Evaluation of members of the TGFβ superfamily as candidates for the oocyte factors that control mouse cumulus expansion and steroidogenesis

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Oocytes secrete factors that control cumulus and granulosa functions, including cumulus expansion and steroid hormone production. Some members of the transforming growth factor β (TGFβ) superfamily influence these activities, yet it is still not determined conclusively whether any of these superfamily members are the previously reported oocyte-secreted factors. The aim of this study was to examine the effects of TGFβ1 and growth differentiation factor 9 (GDF-9) on cumulus expansion and progesterone production by mouse oocytectomized (OOX) complexes in culture. TGFβ1 mimics the effects of oocytes by both enabling cumulus expansion and inhibiting progesterone production; however, neutralizing antibodies to TGFβ1 in cultures of cumulus-oocyte complexes (COCs) or in co-cultures of OOX complexes failed to inhibit the ability of oocytes to enable cumulus expansion or inhibit progesterone production. Activin A had no effect on progesterone production by OOX complexes. In experiments using oocytes obtained from mice with deficient expression of GDF-9, OOX complexes cultured in the presence of heterozygous oocytes were capable of full expansion, whereas OOX complexes cultured with oocytes from GDF-9 null mice did not expand. Similarly, GDF-9 null oocytes failed to suppress FSH-induced progesterone production by OOX complexes. These results support the hypothesis that GDF-9 is the cumulus expansion enabling factor produced by mouse oocytes and that GDF-9 also inhibits cumulus progesterone production; however, the possibility remains that loss of GDF-9 may indirectly affect the ability of oocytes to produce the factors that regulate cumulus cell activity.

Introduction

The process of follicular development is an elaborately orchestrated event that involves bidirectional communication between germ cells and somatic cells. Communication between Oocytes secrete factors that control cumulus and granulosa functions, including cumulus expansion and steroid hormone production. Some members of the transforming growth factor β (TGFβ) superfamily influence these activities, yet it is still not determined conclusively whether any of these superfamily members are the previously reported oocyte-secreted factors. The aim of this study was to examine the effects of TGFβ1 and growth differentiation factor 9 (GDF-9) on cumulus expansion and progesterone production by mouse oocytectomized (OOX) complexes in culture. TGFβ1 mimics the effects of oocytes by both enabling cumulus expansion and inhibiting progesterone production; however, neutralizing antibodies to TGFβ1 in cultures of cumulus-oocyte complexes (COCs) or in co-cultures of OOX complexes failed to inhibit the ability of oocytes to enable cumulus expansion or inhibit progesterone production. Activin A had no effect on progesterone production by OOX complexes. In experiments using oocytes obtained from mice with deficient expression of GDF-9, OOX complexes cultured in the presence of heterozygous oocytes were capable of full expansion, whereas OOX complexes cultured with oocytes from GDF-9 null mice did not expand. Similarly, GDF-9 null oocytes failed to suppress FSH-induced progesterone production by OOX complexes. These results support the hypothesis that GDF-9 is the cumulus expansion enabling factor produced by mouse oocytes and that GDF-9 also inhibits cumulus progesterone production; however, the possibility remains that loss of GDF-9 may indirectly affect the ability of oocytes to produce the factors that regulate cumulus cell activity.

Introduction

The process of follicular development is an elaborately orchestrated event that involves bidirectional communication between germ cells and somatic cells. Communication between
the oocyte and surrounding granulosa cells occurs via both membrane gap junctions (Heller and Schultz, 1980) and paracrine factors (Eppig et al., 1997a). For example, the expression of Kit ligand (KL) by granulosa cells (Manova et al., 1993; Ismail et al., 1996) and KL activation of the Kit tyrosine kinase receptors in oocytes plays a key role in oocyte growth and follicle development (Packer et al., 1994; Yoshida et al., 1997).

Of particular interest in the past decade has been the secretion of paracrine factors by the oocyte that mediate several key functions in the regulation of granulosa cell proliferation and differentiated activity. The suggestion that granulosa cells are dependent upon the oocyte for development and function was evident when ovariectomy of rabbit follicles resulted in the luteinization of granulosa cells (El-Fouly et al., 1970). Additional evidence for such a factor was provided by in vivo and in vitro experiments in rats and rabbits, showing that the removal, absence or destruction of oocytes leads to granulosa cell luteinization (Nekola and Nalbandov, 1971; Hubbard and Erickson, 1988). The procedure of oocytectomy has been used to show that oocyte-secreted factors regulate steroidogenesis in mouse, rat, pig and bovine granulosa cells (Vanderhyden et al., 1993; Coskun et al., 1995; Vanderhyden and Tonary, 1995; Li et al., 2000). Factors produced by oocytes inhibit expression of LH receptors in granulosa cells, which contributes to their actions as luteinization inhibitors (Eppig et al., 1997b). In addition to inhibiting luteinization, oocytes further promote follicle development by secreting a soluble factor that enhances the proliferation of mouse (Vanderhyden et al., 1992; Gilchrist et al., 2001) and bovine (Lanuza et al., 1998; Li et al., 2000) granulosa cells. Finally, soluble factors produced by mouse, rat, pig and bovine oocytes enable the expansion of mouse oocytectomized (O0X) cumulus complexes (Buccione et al., 1990; Vanderhyden, 1993; Ralph et al., 1995) by promoting the ability of cumulus cells to produce hyaluronic acid (Buccione et al., 1990) and by suppressing expression of urokinase plasminogen activator (Canipari et al., 1995).

The identification of the oocyte-secreted factors has been difficult and none are yet confirmed, but members of the transforming growth factor β (TGFβ) superfamily have aroused considerable interest by their ability to mimic the actions of the oocyte in regulating granulosa cell activities, specifically steroidogenesis and enabling of cumulus expansion. Some members of this family, growth differentiation factor 9 (GDF-9), bone morphogenetic protein 15 (BMP-15) and BMP-6, are expressed by oocytes and, therefore, are logical candidates to consider as possible mediators of the effects attributed to the oocyte. The expression of both GDF-9 and BMP-15 in mice begins in oocytes of small primary follicles and continues throughout ovulation (McGrath et al., 1995; Dube et al., 1998), whereas GDF-9 expression in bovine and ovine ovaries is detectable from the primordial stage of follicle development (Bodensteiner et al., 1999), indicating a potential role for GDF-9 in the initiation of folliculogenesis in domestic ruminants. The actions of GDF-9 on steroidogenesis in granulosa cells may be species-dependent and are strongly affected by the presence of gonadotrophins, as recombinant GDF-9 stimulates steroidogenic acute regulatory protein (StAR) expression and progesterone secretion by murine granulosa cells (Elvin et al., 1999a), whereas GDF-9 suppresses FSH-stimulated progesterone and oestradiol production by rat granulosa cells (Vitt et al., 2000). BMP-6 (Otsuka et al., 2001a) and BMP-15 (Otsuka et al., 2000) also suppress FSH-stimulated progesterone production by rat granulosa cells rendering these growth factors possible candidates for the progesterone regulatory factor secreted by oocytes.

Activin, although initially characterized as a gonadal hormone that regulates the pituitary release of FSH (Ling et al., 1986; Vale et al., 1986), may play a role in the regulation of oocyte activity. Activin is produced by granulosa cells (Findlay, 1993), and both oocytes and granulosa cells have activin receptors (Wu et al., 1994; Drummond et al., 2002). However, activin subunits βA and βB transcripts have been detected in Xenopus oocytes (Rebagliati and
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Dawid, 1993) and immunocytochemical studies have shown that activin protein is present in mouse oocytes (Albano et al., 1993). Previous studies have demonstrated the development-dependent effects of activin on granulosa cell steroidogenesis, such that aromatase activity is enhanced, P450scc activity is inhibited, and progesterone production is suppressed as follicular maturation progresses (Miró et al., 1991). As activin is expressed by oocytes and can suppress progesterone production, it too should be investigated as a candidate for one of the oocyte-secreted factors.

In terms of cumulus expansion, TGFβ1 mimics the oocyte-secreted factor in stimulating hyaluronic acid synthesis by rat and mouse cumulus cells in the presence of FSH (Salustri et al., 1990; Tirone et al., 1997). However, TGFβ1 is less effective than the oocyte factor, and TGFβ1 neutralizing antibodies do not inhibit the response to the oocyte factor (Salustri et al., 1990), indicating that TGFβ1 is not the cumulus expansion enabling factor (CEEF). However, because the cumulus cells were plated as monolayers in previous studies, the loss of the three-dimensional organization of the cumulus cells may alter the responsiveness of the cumulus cells to TGFβ1 and, therefore, it is important to determine whether TGFβ1 has activity resembling CEEF using cumulus cells that have maintained their spatial integrity.

GDF-9 has also been shown to enable cumulus expansion in the absence of the oocyte; however, at this time the only evidence to support the hypothesis that GDF-9 is the oocyte-secreted CEEF is the observation that recombinant GDF-9 stimulates cumulus expansion in OOX complexes (Elvin et al., 1999a). Although GDF-9 is expressed in oocytes throughout follicle development, it has not yet been demonstrated that GDF-9 is secreted by oocytes at the appropriate stage of development, or that GDF-9 null oocytes have lost the ability to secrete the expansion enabling activity.

The actions of many of the TGFβ family members on granulosa cell activity seem to be dependent on the context, particularly as it relates to stage of follicle development, the presence of gonadotrophins and the species. Therefore, in the present study, activin-A, TGFβ1 and GDF-9 were assessed for their ability to enable cumulus expansion and regulate steroidogenesis of murine cumulus cells, using the system of oocytectomized complexes that was used in the original studies to determine the presence and activity of the oocyte-secreted factors. Specifically, the objectives of this study were to investigate the effects of TGFβ1 and activin A on cumulus expansion and steroid hormone production by OOX complexes, and to determine the ability of GDF-9 deficient oocytes to regulate cumulus expansion and steroid hormone production by OOX complexes.

Materials and Methods

Isolation and co-culture of denuded oocytes with OOX complexes

Cumulus-oocyte complexes (COCs) were obtained from antral follicles of 24- to 28-day-old (C57BL/6 x Balb/c) F1 mice at 40-44 h after i.p. injection with 5 iu equine chorionic gonadotrophin (eCG). Denuded oocytes and OOX complexes were prepared as described by Vanderhyden et al. (1990) and then cultured in Waymouth media with 5% fetal bovine serum (WAY/FBS). In some experiments, denuded oocytes were obtained from GDF-9-deficient mice (generously provided by M. Matzuk, Baylor College of Medicine; Dong et al., 1996). Southern blot analysis was used to determine the genotype of mice. Wild-type, heterozygous and homozygous oocytes were collected by injecting mice with 5 iu eCG and, 2 days later, preparing denuded oocytes from GDF-9 +/+ and +/− mice as described above. GDF-9-deficient −/− ovaries were placed in WAY/FBS containing collagenase (3 mg ml−1) for approximately 45 min, and intermittent pipetting with a Pasteur pipette was performed to denude the oocytes.
of granulosa cells. Denuded oocytes were washed three times in WAY/FBS, cultured for 4–5 h to allow spontaneous meiotic maturation and then co-cultured with OOX complexes. The general experimental procedure is outlined (Fig. 1).

**Cumulus expansion**

The effects of TGFβ1 on expansion of mouse OOX complexes was tested by culturing ten OOX in 50 μl drops of WAY/FBS under washed oil in 35 mm Petri dishes. Complexes were treated with FSH (100 ng ml⁻¹) or 10 ng TGFβ1 ml⁻¹ (R&D Systems, Minneapolis, MN), or both in combination. Neutralizing antibodies to TGFβ1 (5 μg ml⁻¹; R&D Systems) were added to some drops to determine the specificity of action of TGFβ1 and to confirm the efficacy of the antibodies. The ability of GDF-9-deficient oocytes to enable cumulus expansion was assessed by culturing OOX complexes alone or in the presence of either 25 heterozygous (+/-) or homozygous (-/-) meiotically competent (MC) or incompetent (MI) denuded oocytes (that is, one oocyte per 2 μl). The complexes were cultured for 16 h at 37°C in 5% CO₂:5% O₂:90% N₂ and then assessed for cumulus expansion using a subjective scoring system that ranges from 0 (no response) to +4 (maximal expansion; Buccione et al., 1990).

**Steroidogenesis**

The effects of TGFβ1 and activin A on steroid hormone production by mouse cumulus cells were determined by culturing ten COCs or OOX complexes in 250 μl WAY/FBS. In some wells,
Table 1. Transforming growth factor β1 (TGFβ1) mimics the effects of the cumulus expansion-enabling factor secreted by mouse oocytes

| Treatment                          | Degree of expansion° |
|-----------------------------------|----------------------|
|                                   | COC (n)              | OOX (n)             |
| FSH                               | +3 to +4 (60)        | 0 (70)              |
| TGFβ1                             | -                    | 0 (30)              |
| FSH + TGFβ1                       | +3 to +4 (20)        | +2 to +3 (110)      |
| FSH + TGFβ1-Ab                    | +3 (40)              | 0 (20)              |
| FSH + TGFβ1 + TGFβ1-Ab            | +3 (40)              | 0 (70)              |
| OCM + FSH                          | +3 to +4 (60)        | +3 to +4 (60)       |
| OCM + FSH + TGFβ1-Ab              | +3 (50)              | +3 (70)             |

°Degree of expansion was determined on a scale from 0 (no expansion) to +4 (maximum response with all cumulus layers undergoing expansion).

COC: cumulus–oocyte complexes; OOX: oocytectomized oocyte–cumulus cell complexes; TGFβ1-Ab: neutralizing antibodies to TGFβ1; OCM: oocyte-conditioned media; media collected from plates of denuded oocytes cultured for 24 h; --: not performed.

The OOX complexes were co-cultured with 125 denuded, fully-grown oocytes. Complexes were cultured for 30–48 h in the presence or absence of 10 ng TGFβ1 ml⁻¹ or 7.5–120.0 ng activin A ml⁻¹. Neutralizing antibodies to TGFβ1 (5 μg ml⁻¹; R&D Systems) were added to some wells to determine the specificity of action of TGFβ1. The ability of GDF-9-deficient oocytes to regulate progesterone and oestradiol production by the granulosa cells was assessed by culturing OOX complexes alone or with either 75 heterozygous (+/-) or 75 homozygous (-/-) meiotically competent or incompetent denuded oocytes. Denuded oocytes were cultured for 24 h in 100 μl WAY/FBS before the addition of the OOX complexes. In all experiments, FSH (100 ng ml⁻¹) and testosterone (500 nmol l⁻¹; Sigma, St Louis, MO) were added to the culture media to support oestradiol production. After 48 h, culture media were collected and stored at −20°C until assayed for progesterone and oestradiol by radioimmunoassays as described by Vanderhyden and Tonary (1995).

Statistical analysis

All experiments were performed at least three times, with different pools of ovaries. When comparing multiple groups, data were expressed as mean ± standard error of the mean, and statistical comparison was made using ANOVA with Newman-Keul’s test for multiple comparisons. Statistical comparisons of two groups were made using unpaired, two-tailed t tests for normal distributions. Statistical significance was inferred at \( P < 0.05 \).

Results

TGFβ1 mimics CEEF in OOX complexes

Given that a previous study demonstrated an effect of TGFβ1 on dispersed cumulus cells (Salustri et al., 1990), the first experiment addressed whether cellular associations may alter this response by examining the effect of TGFβ1 on expansion of mouse OOX complexes. FSH stimulated expansion of intact COCs to almost maximal levels (+3 to +4), but oocytectomy rendered the complexes unable to expand in response to FSH (Table 1). TGFβ1 alone had no effect on OOX complexes, but was quite effective at promoting the expansion of FSH-treated OOX complexes, although the overall degree of expansion was not as great as
Effect of transforming growth factor \( \beta1 \) (TGF\( \beta1 \)) on oestradiol and progesterone production by intact cumulus–oocyte cell complexes (COCs) and oocytectomized (OOC) complexes of mice. (a,b) COC (■) and OOC (□) complexes were cultured in the presence of 100 ng FSH ml\(^{-1}\), 10 ng TGF\( \beta1 \) ml\(^{-1}\) or both, and (a) oestradiol and (b) progesterone production after 48 h was determined by radioimmunoassay. All treatments included testosterone as a substrate for oestradiol production. (c,d) OOC complexes were cultured for 48 h in the presence of TGF\( \beta1 \) or denuded oocyte-conditioned media (DO–CM) and in the presence (■) or absence (□) of 5 \( \mu \)g neutralizing antibodies ml\(^{-1}\) to TGF\( \beta1 \). All treatments included FSH and testosterone to support steroid hormone production. Values are mean ± SEM from three experiments and different letters above the bars indicate significant differences \( (P < 0.05) \). Asterisks indicate values significantly different from the same treatment without antibodies \( (P < 0.05) \).

for COCs, or for OOC complexes cultured in oocyte-conditioned media. Neutralizing antibodies to TGF\( \beta1 \) were effective at ablating the response of OOC complexes to TGF\( \beta1 \), but had negligible effects on the expansion of OOC complexes cultured in oocyte-conditioned media, indicating that the TGF\( \beta1 \) antibodies do not interfere with the actions of the oocyte-secreted CEEF.

**Effect of TGF\( \beta1 \) on steroid hormone production by intact and OOC complexes**

Mouse oocytes secrete a factor that inhibits progesterone and enhances oestradiol production by FSH-stimulated cumulus granulosa cells (Vanderhyden et al., 1993). As TGF\( \beta1 \) was able to mimic the effects of the oocyte-secreted CEEF, its actions on cumulus steroid hormone production were then compared with those of oocytes. As previously reported, FSH strongly stimulated oestradiol production (Fig. 2a) in COCs, an effect that was almost completely eliminated by removal of the oocytes. Although TGF\( \beta1 \) alone had no effect on oestradiol production, it enhanced FSH-stimulated oestradiol production by OOC complexes to 62\% of that of COCs. Although TGF\( \beta1 \) mimics the effect of the oocyte in promoting oestradiol
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Fig. 3. Accumulation of progesterone in cultures of intact cumulus-oocyte complexes (COC) and oocyctomized (OOX) complexes and OOX complexes cultured in the presence of denuded oocytes (OOX + DO) or activin A. Complexes were isolated from mice treated with equine chorionic gonadotrophin and cultured for 30 h in the presence of FSH (100 ng ml^{-1}), testosterone (500 nmol l^{-1}) and various concentrations of activin-A (7.5-120.0 ng ml^{-1}). Values are mean ± SEM from four experiments.

production, it does not appear to be the oocyte-secreted factor, as neutralizing antibodies to TGFβ1 could eliminate the effects of TGFβ1 on oestradiol production by OOX complexes (Fig. 2b), but failed to alter the amount of this hormone produced by OOX complexes in the presence of oocyte-conditioned media.

The effects of TGFβ1 on progesterone production were examined in a similar way. OOX complexes yielded significantly higher concentrations of progesterone in response to FSH stimulation compared with COCs, but this progesterone production was almost completely abolished in the presence of TGFβ1 (Fig. 2c). Neutralizing antibodies to TGFβ1 prevented the effects of TGFβ1 on progesterone production by OOX complexes, but had no effect when the OOX complexes were cultured in oocyte-conditioned media (Fig. 2d). Thus, although both oocytes and TGFβ1 promote oestradiol and inhibit progesterone production, the results indicate that TGFβ1 is not the oocyte-secreted steroid regulating factor.

Effect of activin A on progesterone production by OOX complexes

As activin has been reported to inhibit progesterone production by granulosa cells (Miro et al., 1991), the hypothesis that activin might be the oocyte-secreted progesterone inhibitory
factor was tested. FSH stimulated the accumulation of progesterone in cultures of OOX complexes to concentrations fivefold greater than in COCs (Fig. 3), and this accumulation was reduced when OOX complexes were cultured in the presence of denuded oocytes. Addition of activin A did not affect the progesterone production by OOX complexes at any concentration tested.

**Ability of GDF-9-deficient oocytes to enable cumulus expansion of OOX complexes**

GDF-9-deficient oocytes were co-cultured with OOX complexes, to provide additional evidence that GDF-9 may be the oocyte-secreted CEEF that enables expansion of cumulus cells. COCs, OOX and OOX complexes co-cultured with GDF-9 heterozygous oocytes were included as controls in the experiment (Table 2). As expected, COCs expanded (3.3 ± 0.3), whereas no expansion was observed for OOX complexes cultured alone. GDF-9 heterozygous oocytes enabled the expansion of OOX complexes to near maximal levels (3.3 ± 0.2), whereas OOX complexes cultured with meiotically competent and incompetent GDF-9-deficient oocytes did not expand, indicating that the lack of GDF-9 production by these oocytes impairs their ability to enable cumulus expansion.

**Ability of GDF-9-deficient oocytes to regulate cumulus cell steroidogenesis**

Murine oocytes secrete a factor that stimulates cumulus cell oestradiol production, as evident from the significantly reduced production of oestradiol by OOX complexes (24 ± 4.5%) in comparison with the COCs (Fig. 4a). Co-culture of OOX complexes with GDF-9 heterozygous oocytes resulted in a marked increase in oestradiol production to 89% of that of COC. In contrast, GDF-9 homozygous, meiotically competent and incompetent oocytes failed to support oestradiol production by OOX complexes, indicating that, unlike normal oocytes, oocytes from GDF-9-deficient mice do not secrete the oestradiol-stimulatory factor.

COCs produced progesterone at concentrations of only 12% of that of OOX complexes, indicating the secretion of a progesterone-inhibitory factor by the oocyte (Fig. 4b). Similar amounts of progesterone production were observed for OOX complexes co-cultured with

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**Table 2. Effect of growth differentiation factor 9 (GDF-9) +/— and —/— denuded oocytes on cumulus expansion of mouse OOX complexes**

| Control/treatment group | Number of oocytes used for conditioning | Number of complexes assessed | Degree of expansion (0 to +4) |
|-------------------------|----------------------------------------|-----------------------------|-----------------------------|
| COC                     | 0                                      | 15                          | 3.3 ± 0.3                   |
| OOX                     | 0                                      | 15                          | 0                           |
| OOX + GDF-9+/— MC       | 25                                     | 20                          | 3.3 ± 0.2                   |
| OOX + GDF-9—/— MC       | 25                                     | 20                          | 0                           |
| OOX + GDF-9—/— MI       | 25                                     | 25                          | 0                           |

Oocytectomized cumulus-oocyte cell complexes (OOX) were cultured in the presence of GDF-9 heterozygous (+/—) and homozygous (+/+), and homozygous (+/—) oocytes. Control and treatment groups were cultured in 50 µl Waymouth media with 5% fetal bovine serum under washed mineral oil for 16 h in the presence of FSH. The degree of expansion was determined on a scale from 0 (no expansion) to +4 (maximal response with all cumulus layers undergoing expansion). Values represent the mean ± SEM of three experiments.

COC: cumulus-oocyte cell complexes; MC: meiotically competent oocytes; MI: meiotically incompetent oocytes.
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Fig. 4. (a) Oestradiol and (b) progesterone production by intact cumulus-oocyte complexes (COCs) and oocytectomized complexes (OOX), and OOX complexes co-cultured with GDF-9 (+/+, +/-, —/—) denuded oocytes (DO) for 48 h in the presence of FSH and testosterone. Oocytes were separated as meiotically competent (MC) or incompetent (MI) based on their ability to undergo spontaneous meiotic resumption in culture. (b) The ability of GDF-9 +/+ DO and wild-type oocytes from (C57Bl/6 x Balb/c) F1, mice (denoted B6) were compared. Values are mean ± SEM from three experiments and different letters above the bars indicate significant differences (P < 0.05).

(C57Bl/6 x Balb/c) F1 oocytes (27% of that of OOX complexes alone) and GDF-9 wild type (+/+ oocytes (41%). However, the degree of suppression of progesterone production by OOX complexes was reduced in the presence of GDF-9 heterozygous oocytes (70 ± 15.5%). GDF-9-deficient (—/) meiotically competent (94 ± 16.4%) and incompetent (84 ± 8%) oocytes failed to inhibit progesterone production by OOX complexes. These results indicate a progesterone-inhibitory effect of GDF-9 on FSH-stimulated cumulus cells, and indicate that GDF-9 plays an integral role in the regulation of cumulus cell progesterone production.
Discussion

Studies in the past decade have led to considerable advance in the study of folliculogenesis, most particularly because of the identification and early characterization of oocyte proteins involved in this process. Much of our understanding of the specific roles of the oocyte in follicle development is derived from studies that examined granulosa cell function after removal of the oocyte from preantral follicles or COCs. It has been demonstrated, using the procedure of oocytectomy, that oocytes are required for mouse cumulus expansion (Buccione et al., 1990; Vanderhyden et al., 1990), and that oocytes suppress luteinization (Vanderhyden et al., 1993; Vanderhyden and Tonary, 1995), inhibit expression of urokinase plasminogen activator and LH receptors in granulosa cells (Canipari et al., 1995; Eppig et al., 1997b) and regulate KL expression during follicle growth (Joyce et al., 1999).

The various models of germ cell deficiency indicate that the presence of the oocyte is critical for preventing premature progesterone production by rodent follicles. Similar observations have been reported for pigs (Coskun et al., 1995), chickens (Tischkau and Bahr, 1996) and cows (Li et al., 2000) and have also been reported for Xenopus laevis oocytes, in which the presence of early-stage oocytes resulted in the follicle cells producing oestradiol rather than progesterone, an influence not dependent upon physical association with the oocyte (Sretarugsa and Wallace, 1997). Thus, it appears that the default steroidogenic pathway for granulosa cells in many species is the synthesis of progesterone, and the oocyte is key to the inhibition of this pathway.

To date, the molecules that mediate the influence of the oocyte on granulosa cell luteinization have not been identified, and the experiments reported here confirm that, despite the apparent similarities of the action of TGFβ1, activin and the oocyte on granulosa cell progesterone production, neither TGFβ1 nor activin appears to be the oocyte-secreted luteinization inhibitory factor. GDF-9 remains a strong candidate, as GDF-9-deficient oocytes do not have the abilities of normal oocytes to promote oestradiol and suppress progesterone production. This latter observation is in agreement with the previous demonstration that recombinant GDF-9 suppresses FSH-induced progesterone production by rat granulosa cells (Vitt et al., 2000). In addition to its role in the inhibition of FSH-stimulated progesterone production, GDF-9 has been shown to have growth-promoting effects on granulosa cells, an action also shared with oocyte-conditioned media (Vanderhyden et al., 1992). Thus, the diverse actions of GDF-9 could explain many of the regulatory effects of oocytes on granulosa cells. However, it should be noted that there are discrepancies in the action of GDF-9 that distinguish it from the apparent actions of oocytes. Although GDF-9 treatment alone enhances the expression of steroidogenic acute regulatory protein (Elvin et al., 1999a) and progesterone production in cultured granulosa cells, oocytes do not share this effect. In addition, the possibility remains that GDF-9 is not the only factor missing from the oocytes obtained from the GDF-9-deficient mice, and that these oocytes are otherwise deficient as a consequence of their aberrant follicle development. Thus, it cannot yet be concluded that GDF-9 is the oocyte-secreted luteinization inhibitory factor; the development of a neutralizing antibody would greatly facilitate further studies in this area.

Activin, although initially characterized as a gonadal hormone that regulates the pituitary release of FSH (Ling et al., 1986; Vale et al., 1986), may have a role in the regulation of oocyte activity. Activin is produced by granulosa cells (Findlay, 1993) and oocytes (Albano et al., 1993), and both oocytes and granulosa cells have activin receptors (Wu et al., 1994; Drummond et al., 2002). Previous studies have demonstrated the development-dependent effects of activin on granulosa cell steroidogenesis, such that aromatase activity is enhanced...
and progesterone production is suppressed as follicular maturation progresses (Miró et al., 1991). These steroidogenic responses to activin treatment are similar to that of the oocyte-secreted steroid-regulating factor, raising the possibility that activin is the oocyte-secreted factor. However, in contrast to the inhibitory effects of oocytes on cumulus cell progesterone production, this study revealed no effect of activin on either intact or OOX complexes, indicating that activin is less effective on cumulus cells than on granulosa cells. In addition, it appears that the timing of granulosa cell sensitivity to activin is opposite to their sensitivity to the oocyte-secreted factor. Immature granulosa cells were insensitive to activin but gained sensitivity in luteinized granulosa cells (Miró et al., 1991), whereas the process of luteinization reduces the responsiveness to oocytes (Vanderhyden, 1996). This temporal discordance between granulosa cell responsiveness to activin versus oocytes indicates that it is unlikely that activin is the oocyte-secreted factor that inhibits progesterone production.

At least two other members of the TGFβ superfamily have actions that inhibit luteinization: BMP-6 and BMP-15. BMP-6 has no effect on granulosa cell proliferation, but has been shown to produce a marked decrease in FSH-induced progesterone production and, thus, shares a specific activity with oocytes (Otsuka et al., 2001a). Similarly, the ability of BMP-15 to suppress FSH-stimulated progesterone production (Otsuka et al., 2000) warrants further investigation of both of these growth factors as the oocyte-secreted luteinization inhibitor. However promising because of their shared effects with the oocyte in the inhibition of progesterone production, it should be noted that the mechanism of action of the oocyte activity and the BMPs are markedly different. Unlike the oocyte-secreted factors the site of action of which appears to be downstream of the generation of cAMP (Buccione et al., 1990), BMP-6 appears to downregulate FSH-stimulated adenylate cyclase activity (Otsuka et al., 2001a). Likewise, BMP-15 seems to act by reducing the expression of FSH receptors (Otsuka et al., 2001b) and, therefore, neither of these factors are likely to be the oocyte-secreted factor that regulates cumulus expansion or steroid hormone production.

In addition to its ability to regulate progesterone production, TGFβ1 mimics the actions of oocytes in enabling the expansion of OOX complexes. However, as neutralizing antibodies to TGFβ1 fail to prevent the expansion promoting action of oocytes, it is unlikely that TGFβ1 is the oocyte-secreted CEEF. Although the results reported here were confined to mouse oocytes, it should be noted that in preliminary experiments, there was no effect of neutralizing antibodies to TGFβ1 on FSH-induced expansion of pig COCs (n = 30) or OOX complexes (n = 30). In experiments using conditioned media from germinal vesicle-stage pig oocytes and mouse OOX complexes, TGFβ1 neutralizing antibodies did not inhibit FSH-induced expansion of mouse OOX complexes cultured in oocyte-conditioned media. These results are in agreement with previous work on isolated cumulus cells indicating that the oocyte-secreted factor that enables cumulus expansion is not TGFβ1 (Salustri et al., 1990; Tirone et al., 1997).

GDF-9 upregulates expression of HAS-2, the major hyaluronic acid synthase involved in cumulus expansion, and inhibits the protease uPA, an enzyme suppressed during production of the hyaluronic acid extracellular matrix (Elvin et al., 1999a). GDF-9 can also stimulate the expansion of OOX complexes, rendering this growth factor a prime candidate for the oocyte-secreted cumulus expansion enabling factor. The present study adds evidence to support this hypothesis by showing that oocytes from GDF-9-deficient mice are not able to support expansion of OOX complexes. Although most of the current evidence indicates that GDF-9 is the CEEF, the possibility remains, as noted above, that the oocytes obtained from the GDF-9-deficient mice may be otherwise deficient as a consequence of their aberrant follicle development. In addition, there is a discrepancy in the
timing of expression of GDF-9 versus CEEF in oocytes that needs to be explained. GDF-9 is expressed in oocytes very early in follicle development (McGrath et al., 1995), whereas CEEF is not secreted by oocytes until about the time of acquisition of meiotic competence. This study took advantage of the fact that many of the oocytes from GDF-9 deficient mice failed to undergo spontaneous meiotic resumption to compare the ability of meiotically competent versus incompetent oocytes to support cumulus expansion. However, the actions of the two subpopulations of oocytes did not differ. Thus, although GDF-9 remains the primary candidate for CEEF, there are some discrepancies that still need to be resolved.

The present study and other studies have shown that it is not uncommon for several members of the TGFβ superfamily to mimic the actions of the oocytes on granulosa or cumulus cell function in vitro, and it appears that TGFβ1 can mimic several of these actions. However, the fact that the growth factor has the appropriate action is clearly not sufficient to conclude that it plays this role in the physiological context, and more work still needs to be done to determine the specificity of action of the various members of the TGFβ superfamily during follicle development. Another example of this is the regulation of KL expression. Fully grown oocytes suppress KL expression (Joyce et al., 2000), and this action may be mediated by GDF-9, as recombinant GDF-9 can also inhibit KL expression (Joyce et al., 2000) and the ovaries of GDF-9-deficient mice show an increase in the number of KL transcripts (Elvin et al., 1999b). However, TGFβ1 has inhibitory effects on the expression of KL mRNA in ovarian surface epithelial cells (Ismail et al., 1999) and, although its effects on granulosa cells are not yet determined, the possibility remains that both GDF-9 and TGFβ1 elicit the same response in granulosa cells, and the potential interactions between these two growth factors require elucidation. In addition, it is clear that the interactions that need to be studied are not confined to these two growth factors, as there is now evidence that another oocyte-secreted factor, BMP-15, can stimulate KL expression in rat granulosa cells (Otsuka and Shimasaki, 2002). Although these interactions are likely to be quite complex, the consequence of this plethora of newly identified oocyte-secreted factors has been to give potential identity to oocyte factors previously described only by their actions and to turn the investigation of follicle development into a dynamic area of study. The molecular definition of the oocyte–granulosa cell interactions has generated a complex model of the regulatory mechanisms controlling follicle growth that promises to become even more complex (Fig. 5).

The aberrant folliculogenesis found in GDF-9 and BMP-15 deficient mice (Dong et al., 1996; Yan et al., 2001) has led to the investigation of these factors in domestic ruminant and human ovarian function. Both factors are expressed in human oocytes (Teixeira et al., 2002), and there are no mutations associated with these genes in women with premature ovarian failure or polycystic ovary syndrome (Takebayashi et al., 2000); however, the expression of GDF-9 has been reported to be reduced in polycystic ovaries (Teixeira et al., 2002). Treatment of human cortical tissue slices with GDF-9 in vitro enhanced both follicle survival and progression of follicular development to the secondary stage, indicating that this growth factor may have clinical utility in designing culture conditions for human follicles (Hreinsson et al., 2002). Mutations in BMP-15 or BMP receptor type 1B revealed the importance of this pathway in the regulation of follicular development and ovulation in sheep (Galloway et al., 2000; Mulsant et al., 2001; Souza et al., 2001; Wilson et al., 2001), with the interesting observation that BMP-15 mutations caused increased ovulation rate and infertility in a dose-dependent manner (Galloway et al., 2000). The role of oocyte-derived TGFβ superfamily members in sheep folliculogenesis and ovulation are described in detail by K. McNatty et al. (this supplement).
Oocyte factors controlling cumulus cell function

Pituitary gland

Fig. 5. Model showing paracrine and hormonal factors controlling oocyte growth and follicle development. Oocyte-secreted factors, including bone morphogenetic factor 15 (BMP-15), growth differentiation factor 9 (GDF-9) and other factors yet to be identified, act on the granulosa cells to regulate Kit ligand expression and steroid hormone production to enhance follicle development and the inhibition of luteinization. GDF-9 may be the oocyte-secreted factor that inhibits cumulus cell progesterone (P4) production; however, the factor that promotes oestradiol (E2) production is still unknown. The expression of kit ligand (KL) is regulated by several factors, including FSH and at least two factors produced by the oocyte that have opposing actions, one of which is stimulatory (BMP-15) and the other inhibitory (GDF-9). Kit ligand acts through Kit receptors to promote oocyte growth. Green arrows indicate stimulatory actions; red arrows indicate actions that are inhibitory.
Conclusion

The increasing number of growth factors belonging to the TGFβ superfamily and often showing unique expression in the ovary has provided extensive opportunities to explore their expression and function during follicle development, and will ideally reveal the biochemical identities for the oocyte factors that until now have been detectable only by their biological activities. GDF-9 remains the most likely candidate for the oocyte-secreted cumulus expansion enabling factor, and the oocyte-secreted steroid-regulatory factor that inhibits luteinization; however, there are discrepancies that need to be resolved. Future studies will also need to elucidate the cross-modulation between the TGFβ family members and the gonadotrophins, the interplay between the various TGFβ family members, and the modulation of their activity by regulatory proteins, such as follistatin. The investigation of the biochemical interactions and physiological consequences of these molecules in the complex, finely controlled mechanisms regulating follicle growth promises to be a dynamic area of study for some time.

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