors may suppress expression levels of these transcripts in cumulus cells in vivo (Fig. 1A).

To address directly whether levels of these transcripts in cumulus cells are regulated by oocytes in vitro, the effect of oocyte removal (OOX) from COCs on the levels of these transcripts was determined. Cumulus cells were cultured as intact COCs, OOX cumulus cells, or OOX cumulus cells cocultured with denuded, fully grown, germinal vesicle-stage oocytes (2 oocytes/μl) for 20 h, and expression levels of these transcripts in cumulus cells were examined (Fig. 1B). Levels of *Pfkp* mRNA (a known transcript, the expression of which in cumulus cells is promoted by oocytes [42]) as well as levels of *Grem2*, *Htra1*, *Htra3*, and *Nog* mRNA were significantly down-regulated when oocytes were removed from COCs, and the levels found in cumulus cells cultured as intact COCs were restored when OOX cumulus cells were cocultured with oocytes. This indicates that steady-state levels of these transcripts in cumulus cells are promoted by oocytes in vitro. In contrast, levels of *Smad6* and *Spry2* transcripts were up-regulated in OOX cumulus cells compared with those in

TABLE 1. Sequence of PCR primers used for real-time PCR.

Gene	Forward	Reverse
Bambi	GCCACTCCAGCTACTTCTTCATCT	GAGAAGCAGGCACTAAGCTCAGA
Grem2	CGAGGAGGAGGCTTCCATCT	TTCTTCCGTGTTTCAGCTACCTTT
Htra1	GCCATAATCAAGGATGTGGATGA	CAGCAGGACTGGCAGCTTTC
Htra3	GCCAGCTGTGGTCCACATAGA	CCGGCTTCTGACATGATGAAG
Nog	TGAGGTGCACAGACTTGGA	TGTACGCGTGGAATGACCTA
Smad6	CCCCCTATTCTCGGCTGTCT	GAGCAGTGATGAGGGAGTTGGT
Smad7	TCCAGATACCCAATGGATTTTCTC	TAATTCGTTCCCCCGGTTTC
Slc38a3	TATCTTCGCCCCCAACATCTT	TGGGCATGATTCGGAAGTAGA
Spry2	TCGCTGGAAGAAGAGGATTCAA	TCACCCCAGCAGGCTTAGAA
Tob1	CCCGCTGCCCCTTCTC	TCATTCAAAGTGCTGGTATTAGAAGATT
Tob2	CTGGCCAACTGACCGTCTTC	CTTGCGTTTGGCCTTTCTTTT
Twsg1	GATGTGAGCAAATGCCTCATTC	TTCCGAGGGTTGCACATACC

cumulus cells cultured as intact COCs, and these increases were prevented by coculturing OOX cumulus cells with oocytes. Therefore, oocytes suppress expression levels of *Smad6* and *Spry2* transcripts in cumulus cells in vitro.

Taken together, these results strongly suggest that *Spry2* mRNA expression in cumulus cells is suppressed by oocyte-derived factors both in vivo and in vitro. Furthermore, expression of the other sprouty family members, *Spry1*, *Spry3,* and *Spry4,* was not detectable in granulosa cells (data not shown). Although *Smad6* mRNA levels in cumulus cells were suppressed by oocytes in vitro, the expression levels in vivo were not significantly different between cumulus and mural granulosa cells. This suggests that the suppressive effect of oocytes on *Smad6* mRNA expression may be masked by an unknown factor acting on both cumulus and mural granulosa cells. Therefore, we focused on the regulation of *Spry2* expression in the present study.

Localization of Spry2 *mRNA During Follicular Development*

Localization of *Spry2* transcripts during follicular development before the LH surge was determined by in situ hybridization (Fig. 2). *Spry2* mRNA localization was detected

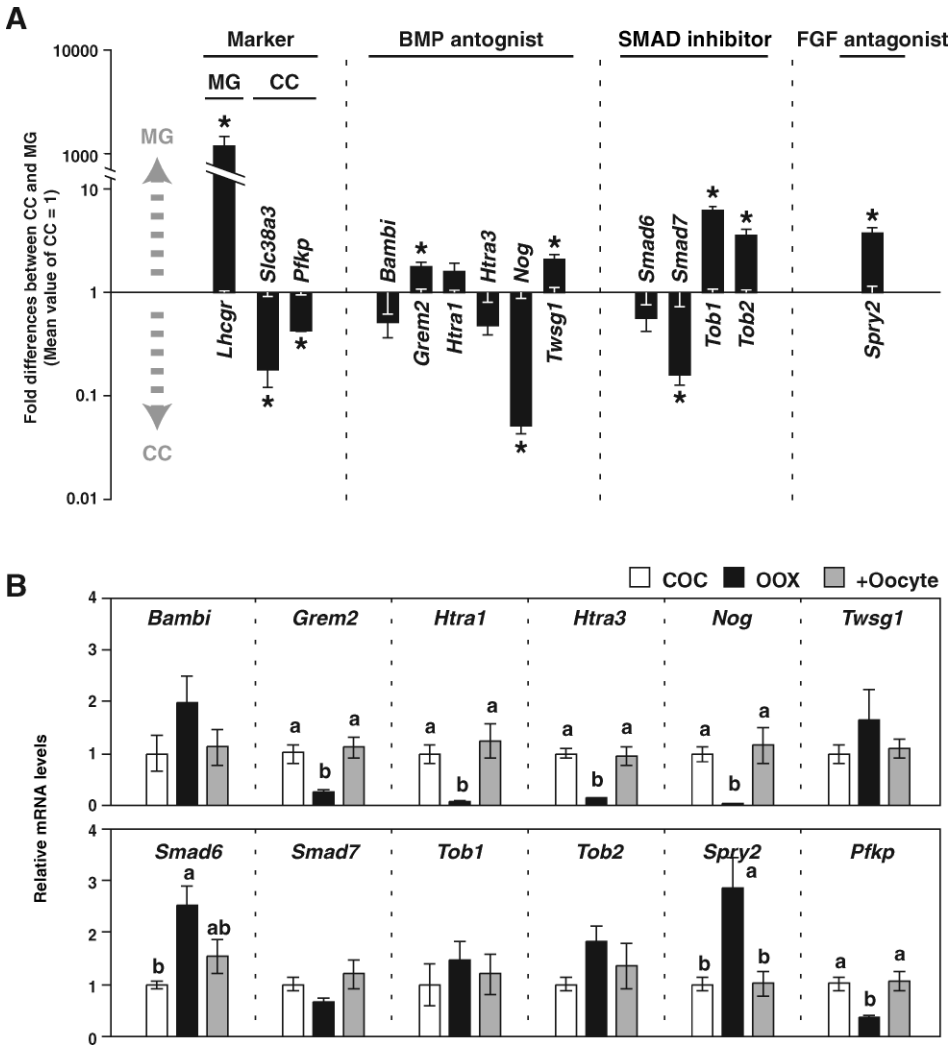

FIG. 1. Expression of transcripts encoding BMP/FGF antagonists in granulosa cells (**A**) and paracrine regulation by oocytes (**B**). **A**) Fold-differences in transcript levels of BMP/FGF antagonist between mural granulosa cells (MG) and cumulus cells (CC) are presented using an exponential scale. Mean values of cumulus cells were normalized to one. Therefore, a value greater than one indicates that the transcript is enriched in mural granulosa cells, and a value smaller than one indicates that the transcript is enriched in cumulus cells. An asterisk indicates that the difference of expression levels between mural granulosa cells and cumulus cells is statistically significant ($P < 0.05$). **B**) Cumulus cells were cultured as intact COCs (COC) or OOX cumulus cells (OOX), or OOX cumulus cells were cocultured with oocytes (+Oocyte) and expression levels of transcripts encoding BMP/FGF antagonists in cumulus cells were examined after 20 h. Values indicated by different lowercase letters (a and b) are significantly different ($P < 0.05$). All values are presented as the mean ± SEM.

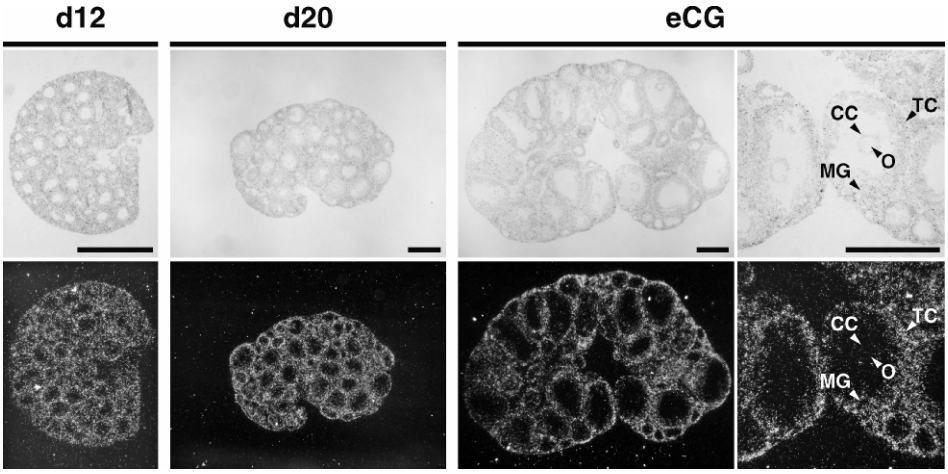

FIG. 2. Localization of *Spry2* mRNA during follicular development before the LH surge. Localization of *Spry2* mRNA in ovaries was detected by in situ hybridization using 12-day-old (d12) and 20-day-old (d20) mice and 22-day-old eCG-primed (eCG) mice. *Spry2* mRNA signals were not detectable in cumulus cells (CC). MG, mural granulosa cells; O, oocytes; TC, theca cells. **Top**) Brightfield images. **Bottom**) Darkfield images. Bar = 500 μm.

in granulosa cells of preantral follicles (Fig. 2, d12) and early antral follicles (Fig. 2, d20). In well-developed antral follicles of ovaries from eCG-primed mice, signals were detected in mural granulosa cells at higher levels than in cumulus cells (Fig. 2, eCG), as was shown by real-time PCR as well (Fig. 1A). *Spry2* mRNA localization also was detected in theca cells but was not detected in oocytes at any stage.

Effect of FGFs, Oocyte Coculture, and TGFβ Superfamily Proteins on Expression Levels of Spry2 *mRNA in OOX Cumulus Cells*

Because *Spry2* expression in some nonovarian cell types is promoted by FGF ligands [36, 37], the effect of recombinant FGFs on levels of *Spry2* mRNA in OOX cumulus cells was determined. OOX cumulus cells were cultured with recombinant FGF1, FGF2, FGF4, and FGF8, and levels of *Spry2* mRNA were examined after 6 h of treatment (Fig. 3A). Treatment with FGF1, FGF2, and FGF8, but not with FGF4, increased *Spry2* mRNA levels in OOX cumulus cells in a dose-dependent manner. These results agree with those of a previous report that FGF2, but not FGF4, promoted *Spry2* expression in human granulosa-lutein cells [47].

The effect of coculture of OOX cumulus cells with oocytes on levels of FGF-induced *Spry2* expression was determined (Fig. 3B). Oocytes suppressed *Spry2* mRNA levels that were elevated by FGF1, FGF2, and FGF8 as well as basal levels of *Spry2* mRNA not stimulated by FGFs (Fig. 3B). Although mouse oocytes produce several FGFs, including FGF8 [9, 11], oocytes suppressed rather than promoted *Spry2* mRNA expression in cumulus cells. Therefore, some other factors likely secreted from oocytes suppress *Spry2* mRNA levels in cumulus cells.

To identify the oocyte factors that suppress FGF8-induced *Spry2* expression in cumulus cells, the effects of the recombinant TGFβ superfamily proteins BMP15, GDF9, TGFB2, BMP4, and BMP6 on FGF8-induced *Spry2* mRNA expression in OOX cumulus cells were determined (Fig. 3, C–E). In rodents, oocytes produce BMP6 [48–50], both oocytes and granulosa cells produce TGFB2 [51, 52], and theca cells

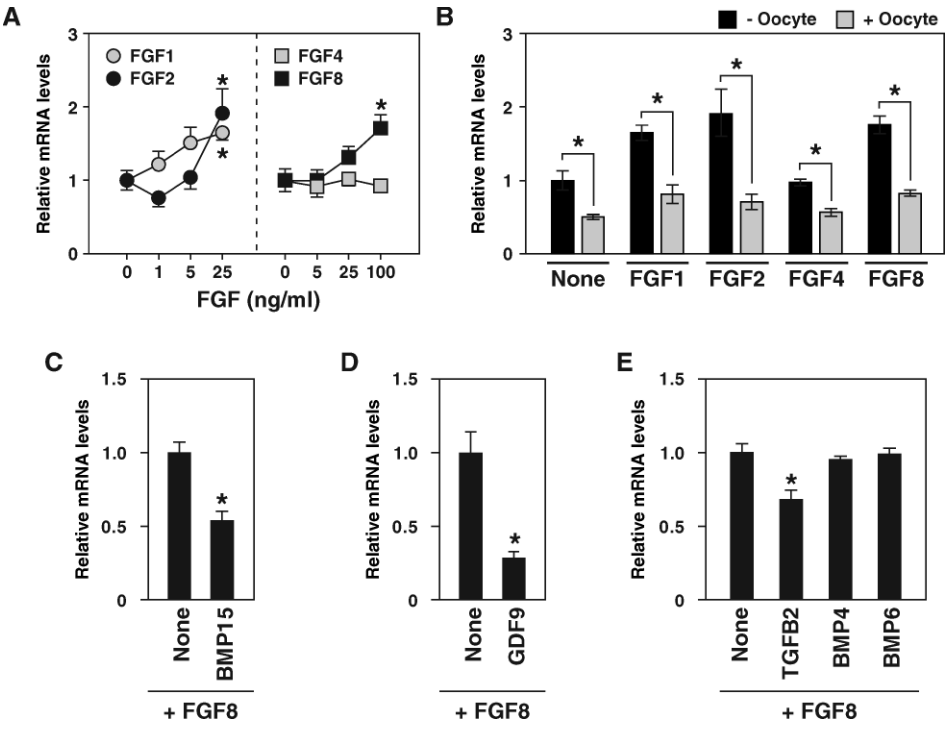

FIG. 3. Effect of FGFs (**A**), oocyte coculture (**B**), and TGFβ superfamily proteins (**C–E**) on expression levels of *Spry2* mRNA in OOX cumulus cells. **A**) OOX cumulus cells were cultured with recombinant FGFs and *Spry2* transcript levels were assessed after 6 h. A value with an asterisk is significantly different from the value of a group that was not treated with FGFs ($P < 0.05$). **B**) OOX cumulus cells were cultured with either FGF1 (25 ng/ml), FGF2 (25 ng/ml), FGF4 (100 ng/ml), or FGF8 (100 ng/ml), and the effect of oocytes (2 oocytes/μl) were examined. An asterisk indicates that the difference of expression levels in OOX cumulus cells cocultured with and without oocytes is statistically significant ($P < 0.05$). **C–E**) OOX cumulus cells were cultured with FGF8 (100 ng/ml) and with or without BMP15 (500 ng/ml), GDF9 (500 ng/ml), TGFB2 (10 ng/ml), BMP4 (100 ng/ml), and BMP6 (100 ng/ml), and *Spry2* expression levels were assessed with real-time PCR. A value with an asterisk is significantly different from the value of a control group (None; $P < 0.05$). Values are presented as the mean ± SEM.

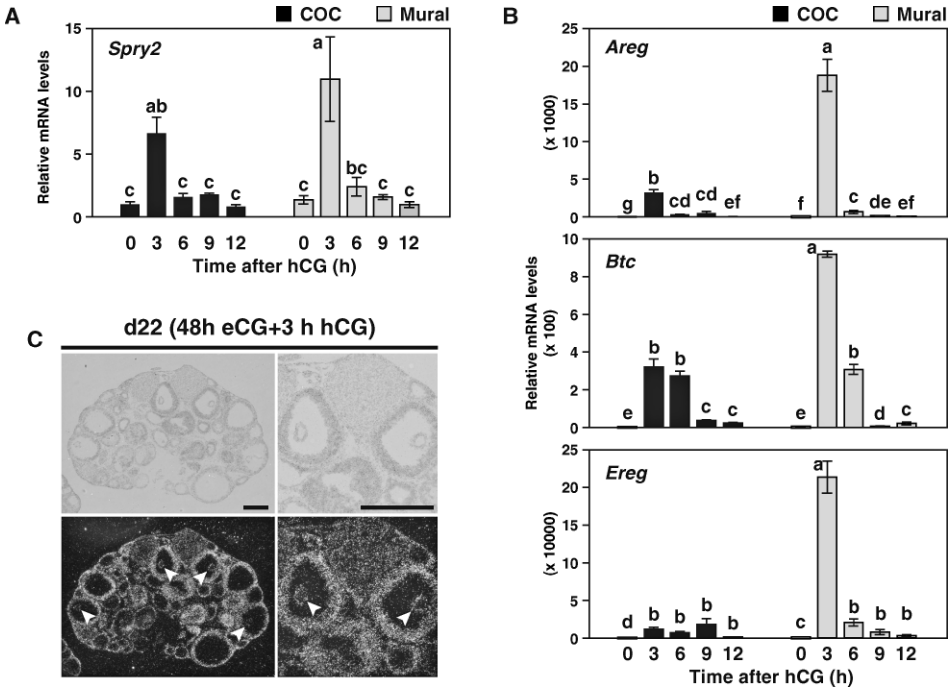

FIG. 4. Correlation of *Spry2* mRNA expression with transcripts encoding EGF-like growth factors. The eCG-primed mice were injected with hCG, and (**A**) kinetics of *Spry2* expression levels as well as (**B**) *Areg, Btc,* and *Ereg* mRNA expression in COCs and mural granulosa cells were determined. Values indicated by different lowercase letters (a–g) are significantly different ($P < 0.05$). Also shown is (**C**) localization of *Spry2* mRNA in ovaries 3 h after hCG treatment. **Top**) Brightfield images. **Bottom**) Darkfield images. Arrows indicate cumulus cells. d22 = Day 22. Bar = 500 μm.

produce BMP4 [53]. OOX cumulus cells were cultured with FGF8 alone or with both FGF8 and one of the TGFβ superfamily proteins, and *Spry2* mRNA levels in cumulus cells were measured after 20 h of treatment. BMP15, TGFB2, and GDF9 suppressed *Spry2* mRNA levels in FGF8-treated OOX cumulus cells, whereas BMP4 and BMP6 had little or no effect (Fig. 3, C–E). These results indicate that one or more oocyte-derived TGFβ superfamily ligands suppress *Spry2* mRNA expression in cumulus cells before the LH surge.

Correlation of Spry2 *mRNA Expression with Transcripts Encoding EGF-Like Growth Factors*

During the ovulatory period, the LH surge induces robust expression of EGF-like growth factors, amphiregulin (AREG), betacellulin (BTC), and epiregulin (EREG), in granulosa cells. These EGF-like growth factors play critical roles in mediating LH action within the follicles [38, 39, 41]. In addition to FGFs, EGF receptor signals also induce *Spry2* expression in some cell types, including human granulosa-lutein cells [47]. Therefore, it was of interest to determine the pattern of *Spry2* expression after the LH surge, and the effect of hCG-treatment, which mimics the LH surge, on *Spry2* mRNA levels was examined as well (Fig. 4). The eCG-primed mice were injected with hCG, and kinetics of *Spry2*, *Areg*, *Btc*, and *Ereg* mRNA expression in COCs and mural granulosa cells were determined (Fig. 4, A and B). *Spry2* mRNA levels were low before hCG treatment (Fig. 4A, 0 h), but within 3 h of hCG treatment, dramatically increased *Spry2* transcript levels were observed in both COCs and mural granulosa cells. Subsequently, *Spry2* mRNA levels decreased by 12 h after hCG treatment to a level comparable with that found before hCG treatment. This pattern of *Spry2* mRNA expression correlated well with those of *Areg*, *Btc*, and *Ereg* transcripts, suggesting that *Spry2* mRNA expression after the LH surge may be regulated by these EGF-like growth factors (Fig. 4B).

Because *Spry2* mRNA levels in COCs were greatest after 3 h of hCG treatment, localization of *Spry2* transcripts in the ovary after 3 h of hCG treatment was determined by in situ hybridization (Fig. 4C). In contrast to eCG-primed ovary (Fig. 2, eCG), in the ovary 3 h after hCG treatment, comparable levels of *Spry2* localization signals were found in both cumulus and mural granulosa cells in all antral follicles observed (Fig. 4C), and this was confirmed by real-time PCR (Fig. 4A, compare COC and mural granulosa cell at 3 h). These results suggest that oocytes may not suppress *Spry2* mRNA expression in cumulus cells after the LH surge.

Effect of EGF, Oocyte Coculture, and TGFβ Superfamily Proteins on Expression Levels of Spry2 *mRNA in OOX Cumulus Cells*

To test further the idea that EGF receptor signal regulates *Spry2* mRNA levels in cumulus cells, the effect of EGF on *Spry2* transcript levels in OOX cumulus cells was determined in vitro (Fig. 5A). OOX cumulus cells were cultured with recombinant EGF, and the steady-state levels of *Spry2* mRNA were examined after 6 h of treatment (Fig. 5A). A dose-dependent increase of *Spry2* mRNA levels in OOX cumulus cells was observed up to 0.5 ng/ml of EGF. Higher concentrations of EGF (up to 10 ng/ml) did not further stimulate *Spry2* expression; therefore, the effect of EGF on *Spry2* mRNA expression appeared to be saturated at concentrations of 0.5 ng/ml and higher.

Effect of coculture of OOX cumulus cells with oocytes on expression levels of EGF-stimulated *Spry2* expression also was determined (Fig. 5B). Surprisingly, in contrast to FGF-induced *Spry2* expression, EGF-simulated *Spry2* expression in OOX cumulus cells was augmented by coculture with oocytes. This positive effect of oocytes on EGF-stimulated *Spry2* mRNA expression was observed even when a concentration of EGF (10 ng/ml) higher than saturation was used (Fig. 5B).

To identify the oocyte factors that promote EGF-induced *Spry2* expression in cumulus cells, effects of recombinant TGFβ superfamily proteins on EGF-induced *Spry2* mRNA expression in OOX cumulus cells were determined (Fig. 5, C and D). Whereas TGFB2 had no effect, BMP15, BMP4, and BMP6 (Fig. 5C), as well as GDF9 (Fig. 5D), promoted *Spry2* expression in OOX cumulus cells. Therefore, members of TGFβ superfamily derived from oocytes as well as from

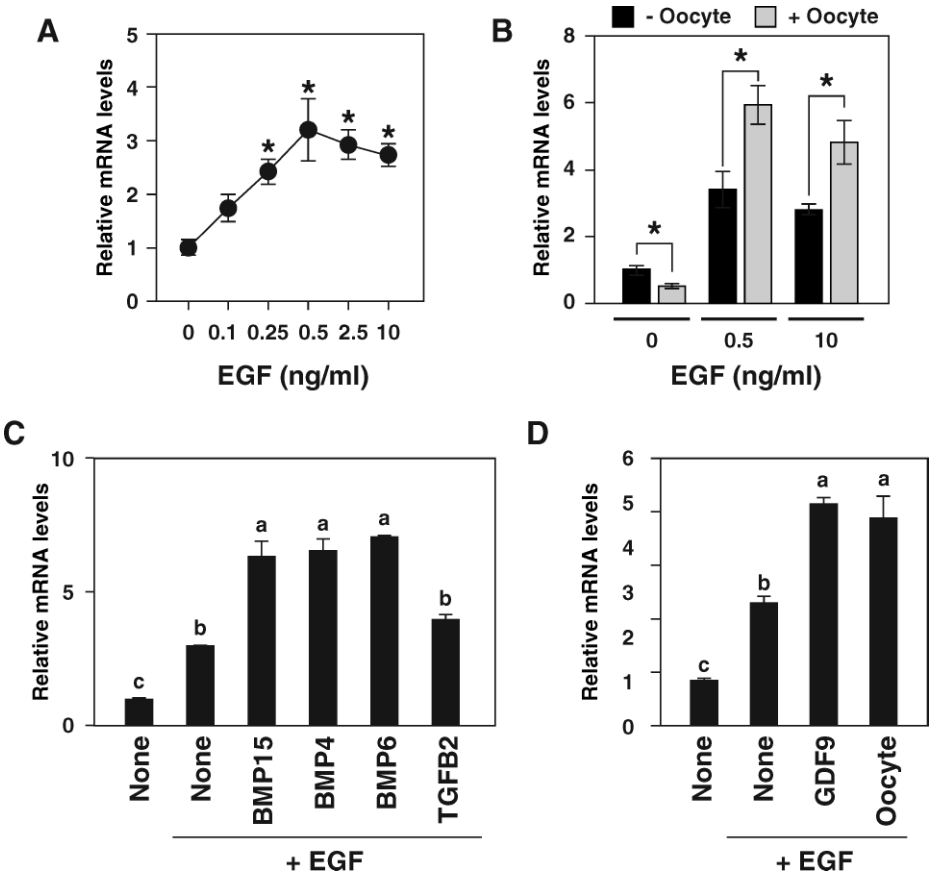

FIG. 5. Effect of EGF (**A**), oocyte coculture (**B**), and TGFβ superfamily proteins (**C** and **D**) on expression levels of *Spry2* mRNA in OOX cumulus cells. **A**) OOX cumulus cells were cultured with recombinant EGF, and *Spry2* transcript levels were assessed after 6 h. A value with an asterisk is significantly different from the value of the group which was not treated with EGF ($P < 0.05$). **B**) OOX cumulus cells were cultured with EGF, and the effect of oocytes (2 oocytes/μl) was examined. An asterisk indicates that the differences of expression levels in OOX cumulus cells cocultured with and without oocytes are statistically significant ($P < 0.05$). **C** and **D**) OOX cumulus cells were cultured with EGF (10 ng/ml) and with or without BMP15 (500 ng/ml), BMP4 (100 ng/ml), BMP6 (100 ng/ml), TGFB2 (10 ng/ml), and GDF9 (500 ng/ml), and *Spry2* expression levels were assessed with real-time PCR. The values indicated by different lowercase letters (a–c) are significantly different ($P < 0.05$). Values are presented as the mean ± SEM.

follicular somatic cells augment EGF-induced *Spry2* expression in cumulus cells in vitro.

DISCUSSION

Precise coordination of multiple endocrine, autocrine, and paracrine growth factor signals is required for normal ovarian follicular development. However, the interactions of oocyte- and somatic cell-derived signaling pathways are far from well understood. The present results showed that several transcripts encoding antagonists of either BMP or FGF signaling are expressed in granulosa cells, and some of these are regulated by oocytes. Furthermore, before the LH surge, *Spry2* mRNA was differentially expressed between cumulus and mural granulosa cells, probably because paracrine factors secreted from oocytes suppress *Spry2* expression in cumulus cells. Although both FGFs and EGF promoted *Spry2* mRNA expression in cultured cumulus cells, the effects of oocytes on FGF- and EGF-stimulated *Spry2* mRNA expression were surprisingly different: Oocytes suppressed FGF-induced *Spry2* mRNA expression in cumulus cells, but EGF-induced *Spry2* mRNA expression was promoted by oocytes. Recombinant BMP15 and GDF9, as well as some of other TGFβ superfamily members, mimicked these effects of oocytes, suggesting that the oocyte factors affecting *Spry2* expression in cumulus cells probably are members of the TGFβ superfamily, including BMP15 and GDF9. Therefore, we have concluded that oocyte-derived TGFβ superfamily members cooperate with FGFs and EGF receptor signals to regulate *Spry2* mRNA levels in cumulus cells. Regulation of *Spry2* expression by TGFβ superfamily signals may be one of the mechanisms by which oocyte-derived TGFβ superfamily signals cooperate with FGF/EGF signals in granulosa cells during ovarian follicular development.

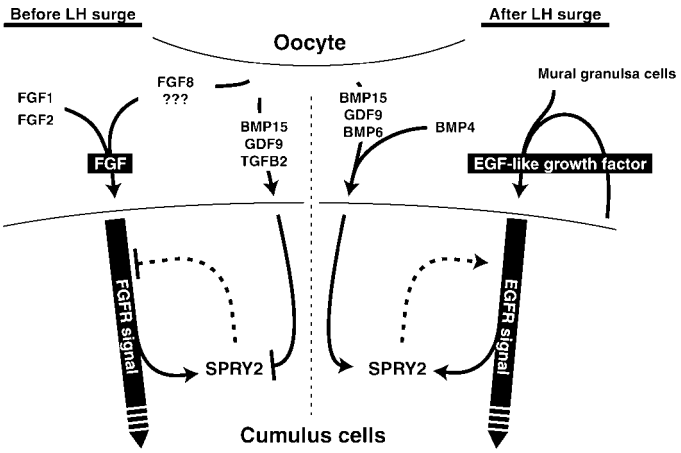

FIG. 6. Schematic diagram of crosstalk between FGF/EGF signals and TGFβ superfamily signals in cumulus cells (see text for details).

The *sprouty* gene was first described in *Drosophila* sp. and the proteins characterized as antagonists of FGF receptor-mediated tracheal branching [54]. Subsequent studies have shown that sprouty proteins generally are inhibitors of receptor tyrosine kinase signals in both vertebrates and invertebrates [37]. In mammals, four sprouty genes, sprouty homolog 1 to sprouty homolog 4, have been identified. The present study focused on *Spry2* mRNA regulation, because expression of the other members of sprouty family was not detectable in mouse granulosa cells (Sugiura and Eppig, unpublished results). *Spry2* null mice exhibit a severe gastrointestinal phenotype, and approximately half of them die within 6 wk after birth [55, 56]. Remaining *Spry2* null mice survived but were significantly

smaller than wild-type littermates, and to our knowledge, their ovarian morphology and fertility have not been studied. In *Drosophila* ovaries, *sprouty* mRNA is expressed in follicle cells in a manner correlated with EGF receptor signals, and *sprouty* mRNA expression is reduced in mutants of *gurken*, one of the ligands of EGF receptor [57, 58]. Whereas overexpression of *sprouty* mRNA in follicle cells resulted in a defective ovary that resembled that of a reduced EGF receptor pathway, *sprouty* mutation resulted in a phenotype consistent with an increased activity of EGF receptor signals [58]. Therefore, *Drosophila sprouty* protein appears to be a key negative regulator of EGF receptor signals during ovarian development. Interestingly, in contrast to *Drosophila sprouty* protein, which inhibits EGF receptor signals [58–60], mammalian *sprouty* proteins appear to potentiate EGF receptor signals. This agonistic effect of mammalian SPRYs on EGF receptor signals is achieved by preventing ubiquitylation and degradation of activated EGF receptors [61–64]. Therefore, mammalian SPRY2 likely has a role distinct from that of *Drosophila sprouty* protein during ovarian follicular development.

How do members of TGFβ superfamily regulate *Spry2* expression in cumulus cells? The present results showed that basal and FGF-induced *Spry2* mRNA expression levels were down-regulated by members of TGFβ superfamily, including BMP15, GDF9, and TGFB2. Similar results have been reported using mesenchymal cells in which *Spry2* mRNA expression was suppressed by TGFB1 [65]. That report also showed that inhibitors of histone deacetylases (HDACs) prevented TGFB1-induced *Spry2* mRNA down-regulation, suggesting that HDAC activity was required for *Spry2* down-regulation by TGFBs. Therefore, similar HDAC-dependent mechanisms may exist in cumulus cells. Furthermore, a recent study has shown that *Spry2* is a direct target of forkhead transcription factors (FOXOs) in endothelial cells [66]. In addition, SMADs, mediators of TGFβ superfamily signals, are reported to associate with FOXOs and to promote gene expression in neuroepithelial and glioblastoma cells [67]. Therefore, up-regulation of EGF-stimulated *Spry2* expression induced by members of TGFβ superfamily in cumulus cells may involve a similar FOXO-dependent mechanism. The reasons why the effects of TGFβ superfamily signals are different between FGF-induced and EGF-induced *Spry2* expression remain to be resolved.

Based on the present results, together with well-established functions of mammalian sprouty proteins [37, 68], we suggest that SPRY2 may be a key regulator of ovarian follicular development. A working model explaining how oocyte-derived TGFβ superfamily signals might affect FGF and EGF receptor signals in cumulus cells is presented in Figure 6. Before the LH surge, oocyte-derived FGF8 and, perhaps, other FGFs produced by oocytes and somatic cells comprise a total FGF signal and activate FGF receptors on cumulus cells to affect development and function. However, at same time, these FGF signals induce elevated SPRY2 expression, which negatively affects the FGF signals. Consequently, oocyte-derived TGFβ superfamily proteins, including BMP15 and GDF9, suppress *Spry2* expression; therefore, FGF signals can continue to affect the cumulus cell functions. On the other hand, after the LH surge, EGF-like growth factors produced by cumulus and mural granulosa cells act together to activate EGF receptor signals in cumulus cells. However, activated EGF receptor may be rapidly down-regulated by its degradation, which would negatively affect EGF-dependent cellular functions [69, 70]. Oocyte-derived TGFβ superfamily proteins, including BMP15 and GDF9, now promote *Spry2* expression in cumulus cells and, therefore, prevent degradation of activated EGF receptor, which leads to a prolonged EGF signal in cumulus cells. This regulatory mechanism could explain why both FGFs and BMP15 are required for some cumulus cell function, such as elevated glycolysis, before the LH surge [9] and why both oocyte-derived factors and EGF receptor signals are required for post-LH-surge events, such as cumulus expansion. Genetic experiments are in progress to test this working model.

Although SPRY2 may be one of the critical components involved in the cooperative action of TGFβ superfamily and FGF/EGFs during follicular development, other participants are likely to exist. In fact, the present results (Fig. 1) showed that several other BMP antagonists are expressed in granulosa cells, suggesting involvement of other BMP/FGF antagonists in this cooperation. In addition, agonists or receptors of TGFβ superfamily or FGFs may be involved. Furthermore, extracellular regulated kinases (MAPK3/1) downstream of receptor tyrosine kinases, such as the FGF and EGF receptors, can negatively affect transcriptional activity of SMADs by directly phosphorylating the linker region of SMADs in cultured epithelial cells [71], which may add further complexity in this model. Future studies of these signal interactions are required to resolve the complex cooperative pathways participating in oocyte control of follicular development.

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