Species differences in the regulation of cumulus expansion by an oocyte-secreted factor(s)

B. C. Vanderhyden

Department of Medicine, University of Ottawa, 451 Smyth Road, Ottawa, Ontario,
Canada K1H 8M5

The expansion of the mouse cumulus oophorus *in vitro* in response to FSH is dependent upon the presence of an enabling factor secreted by the oocyte. The purpose of this study was to determine whether the expansion of cumulus cells from rats and pigs were similarly dependent upon an oocyte-secreted enabling factor. Mouse and rat oocyte–cumulus cell complexes were isolated from pregnant mares' serum gonadotrophin (PMSG)-stimulated animals; pig oocyte–cumulus cell complexes with the attached piece of mural granulosa were obtained from either prepubertal gilts or cyclic sows. FSH ($25\text{--}1000\text{ ng ml}^{-1}$) or dibutyryl cyclic adenosine monophosphate (dbcAMP; $0.05\text{--}2\text{ mmol l}^{-1}$) induced a dose-dependent expansion of the oocyte–cumulus complexes from rats and pigs. The hypothesis that the oocyte plays a role in the regulation of cumulus expansion was tested by microsurgically removing oocytes from oocyte–cumulus complexes and the oocyctomized complexes were tested for their ability to undergo expansion in response to FSH. FSH did not induce cumulus expansion in oocyctomized mouse complexes; however, expansion occurred in rat and pig oocyctomized complexes. The pieces of mural granulosa, detached from the pig complexes, also expanded in response to FSH stimulation. Rat and pig oocyctomized complexes were then held in culture for up to 48 h before stimulation by FSH. The degree of expansion in rat oocyctomized complexes decreased as the delay before FSH stimulation increased such that, with an 8 h delay, oocyctomized complexes did not expand. Pig oocyctomized complexes expanded fully even with a 32 h delay before FSH stimulation, and in response to dbcAMP or epidermal growth factor (EGF). Finally, when rat or mouse oocyctomized complexes were cocultured with germinal vesicle-stage mouse, rat or pig oocytes, FSH stimulated expansion by all oocyctomized complexes. These results indicate that a factor(s) secreted by mouse and rat oocytes is necessary for the cumulus cells to undergo expansion in response to FSH, EGF or dbcAMP. Pig oocytes can secrete a cumulus expansion-enabling factor, but expansion of pig complexes does not depend upon this factor.

Introduction

During the development of the ovarian follicle, the granulosa cells proliferate to form a multilayered epithelium around the growing oocyte. Towards the end of the growth phase, the granulosa cells differentiate into two subpopulations organized as pseudostratified epithelia: mural granulosa cells in contact with the basal lamina that circumscribes the follicle, and cumulus granulosa cells enclosing the oocyte and coupled by gap junctions to both the oocyte and the surrounding mural granulosa cells (Anderson and Albertini, 1976; Heller and Schultz, 1980; Moor *et al.*, 1980). These two cell types differ in their distribution of receptors (Amsterdam *et al.*, 1975; Bortolussi *et al.*, 1979; Lawrence *et al.*, 1980) and their steroidogenic capabilities (Hillensjo *et al.*, 1981; Zoller and Weisz, 1978, 1979). In addition, these cells show a dramatic difference in their response to the preovulatory surge of gonadotrophins. Gonadotrophins

stimulate cumulus granulosa cells to produce and secrete hyaluronic acid that disperses the cumulus cells, a process called expansion or mucification (Dekel and Kraicer, 1978; Eppig, 1979a; Salustri *et al.*, 1989). Mural granulosa cells do not undergo expansion but become luteal cells.

Recent studies have demonstrated that the proliferation, differentiation and function of mouse granulosa cells are dependent upon the oocyte (Buccione *et al.*, 1990; Salustri *et al.*, 1990; Vanderhyden *et al.*, 1990, 1992). A microsurgical procedure whereby the oocyte can be removed from the oocyte–granulosa cell complex (oocyctomy) was used and continual contact of the granulosa cells with the oocyte was found to be necessary for the differentiation of granulosa cells into functional cumulus cells (Vanderhyden *et al.*, 1990). Furthermore, soluble factors secreted by mouse oocytes promoted granulosa cell proliferation and helped to maintain the structural organization of the follicle (Vanderhyden *et al.*, 1990, 1992). A specific role of the oocyte in the regulation of cumulus cell function *in vitro* has also been demonstrated (Buccione *et al.*, 1990; Salustri

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et al., 1990; Vanderhyden *et al.*, 1990). Cumulus expansion is induced *in vitro* by FSH (Thibault, 1972; Dekel and Kraicer, 1978; Eppig, 1979a, b; Hillensjo and Channing, 1980; Ball *et al.*, 1985; Salustri *et al.*, 1989), via a mechanism that appears to be mediated by cAMP (Dekel and Kraicer, 1978; Eppig 1979b). Epidermal growth factor (EGF) is also a potent stimulator of cumulus expansion (Downs *et al.*, 1988). However, separation of the oocytes from the cumulus cells impaired the ability of the cumulus cells to synthesize hyaluronic acid and undergo cumulus expansion *in vitro*, indicating that mouse oocytes secrete a specific, developmentally regulated cumulus expansion-enabling factor that allows cumulus cells to undergo expansion in response to FSH, 8-bromo-cAMP or EGF (Buccione *et al.*, 1990; Salustri *et al.*, 1990; Vanderhyden *et al.*, 1990). The action of this oocyte-secreted factor occurs downstream from the FSH-induced increase in intracellular cAMP (Buccione *et al.*, 1990).

A recent study using oocyctomized pig complexes has determined that, in contrast to mouse cumulus expansion, the expansion of pig oocyte–cumulus complexes *in vitro* is not dependent upon the oocyte (Procházka *et al.*, 1991). This observation suggests that pig oocytes do not secrete a cumulus expansion-enabling factor and, therefore, that there are differences among species in the factors that regulate cumulus expansion. In this study, these possibilities were investigated by comparing the ability of factors secreted by mouse, rat and pig oocytes to enable cumulus expansion in response to FSH, dibutyryl cAMP (dbcAMP) and EGF. The specific aims of the study were (1) to determine whether the expansion of rat oocyte–cumulus cell complexes is dependent upon a cumulus expansion-enabling factor secreted by the oocyte, (2) to determine whether pig and rat oocytes secrete a cumulus expansion-enabling factor, and (3) to determine the species specificity of the activity of cumulus expansion-enabling factors secreted by mouse, rat and pig oocytes.

Materials and Methods

Isolation of oocyte–cumulus cell complexes

Mouse ovaries were obtained from 22- to 24-day-old BALB/c mice injected 44 h previously with 5 iu pregnant mares' serum gonadotrophin (PMSG). Sprague–Dawley rats (26- to 28-day-old) primed with 7.5 iu PMSG provided rat ovaries. With these treatments, the ovaries yield 40–50 oocyte–cumulus cell complexes per animal. Mouse and rat oocyte–cumulus cell complexes were isolated by puncturing antral follicles with 25-gauge needles in Waymouth MB 752/1 (WAY) containing 75 mg penicillin-G l^{-1} , 50 mg streptomycin sulfate l^{-1} and 25 mg sodium pyruvate l^{-1} (all from Sigma Chemical Co., St Louis, MO). The culture medium was supplemented with 5% fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT) and also contained 0.1 mmol 3-isobutyl-1-methylxanthine l^{-1} (IBMX; Aldrich Chemical Co., Milwaukee, WI) to inhibit meiotic maturation of the oocytes. After isolation, the complexes were washed three times in fresh medium.

Pig ovaries were obtained from prepubertal Landrace gilts (5–6 months old) or cyclic sows (2 years old) from a local abattoir. Ovaries were transported on ice in Dulbecco's phosphate-buffered saline supplemented with 5% FBS and 0.1 mmol IBMX l^{-1} and

1.25 μ g fungizone ml^{-1} (Gibco Laboratories, Grand Island, NY). Within 45 min, the ovaries were transferred into pre-warmed WAY/FBS/IBMX. Each antral follicle (2–6 mm in diameter) was opened and a piece of mural granulosa with the attached oocyte–cumulus cell complex was scraped from the wall of the follicle. The complexes were then washed three times in fresh medium. Although the attached pieces of mural granulosa varied in size, the differences had no effect on the response to the treatments given in this study. In addition to assessing the response to treatments qualitatively, as described below, quantitative measurements were made by estimating the area of each piece of mural granulosa before and after treatment. Since the responses of complexes from the antral follicles of prepubertal or cyclic pigs did not differ in these experiments, they were treated as one group.

Oocyctomy

Oocytes were removed microsurgically from the isolated complexes with the use of two Leitz micromanipulators and a Leitz Labovet inverted microscope using a technique described previously (Buccione *et al.*, 1990; Vanderhyden *et al.*, 1990). Briefly, each complex was held with the holding pipette. The lancing pipette was then pushed through the cumulus cells and the oocyte into the holding pipette. Upon withdrawal of the lancing pipette, the negative pressure in the holding pipette aspirated the oocyte, leaving the partially collapsed zona pellucida *in situ*. When released from the holding pipette, the oocyctomized complex returned to a normal spherical shape. The time required to oocyctomize a set of 30 complexes was approximately 15 min.

Culture of intact and oocyctomized complexes

Intact and oocyctomized complexes were transferred to drops of 25 μ l (mouse and rat) or 100 μ l (pig) WAY/FBS/IBMX that were covered with washed mineral oil in Falcon culture dishes (Becton Dickinson, Lincoln Park, NJ). Light mineral oil (Fisher Scientific, Fair Lawn, NJ), 200 ml, was equilibrated with 20 ml WAY/FBS by stirring for 4 days, with one media change after 2 days. After allowing the media to settle, the equilibrated oil was sterilized by filtration through a 0.45 μ m filter. In all experiments FSH (NIADDK-oFSH-17; 25–1000 ng ml^{-1}), dbcAMP (Sigma; 0.05–2 mmol l^{-1}) or EGF (from submaxillary glands, Boehringer-Mannheim, Laval, Quebec; 2 ng ml^{-1}) was added to the drops of medium to stimulate cumulus expansion. The complexes were then cultured for up to 24 h for mouse and rat complexes and up to 48 h for pig complexes at 37°C in 5% CO₂: 5% O₂: 90% N₂, before being assessed for the degree of cumulus expansion.

Delay of stimulation after oocyctomy

Tissue culture plates (24 wells; Corning Glass Works, Corning, NY) were coated with 200 μ l 2% agarose (ultra pure grade, Bethesda Research Laboratories, Gaithersburg, MD) and equilibrated with WAY/FBS/IBMX. Intact and oocyctomized rat and pig complexes were prepared as described previously and transferred to the wells (20–50 complexes per well). The

agarose coating prevented the complexes from adhering to the tissue culture plate. The complexes were cultured for 1–48 h before transfer to drops of WAY/FBS/IBMX under oil and treatment with FSH ($1 \mu\text{g ml}^{-1}$), dbcAMP (2 mmol l^{-1}) or EGF (2 ng ml^{-1}). Complexes were assessed for the degree of cumulus expansion after 24 (rat) or 32 h (pig); the times were determined in preliminary experiments to achieve the maximum degree of cumulus expansion.

Coculture of oocyctomized complexes with denuded oocytes

Denuded oocytes were prepared by repeated pipetting of mouse, rat or pig oocyte–cumulus cell complexes with a Pasteur pipette. Groups of 50 denuded germinal vesicle-stage oocytes were transferred to 25 μl drops of WAY/FBS/IBMX under oil in Falcon culture dishes and cultured for 24 h; oocyte-free drops were also prepared. The next day, intact and oocyctomized complexes from mice, rats or pigs were transferred, in groups of 12–25 in minimum volume, to the drops of unconditioned or oocyte-conditioned media. In some treatments, complexes were added to media conditioned by oocytes from the same species. In other treatments, complexes were added to drops of denuded oocytes from a different species. FSH ($1 \mu\text{g ml}^{-1}$), dbcAMP (2 mmol l^{-1}) or EGF (2 ng ml^{-1}) was then added to the cultures and the degree of expansion was scored after the appropriate period of culture.

Preliminary characterization of the enabling factor

The biochemical nature of the cumulus expansion-enabling factor was investigated in experiments in which medium was conditioned with denuded pig oocytes (two oocytes per μl) as described above and subjected to one of three treatments: (1) maintained at 65°C for 30 min, (2) treated with proteinase K (Bethesda Research Laboratories; $100 \mu\text{g ml}^{-1}$) for 7 h at 55°C , which was then inactivated with phenylmethylsulfonylfluoride (PMSF, Boehringer-Mannheim; $200 \mu\text{g ml}^{-1}$), or (3) addition of PMSF ($200 \mu\text{g ml}^{-1}$). Because the first two treatments would inactivate the serum proteins, as well as any oocyte-secreted proteins, the original concentration of FBS (5%) was restored to the conditioned media following these two treatments. Groups of 20 oocyctomized mouse complexes were transferred to 25 μl drops of unconditioned media, untreated oocyte-conditioned media, or the media that had been treated after conditioning. FSH ($1 \mu\text{g ml}^{-1}$) was added to the drops and the degree of expansion was assessed after 20–24 h of culture.

Assessment of cumulus expansion

At the end of the culture period, the complexes were assessed for expansion according to a subjective scoring system described by Vanderhyden *et al.* (1990). In this system the degree of expansion is ranked from +1 to +4: +1 indicates the minimum observable response, where cumulus cells have a glistening appearance and are not adhering to the dish; +4 indicates the maximum degree of expansion where all layers of cumulus cells, including the corona radiata, have expanded. A score of 0 indicates no expansion; the cells are adhering to the

Table 1. The effect of FSH and dibutyryl cAMP (dbcAMP) on the expansion of porcine and rat oocyte–cumulus cell complexes *in vitro*

Culture conditions	Degree of expansion	
	Rat ^a	Pig ^b
Control	0	0
FSH (ng ml^{-1})		
25	+1 to +2	+2
50	+3	+2 to +3
100	+3	+3
250	+3 to +4	+4
500	+3 to +4	+4
1000	+3 to +4	+4
dbcAMP (mmol l^{-1})		
0.05	0	0
0.1	0	0
0.5	+1	+2 to +3
1.0	+1 to +2	+3 to +4
1.5	+2 to +3	+4
2.0	+3 to +4	+4

^a60 rat oocyte–cumulus cell complexes were assessed for each treatment in a total of three experiments.

^b45 pig oocyte–cumulus cell complexes were assessed for each treatment in a total of three experiments.

dish and spreading away from the complex. To ensure that the cumulus expansion induced in these complexes is a result of hyaluronic acid-synthesizing activity, samples of intact and oocyctomized expanded complexes from all treatment groups were exposed to 200 U *Streptomyces* hyaluronidase ml^{-1} (Sigma) and the dissociation of cumulus cells was monitored for 10 min. Since *Streptomyces* hyaluronidase specifically degrades hyaluronic acid, cumulus cell dispersion in the presence of this enzyme indicates that hyaluronic acid is a component of the expanded complexes.

Results

FSH and dbcAMP stimulation of rat and pig cumulus expansion

FSH and dbcAMP induced expansion in rat and pig oocyte–cumulus complexes in a dose-dependent manner (Table 1). The degree of expansion achieved by more than 80% of the rat complexes was +3 to +4 when they were cultured in at least 250 ng FSH ml^{-1} or 2.0 mmol dbcAMP l^{-1} for 24 h. Pig oocyte–cumulus complexes underwent complete expansion (+4) at similar concentrations of FSH or dbcAMP after 32 h of culture. Shorter periods of culture resulted in lesser degrees of expansion (data not shown). FSH-stimulated expansion of oocyte–cumulus complexes is shown (Fig. 1a, b (pig) and Fig. 2a, b (rat)). The pieces of mural granulosa attached to pig complexes also expanded in response to stimulation by FSH ($1 \mu\text{g ml}^{-1}$) or dbcAMP (2 mmol l^{-1}), even when they were detached from the complexes before treatment with the hormone (Fig. 3). In response to FSH, the area of each piece of

Fig. 1. Photomicrographs of (a, b) intact and (c, d, e) oocyte-tomized porcine oocyte-cumulus cell complexes with attached pieces of mural granulosa. Neither (a) intact nor (c) oocyte-tomized complexes expanded in the absence of gonadotrophic stimulation; however, both (b) intact and (d) oocyte-tomized complexes were expanded 32 h after stimulation by FSH ($1 \mu\text{g ml}^{-1}$). (e) Expansion of oocyte-tomized complexes incubated for 24 h before treatment with FSH. (f) Hyaluronidase treatment of two intact complexes and one oocyte-tomized complex (indicated by the arrow) following expansion induced 24 h earlier by FSH. The pieces of mural granulosa and the cumulus cells, except the corona radiata, were dispersed within minutes. Scale bars = 250 μm .

mural granulosa increased at least tenfold ($0.181 \pm 0.008 \text{ mm}^2$ versus $2.104 \pm 0.109 \text{ mm}^2$). FSH ($1 \mu\text{g ml}^{-1}$) stimulated cumulus expansion of intact rat and pig complexes subjected to sham micromanipulation where the oocyte was penetrated but not aspirated or killed by the lancing pipette (data not shown). Expanded rat and pig complexes and the attached pieces of mural granulosa were dissociated within 4–5 min by the addition of *Streptomyces* hyaluronidase. Hyaluronidase-treated pig complexes are shown (Fig. 1f).

Expansion of intact and oocyte-tomized mouse, rat and pig complexes

The dependence of FSH-induced cumulus expansion upon an oocyte-derived factor was investigated by removing oocytes from oocyte-cumulus complexes and stimulating the oocyte-tomized complexes with FSH. Whereas FSH induced +3 to +4 expansion in intact rat, pig (Table 2) and mouse (Table 3) oocyte-cumulus complexes, oocyte-tomized complexes of the different species

Fig. 2. Photomicrographs of (a, b) intact and (c, d) oocytectomized rat oocyte-cumulus cell complexes. Oocytectomized complexes were incubated for 5 h after oocytectomy before further treatment. Intact complexes were preincubated for an equivalent period. (a) In unstimulated, intact complexes, cumulus cells do not expand but, instead, adhere to the substrate and begin to migrate away from the oocyte. FSH ($1 \mu\text{g ml}^{-1}$) induced the expansion of (b) intact but not (c) oocytectomized complexes. (d) Expansion of oocytectomized rat complexes cocultured overnight with porcine denuded oocytes ($2 \text{ oocytes } \mu\text{l}^{-1}$) in the presence of FSH. Scale bars = $100 \mu\text{m}$.

were more variable in their response to treatment. Oocytectomized mouse complexes failed to expand in response to FSH. Whereas FSH-stimulated oocytectomized rat complexes showed some expansion (+2; Table 2), the degree of expansion was less than that of intact complexes (+3 to +4; Table 2). In contrast, pig complexes were unaffected by the removal of the oocyte, and exhibited expansion comparable to that of intact complexes (+4, Table 2).

Delay of stimulation after oocytectomy

The ability of rat and pig complexes to undergo FSH-induced cumulus expansion after oocytectomy suggests that expansion of the complexes of these species does not depend upon a factor produced by the oocyte. Alternatively, these results may be explained if the cumulus expansion-enabling factor from these species had a longer half-life than the mouse enabling factor, or if there was a delay in the loss of oocyte-induced biological effects on the cumulus cells.

To differentiate between these possibilities, oocytectomized rat and pig complexes were cultured for various periods after oocytectomy to ensure that the cumulus cells were not responding to previous stimulation by oocyte-secreted factors. Although intact rat complexes maintained their ability to expand in response

to FSH over 24 h, oocytectomized complexes demonstrated a decreasing ability to undergo expansion as the duration of culture after oocytectomy increased (Table 4 and Fig. 2c). By 4–8 h after oocytectomy, the oocytectomized complexes had completely lost their ability to undergo FSH-stimulated cumulus expansion. In addition, the oocytectomized complexes were unable to expand in response to dbcAMP or EGF, stimuli that still induced cumulus expansion in intact complexes after a 5–6 h delay before stimulation (Table 5).

In contrast to the results observed in rat complexes, both intact and oocytectomized pig complexes maintained their ability to expand in response to FSH stimulation for up to 48 h (Table 4 and Fig. 1e). Similarly, the degree of expansion of oocytectomized complexes stimulated by dbcAMP or EGF was not affected when the stimulation was delayed by 24 h (Table 5).

Coculture of oocytectomized complexes with denuded oocytes

The decreased ability of rat oocytectomized complexes to undergo expansion when the FSH stimulation is delayed suggests that the cumulus cells do depend upon an oocyte-secreted factor. Oocytectomized rat complexes were added to rat oocyte-conditioned medium, with or without FSH, dbcAMP

Fig. 3. Expansion of the piece of mural granulosa detached from a pig oocyte–cumulus cell complex. The piece of mural granulosa was incubated for 24 h before FSH ($1\text{ }\mu\text{g ml}^{-1}$) was added. Expansion was assessed after 32 h. In this experiment, some intact rat oocyte–cumulus cell complexes (open arrows) were included in the culture to demonstrate cumulus expansion. Solid arrows indicate the boundary separating the cells of the piece of mural granulosa that undergo expansion from those that do not expand. Scale bar = 250 μm .

Table 2. Expansion of FSH-stimulated intact and oocyctomized complexes

Culture conditions	Type of complex	Degree of expansion	
		Rat ^a	Pig ^b
Control	Intact	0	0
	Oocyctomized	0	0
FSH ($1\text{ }\mu\text{g ml}^{-1}$)	Intact	+ 3 to + 4	+ 4
	Oocyctomized	+ 2 ^c	+ 3 to + 4

^a135 rat complexes were assessed for each treatment in a total of seven experiments.
^b50 to 58 pig complexes were assessed for each treatment in a total of five experiments.
^cThe degree of expansion in this group varied between 0 and + 3, but the majority (> 70%) was assessed as + 2.

or EGF, to determine whether cumulus-free germinal vesicle-stage oocytes could promote FSH-, dbcAMP- or EGF-induced cumulus expansion. As shown in Table 5, FSH, dbcAMP and EGF all stimulated cumulus expansion of the oocyctomized complexes cocultured with denuded oocytes. The degree of expansion of the oocyctomized complexes in oocyte-conditioned medium was similar to that of intact complexes. Expansion of oocyctomized complexes did not occur in control drops of unconditioned medium with any stimuli, or in conditioned medium in the absence of stimuli (Table 5).

Cumulus-free mouse, rat and pig oocytes were cocultured with intact and oocyctomized mouse and rat complexes to determine

whether pig oocytes secrete a factor(s) that could affect the oocyte-dependent cumulus expansion of complexes from rats and mice. Rat complexes were preincubated for 5 h before treatment as described above. FSH stimulated cumulus expansion by all oocyctomized complexes cocultured with denuded oocytes (Table 3). Rat and mouse oocyctomized complexes cocultured with pig oocytes underwent FSH-induced expansion to degrees comparable to those in conspecific cocultures. For each culture condition, the degree of expansion of oocyctomized complexes was similar to that of the corresponding intact complexes.

Preliminary characterization of the enabling factor

The observation that mouse oocyctomized complexes expand in pig oocyte-conditioned media in response to FSH was used and the biochemical nature of the pig enabling factor was investigated by inactivating any proteins secreted by the oocyte. Treatment of pig-oocyte conditioned media with proteinase K for 7 h, or by heating at 65°C for 30 min depleted the media of its ability to enable expansion of mouse oocyctomized complexes in the presence of FSH (0 expansion). Oocyctomized complexes in untreated oocyte-conditioned media, or in media to which PMSF had been added were able to undergo expansion to + 3 levels in response to FSH.

Discussion

The experiments reported here demonstrate that mouse, rat and pig oocytes all secrete a cumulus expansion-enabling factor(s) that allows the cumulus cells to undergo expansion in response to FSH or EGF. Whereas the expansion of mouse and rat cumulus cells requires the presence of this factor, the expansion of pig complexes *in vitro* does not depend upon an oocyte-secreted enabling factor. These results were obtained in experiments that demonstrated that microsurgical removal of the oocyte impairs the ability of mouse and rat oocyte–cumulus cell complexes to undergo expansion in response to FSH or dbcAMP. EGF, which stimulates cumulus expansion of intact complexes by a mechanism that, at least initially, is different from FSH, also failed to stimulate cumulus expansion after oocyctomy. When oocyctomized rat complexes were cultured in oocyte-conditioned medium, the cumulus cells underwent expansion after stimulation with FSH, dbcAMP or EGF to the same extent as in intact complexes. In contrast, expansion of pig oocyte–cumulus complexes was not affected by oocyctomy. Medium conditioned by pig oocytes, however, enabled the expansion of rat and mouse oocyctomized complexes in response to FSH.

Expansion of the mouse cumulus oophorus *in vitro* was found to be dependent upon regulation of hyaluronic acid synthesis by the oocyte (Buccione *et al.*, 1990; Salustri *et al.*, 1990). In the present study, cumulus cells in the expanded complexes from rat, mouse and pig were dispersed in the presence of *Streptomyces* hyaluronidase. The sensitivity of all expanded complexes to this highly specific hyaluronidase indicates that hyaluronic acid is a component of the expanded complexes of all three species.

In initial experiments, oocyctomy of rat oocyte–cumulus complexes resulted in a somewhat variable response to FSH stimulation. By allowing a period before FSH stimulation, a more consistent, and oocyte-dependent, result was obtained

Table 3. Expansion of intact and oocyctomized complexes cultured in unconditioned medium or medium conditioned by oocytes of the same or other species

Culture conditions ^a		Degree of expansion			
		Mouse ^b		Rat ^c	
		Intact	Oocyctomized	Intact	Oocyctomized
Conditioned by	FSH				
—	—	0	0	0	0
—	+	+3 to +4	0	+3 to +4	0 to +1
Mouse oocytes	—	0 to +1	0	0	0
Mouse oocytes	+	+4	+3 to +4	+3 to +4	+3
Rat oocytes	—	0 to +1	0	0	0
Rat oocytes	+	+4	+3 to +4	+3 to +4	+3
Pig oocytes	—	0	0	0	0
Pig oocytes	+	+4	+3	+3 to +4	+3

^aConditioned media were prepared by culturing 50 denuded germinal vesicle-stage oocytes in 25 µl of medium for 24 h before addition of intact or oocyctomized complexes. FSH was added at a concentration of 1 µg ml⁻¹.

^b60 mouse complexes were assessed for each treatment in a total of three experiments.

^c60 to 75 rat complexes were assessed for each treatment in three (mouse and rat oocyte-conditioned media) or five (rat and pig oocyte-conditioned media) experiments. Rat complexes were incubated for 5 h before treatment. Mouse complexes had no incubation period before treatment.

Table 4. Effect of a delay between oocyctomy and FSH stimulation on the expansion of rat and pig cumulus complexes

Delay ^a (h)	Degree of expansion			
	Rat ^b		Pig ^c	
	Intact	Oocyctomized	Intact	Oocyctomized
0	+3 to +4	+2 to +3	+4	+4
1	+3 to +4	+1 to +3	—	—
2	+3 to +4	0 to +2	—	—
4	+3 to +4	0 to +1	+4	+4
8	+3	0	+4	+4
24	+3	0	+4	+4
32	—	—	+4	+4
48	—	—	+3	+3

—: not performed.

^aFor oocyctomized complexes, the delay is the period of culture between the time of oocyctomy and the time when FSH was added to the cultures. Intact complexes were cultured for an equivalent period before FSH stimulation. FSH was added at a concentration of 1 µg ml⁻¹.

^b55 rat complexes were assessed for each treatment in a total of three experiments.

^c60 pig complexes were assessed for each treatment in a total of five experiments.

with a 5–6 h preincubation for rat oocyctomized complexes. The need for a delay before rat cumulus cells demonstrated a dependence on the oocyte-secreted factor suggests at least two differences between mouse and rat oocyte-derived factors in their ability to enable cumulus expansion in response to FSH. Rat cumulus cells may have a prolonged responsiveness to

Table 5. Effect of various stimuli on the expansion of intact and oocyctomized complexes cultured in unconditioned or oocyte-conditioned medium

Culture conditions	Type of complex	Degree of expansion	
		Rat ^a	Pig ^b
Control	Intact	0	0
	Oocyctomized	0	0
FSH (1 µg ml ⁻¹)	Intact	+3 to +4	+4
	Oocyctomized	0	+4
dbcAMP	Intact	+3 to +4	+4
(2 mmol l ⁻¹)	Oocyctomized	0	+4
EGF ^c (2 ng ml ⁻¹)	Intact	+3 to +4	+4
	Oocyctomized	0	+4
Coculture ^d	Intact	0	—
	Oocyctomized	0	—
Coculture + FSH	Intact	+4	—
	Oocyctomized	+3	—
Coculture + dbcAMP	Intact	+4	—
	Oocyctomized	+3 to +4	—
Coculture + EGF	Intact	+4	—
	Oocyctomized	+3 to +4	—

^a95 rat complexes were assessed for each treatment in a total of seven experiments. Complexes were incubated for 5–6 h before treatment.

^b60 pig complexes were assessed for each treatment in a total of five experiments. Complexes were incubated for 24 h before treatment.

^cEGF: epidermal growth factor.

^dCocultures were prepared by culturing 50 denuded rat germinal vesicle-stage oocytes in 25 µl medium for 24 h. Intact or oocyctomized complexes were then added with or without FSH, dbcAMP or EGF. Rat complexes were incubated for 5–6 h before adding them to the oocyte-conditioned medium. Since pig complexes expanded fully even after oocyctomy, it was unnecessary to use these complexes in coculture experiments.

FSH after oocyectomy if the rat oocyte-secreted factor has a longer half-life within the complex than does the mouse enabling factor. Since cumulus expansion-enabling factor is secreted constitutively by the oocyte (Vanderhyden *et al.*, 1990), an alternative explanation may be that rat cumulus cells require a longer period after oocyectomy to lose the biological effects induced by stimulation before oocyectomy. Despite the need for a delay of 5–6 h before FSH stimulation, these experiments have shown that the expansion of rat cumulus cells, like mouse cumulus cells, is dependent upon a factor secreted by the oocyte.

In contrast to the rat, preincubations of up to 48 h were ineffective in demonstrating a dependence of pig cumulus cell expansion on the oocyte. Procházka *et al.* (1991) demonstrated the lack of effect of oocyectomy on the expansion of pig complexes in response to FSH and suggested that pig oocytes do not secrete a cumulus expansion-enabling factor. The experiments presented here, however, demonstrate that pig oocytes do secrete a factor that enables the expansion of mouse and rat oocyectomized complexes. It appears that the function of this factor in promoting cumulus expansion is different in rodent and pig oocyte–cumulus cell complexes, and this difference may be simply temporal. Previous experiments have determined that, in mice, only meiotically-competent oocytes can secrete the cumulus expansion-enabling factor (Vanderhyden *et al.*, 1990). As the acquisition of meiotic competence and differentiation of granulosa cells into functional (i.e. able to expand) cumulus cells normally occurs at the same time, perhaps during differentiation, the pig enabling factor induces a permanent change in the cumulus cells that enables them to undergo expansion whenever the appropriate hormonal stimulation occurs. In contrast, the secretion of enabling factor in rodent complexes may not induce permanent changes during differentiation, and, therefore, they require the immediate presence of the enabling factor at the time of exposure to FSH. It is also conceivable that the cumulus expansion-enabling factor secreted by pig oocytes is not necessary for the expansion of pig complexes, but plays some other role in the development or function of cumulus cells.

Since the mouse and rat oocyte–cumulus complexes used in these experiments were obtained from PMSG-primed animals, whereas the pig complexes were collected from unprimed animals, it is possible that a difference in physiological/hormonal environment is responsible for the species differences noted here. However, in a previous study (Vanderhyden *et al.*, 1990) using primed and unprimed mice, oocytes isolated from the early antral follicles of unprimed 16-day-old mice could secrete the cumulus expansion-enabling factor and, further, the cumulus cells from these mice could expand if cultured in the presence of sufficient enabling factor. Similar results have since been obtained using rat complexes from unprimed animals (B. C. Vanderhyden, unpublished observations). In addition, Procházka *et al.* (1991) demonstrated the lack of effect of oocyectomy on the expansion of pig complexes obtained from PMSG-primed animals. These observations indicate that priming has no apparent effect on the ability of oocytes to secrete the cumulus expansion-enabling factor, nor on the ability of cumulus cells to respond to this factor.

Investigation of the biochemical nature of the oocyte-secreted cumulus expansion-enabling factor may prove to be somewhat

difficult. Denuded oocytes cannot be easily obtained, especially in the pig, in the numbers needed to condition sufficient media for biochemical analysis. This problem is compounded by the fact that the enabling factor is very unstable, and loses activity when stored under any of the conditions tested to date (J. J. Eppig and B. C. Vanderhyden, unpublished observations). Preliminary characterization of the mouse enabling factor by Eppig *et al.* (in press) has revealed that the factor is heat labile (65°C for 15 min), and proteinase K sensitive, and its activity is retained in the fraction with molecular mass > 100 kDa during fractionation. In the present study, similar experiments using media conditioned by pig oocytes have demonstrated that the pig oocyte-secreted factor is also heat labile and proteinase K sensitive. Because large quantities of oocytes are necessary to condition the medium, biochemical analysis is far more feasible with rodents, where large numbers of oocytes can be grown *in vitro* (Eppig *et al.*, in press). Further characterization of the enabling factor will allow us to investigate the role of this factor in porcine follicle development.

Contact of granulosa cells with a collagen-containing basal lamina affects cell structure, synthesis and organization of cytoskeletal components, and the development of functional LH receptors (Amsterdam *et al.*, 1989). In comparison, the differentiation of mouse cumulus cells, and their ability to undergo cumulus expansion depend upon contact with the oocyte or oocyte-secreted factors (Buccione *et al.*, 1990; Vanderhyden *et al.*, 1990). These observations suggest that the oocyte and the basal lamina play critical roles in the differentiation and function of subpopulations of granulosa cells. It is not known which of these two substrates has the dominant effect on the differentiation and function of the piece of mural granulosa that connects the mural granulosa cells to the cumulus granulosa cells. However, the clear delineation between granulosa cells that can expand and those that cannot suggests that the boundaries indicating the limit of influence of the substrates are very well defined. If oocyte-secreted factors promote the ability of granulosa cells to undergo expansion, and if the concentration of these factors decreases in a gradient away from the oocyte, then perhaps a minimum concentration of this factor is necessary to induce the responsiveness of the cumulus cells to FSH.

In mice, the granulosa cells comprising the stalk between the oocyte–cumulus cell complex and the collagen membrane upon which the complex was grown *in vitro* do not undergo expansion in response to FSH, suggesting that the granulosa cells of the stalk resemble mural granulosa cells (Vanderhyden *et al.*, 1990). In contrast, the experiments reported here and in a previous study (Procházka *et al.*, 1991) demonstrated that, in the pig, pieces of membrana granulosa could undergo expansion even when separated from the oocyte–cumulus cell complex, suggesting that these cells resemble cumulus granulosa cells. These observations indicate that there may be differences between species in the function of the granulosa cells that are most closely connected to the cumulus cells or that the range of oocyte-secreted factors that enable cumulus expansion may extend further in pig follicles than in mouse or rat follicles. However, the mouse oocyte–granulosa cell complexes grown *in vitro* were supported on a stalk of granulosa cells that adhered directly to a collagen-coated membrane as opposed to the normal mural granulosa cells. This deviation from the normal cell–cell associations in favour of cell–substrate interactions may

have directed the stalk of granulosa cells along a path of differentiation similar to mural granulosa cells.

These experiments have shown that expansion of mouse and rat cumulus cells depends upon the presence of an oocyte-secreted enabling factor. The expansion of pig complexes *in vitro* appears to be independent of the oocyte or any oocyte-secreted factors, suggesting that the regulation of cumulus expansion in pig complexes is different from that in rodents. Although the magnitude of the differences between these species in the regulation of cumulus expansion will only be known when the function of the porcine enabling factor has been identified, the observations that rat, mouse and pig oocytes all secrete a cumulus expansion-enabling factor and that complexes from all these species undergo expansion in response to FSH and EGF suggests that there are important cross-species similarities in the factors that regulate cumulus expansion. These similarities suggest that there may be only subtle (for example, temporal) species-dependent differences in the specific function of the cumulus expansion-enabling factor or the responsiveness to gonadotrophins that it induces in cumulus cells.

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