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Activation of primordial follicles *in vitro*

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The resting pool of primordial follicles in mammalian ovaries is a potential resource for the genetic manipulation of domestic animals, the preservation of endangered species, and the amelioration of some forms of infertility in humans. Exploitation of this large reservoir of follicles depends on the development of methods for activating primordial follicles to begin growth *in vitro* and of methods for sustaining follicular growth to the stage at which oocytes are capable of meiotic maturation, fertilization and development to live young. It has been shown that primordial follicles of rodents, cattle and primates can initiate growth *in vitro*, even in serum-free medium. The signals that cause primordial follicles to leave the resting pool or remain quiescent are unknown. However, of interest is the observation that in cultures of whole rodent ovaries an apparently normal number of follicles leaves the resting pool and begins to grow, whereas in cultures of isolated bovine or primate ovarian cortex almost all primordial follicles activate and develop into primary follicles. This finding suggests that non-cortical portions of the ovary may regulate the flow of follicles from the resting reservoir. In cattle, it has been difficult to sustain follicular growth beyond the primary stage and the development of methods for doing so are critical for achievement of the practical goal of use of the primordial pool for embryo production. However, the development of murine follicles *in vitro* from the primordial stage through oocyte maturation and fertilization, and the birth of one pup, provides encouragement for efforts to achieve similar results in large mammals.

Introduction

Mammalian ovaries contain a reservoir of non-growing primordial follicles. Primordial follicles are formed when primary oocytes become invested with a layer of flattened pre-granulosal cells. These follicles constitute a pool from which follicles will be drawn gradually to begin growth, starting soon after follicle formation and continuing throughout the reproductive life span. What causes follicles to leave (or remain in) the resting pool and how a continuous 'trickle' of exiting follicles is achieved are unknown. The regulation of follicular quiescence versus growth is currently perhaps the most intriguing question in the area of regulation of ovarian follicular development. Although some progress has been made and will be reviewed in this manuscript, the most fundamental questions remain to be answered.

Two types of mammalian species have been used as models to address questions of the initiation of follicular growth – rodents and larger mammals, especially cattle and primates. In rats and mice follicle formation occurs at a specific time, one or two days after birth, depending on the species or strain. After follicle formation, follicles begin immediately to leave the resting pool and the first cohort reaches the antral stage after about two weeks (Hirshfield, 1991). There are several important advantages to rodents as models for exploring the mechanisms that govern the initiation of follicular growth, a process also referred to as follicle activation. The ovaries of newborn animals are small and

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soft, and they can be cultured as whole ovaries, thus maintaining relationships among ovarian components, or they can be dissociated with enzymes to allow retrieval and culture of individual follicles (see for example Eppig and O'Brien, 1996). In addition the presence of only primordial follicles in newborn rodents allows analysis, during the first few weeks of life, of a synchronous cohort, the first follicles that leave the resting reservoir and initiate growth.

In larger mammals, such as ruminants and primates, the formation of primordial follicles occurs during fetal life over a much more protracted period, compared with that in rodents, of weeks or months (Henricson and Rajakoski, 1959; van Wagenen and Simpson, 1965; Russe, 1983). Some follicles begin to leave the resting pool before others have been formed, so that in these species follicular formation and the initiation of growth are occurring simultaneously within the same ovary. This offers the advantage that fetal ovaries, which may be more readily available and more economical and which have large numbers of primordial follicles, can be used to study follicle growth initiation. However, there are two distinct disadvantages to ruminants and primates as animal models for studies of activation of primordial follicles. First, since some follicles leave the resting pool before other primordial follicles have been formed, there is no easily identifiable time during development when the ovary contains only primordial follicles in large numbers. Second, the stroma of ruminant and primate ovaries is much denser and tougher than the stroma of rodent ovaries, making enzymatic dissociation or mechanical dissection of component parts difficult. This difficulty can be partially obviated by the use of fetal ovaries, which are much softer. However, bovine oocytes are more sensitive to enzymatic treatments that are tolerated well by rodent oocytes and can be easily damaged by enzymatic dissociation of ovarian tissue (Wandji *et al.*, 1996a).

Despite the difficulties of studying the regulation of follicle activation in large mammals, they have been used as models because of the practical benefits that could result from greater knowledge of the signals that regulate follicular growth and differentiation. The resting pool of primordial follicles is a potential resource that could be tapped to increase the reproductive potential of valuable domestic animals, members of endangered species, and women with fertility problems. The similarity of bovine and primate ovaries makes cattle excellent models for humans, as well as important models in themselves because of their agricultural importance. The ultimate goal of studies on the activation of primordial follicles *in vitro* is to develop conditions that will sustain follicular development to the stage where the oocyte is capable of meiotic maturation, fertilization and normal development.

Initiation of Follicular Growth *In Vitro*

The initiation of follicular growth has been achieved *in vitro* for several species. Blandau *et al.* (1965) cultured fetal mouse ovaries in serum-containing medium and reported that some oocytes grew in culture, specifically those that were surrounded by one or more layers of somatic cells. Eppig and O'Brien (1996) cultured ovaries obtained from newborn mice, which contain only newly formed primordial follicles, in medium containing 10% serum for 8 days. During this interval, follicular development *in vitro* was qualitatively similar to development *in vivo*, in that some primordial follicles initiated growth and developed to the secondary stage. More remarkably, these authors then isolated growing preantral follicles from the ovaries after 8 days in culture and grew them to the stage of oocyte competence for meiotic maturation, fertilization and embryonic development. One live pup was produced after embryo transfer. These experiments showed that murine primordial follicles can initiate growth *in vitro*, at least in the presence of 10% serum.

Several years ago, our laboratory began experiments to determine whether bovine primordial follicles could be activated *in vitro* in serum-free medium. Since the large size of bovine ovaries precludes whole-organ cultures, we isolated small pieces of ovarian cortex (about 0.5 mm × 0.5 mm × 0.3 mm). Because primordial follicles are located in the cortical region of the ovary, cortical pieces are rich in primordial follicles. Since follicle formation in cattle begins in mid-gestation and fetal ovaries are much softer and easier to dissect than adult ovaries, fetuses in the third trimester of gestation were used. Pieces of cortex were cultured on transwell membrane inserts in Waymouth MB

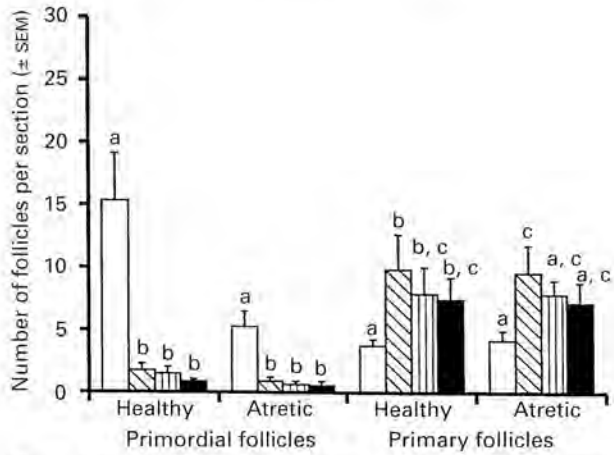

Fig. 1. Numbers of healthy and atretic primordial and primary follicles (mean per histological section ± SEM, $n = 4$ fetuses, with 59–71 sections examined per fetus) in fetal bovine ovarian cortex after 0 (□), 2(▨), 4 (▥) or 7 (■) days in culture. Within each group of four bars, bars with no common superscript are significantly different (a,b $P < 0.01$; a,c $P < 0.05$). (Reprinted from Wandji *et al.*, 1996b with permission.)

752/1 medium containing antibiotics and ITS+ (insulin, transferrin, selenium, BSA and linoleic acid) for up to 7 days (Wandji *et al.*, 1996b). At the initiation of culture (day 0), the cortical pieces contained mostly primordial follicles (Fig. 1), characterized by an oocyte surrounded by a single layer of flattened granulosal cells. As early as the second day of culture the number of primordial follicles had declined markedly, whereas the number of primary follicles, characterized by a single layer of cuboidal granulosal cells, had increased (Wandji *et al.*, 1996b). In addition, the diameter of primary follicles and their oocytes increased gradually throughout the 7 day culture (Fig. 2; Wandji *et al.*, 1996b). These results indicate that bovine primordial follicles can activate *in vitro* in serum-free medium and differentiate into primary follicles. This indicates, as the experiments with rodents had implied, that the activation of primordial follicles does not depend on specific endocrine signals. The experiments with cattle further support the contention that signals from non-cortical components of the ovary are not needed to stimulate initiation of follicle growth.

What is puzzling about the results presented in Fig. 1 is that such a large percentage of the primordial follicles in pieces of fetal bovine cortex initiated growth; almost all of them became activated *in vitro*. This mass exodus of follicles from the resting stage was not due to the fetal origin of the cultured cortical pieces, since Braw-Tal and Yossefi (1997) reported a similar loss of follicles from the primordial pool and an increase in primary follicles after 2 day cultures of ovarian cortical pieces from adult cattle (Table 1). Hence, something about the conditions of culture obviated the mechanisms that normally 'tell' each follicle when its 'turn' has come to leave the resting pool. The serum-free culture of ovarian cortical pieces provides a system that may be used to explore some of the factors that regulate follicle activation. Some potential regulators will be discussed below. The mass movement of follicles out of the resting pool and their development and growth as primary follicles under these experimental conditions is not unique to cattle. We have used identical methods to isolate and culture cortical pieces from ovaries from baboon fetuses and obtained similar results (Wandji *et al.*, 1997). In addition the results of Hovatta *et al.* (1997) indicate that primordial follicles may activate in cultured slices of human ovarian tissue.

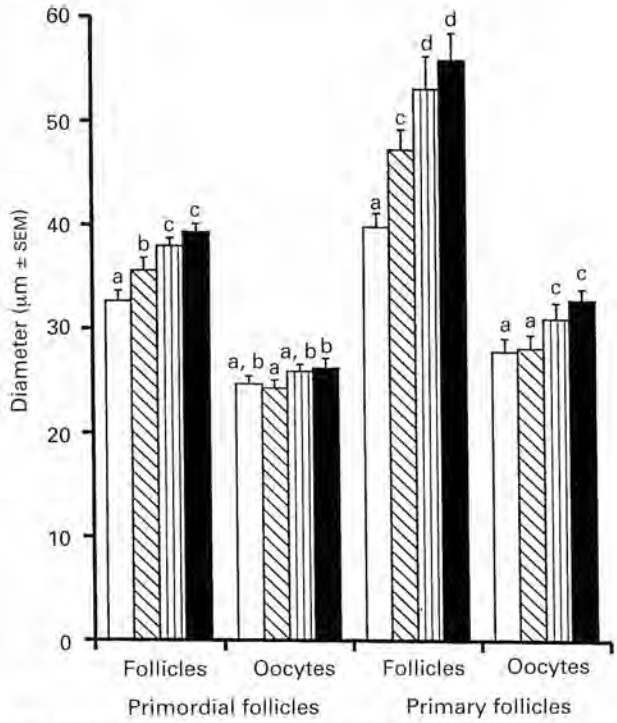

Fig. 2. Mean diameter (μm ± SEM) of healthy primordial and primary follicles and oocytes in pieces of fetal bovine ovarian cortex after 0 (□), 2 (▨), 4 (▥) or 7 (■) days in culture ($n = 4$ fetuses, with 107–204 primordial and 231–346 primary follicles/oocytes measured per fetus). Within each group of four bars, bars with no common superscript are significantly different (a,c,d $P < 0.01$; a,b $P < 0.05$). (Reprinted from Wandji *et al.*, 1996b, with permission.)

What Regulates the Activation of Primordial Follicles?

As discussed above, experiments to date with rodent, bovine and primate ovaries show that primordial follicles can activate in whole ovaries or ovarian cortical pieces maintained *in vitro* even, in the case of experiments with cattle and baboons, in serum-free medium. Thus, gonadotrophins or other blood-borne factors do not appear to be necessary for initiation of follicle growth. Of interest is the observation that in cultures of whole rodent ovaries only some of the primordial follicles initiate growth *in vitro*, as occurs *in vivo* (Eppig and O'Brien, 1996), whereas in isolated pieces of bovine or baboon ovarian cortex the vast majority of primordial follicles is activated (Wandji *et al.*, 1996b; Braw-Tal and Yossefi, 1997; Wandji *et al.*, 1997). It is therefore possible that the more central, medullary portion of the ovary regulates the flow of primordial follicles into the pool of growing preantral follicles by secreting an inhibitory factor(s) that keeps most primordial follicles quiescent. Alternatively, the artificial reduction in the size of the pool of primordial follicles in the cortical pieces may remove some inhibitory factor(s) that normally emanates from that compartment of the ovary. There is evidence that a higher percentage of primordial follicles becomes activated if the size of the resting pool is reduced (Krarup *et al.*, 1969; Hirshfield, 1994). Another possibility is that the conditions *in vitro* for cultured bovine and baboon cortical pieces are richer in some way(s) than their situation *in vivo*. For example, the ovarian cortex is known to be poorly vascularized (Guraya,

Table 1. Effect of culture and FSH (100 ng ml^{-1}) on development of bovine follicles *in vitro*

Day of culture (*n*)	FSH	Primordial follicles (% of total)	Primary or transitory follicles (% of total)	Preantral follicles (% of total)	Follicle diameter (mean ± SEM, μm)	Oocyte diameter (mean ± SEM, μm)	Number of granulosal cells*
0 (69)	–	50[a] (72.0)	17[a] (25.6)	2 (2.9)	37.94 ± 1.54[a]	28.50 ± 0.49[a]	9.81 ± 1.48[a]
2 (58)	–	6[b] (10.3)	50[b] (86.2)	2 (3.4)	50.83 ± 2.16[b]	27.91 ± 0.52[a]	11.47 ± 1.22[a]
2 (57)	+	3[b] (5.3)	53[b] (93.0)	1 (1.7)	50.77 ± 2.67[b]	27.91 ± 0.53[a]	10.84 ± 1.85[a]

Within columns, values with different superscripts are significantly different ($P < 0.05$).
n: number of non-atretic follicles examined.
*Largest cross-section of the follicle.
(Reprinted with permission from Braw-Tal and Yossefi, 1997).

1985; van Wezel and Rodgers, 1996), so primordial follicles may have better access to nutrients *in vitro* and/or a higher oxygen concentration than *in vivo*.

The only specific factor that has thus far been linked to the activation of primordial follicles is kit ligand (also called stem cell factor or steel factor). Kit ligand is produced by granulosal cells, whereas primordial germ cells, oocytes and theca cells express the receptor for kit ligand, c-kit (Manova *et al.*, 1993; Motro and Bernstein, 1993). Yoshida *et al.* (1997) injected mice with a function-blocking antibody to c-kit at various times during the first two weeks of life and concluded that kit ligand is needed for the activation of primordial follicles, but not for their formation. Parrott and Skinner (1997) reported that addition of kit ligand to cultures of rat ovaries induced primordial follicles to begin development, whereas an antibody that blocks the function of c-kit (the receptor for kit ligand) blocked spontaneous activation of primordial follicles. Although workers in our laboratory did not observe any effects of kit ligand on the activation of primordial follicles or subsequent growth of primary follicles (Wandji and Fortune, unpublished), in our experimental model (isolated cortex) almost all of the primordial follicles activate spontaneously. Hence further studies on the potential role of kit ligand and c-kit in the initiation of follicle growth will be of interest.

Can Follicular Growth be Maintained *In Vitro*?

The previous section summarized current limited knowledge of the signals that regulate the movement of primordial follicles into the growing pool. Clearly this question is of interest if we are to understand this critical first step in follicular growth and differentiation. However, determining factors and conditions that will maintain the growth of follicles once they have been activated *in vitro* is also of interest. In cultures of bovine or baboon ovarian cortex, growth beyond the primary stage was rare (Wandji *et al.*, 1996b, 1997), in contrast to the development of secondary follicles in organ cultures of whole newborn mouse or rat ovaries (Eppig and O'Brien, 1996; Mayerhofer *et al.*, 1997). Sustained follicular growth after activation of primordial follicles in ovarian cultures from larger mammals is a necessary step if the pool of primordial follicles is to provide oocytes that can be fertilized. The sections below discuss factors or conditions which may enhance the development of small preantral follicles *in vitro*.

Culture media

Recently we have conducted experiments to determine whether media containing fetal bovine serum (FBS) or a combination of FBS and ITS+ in various proportions would support the growth of

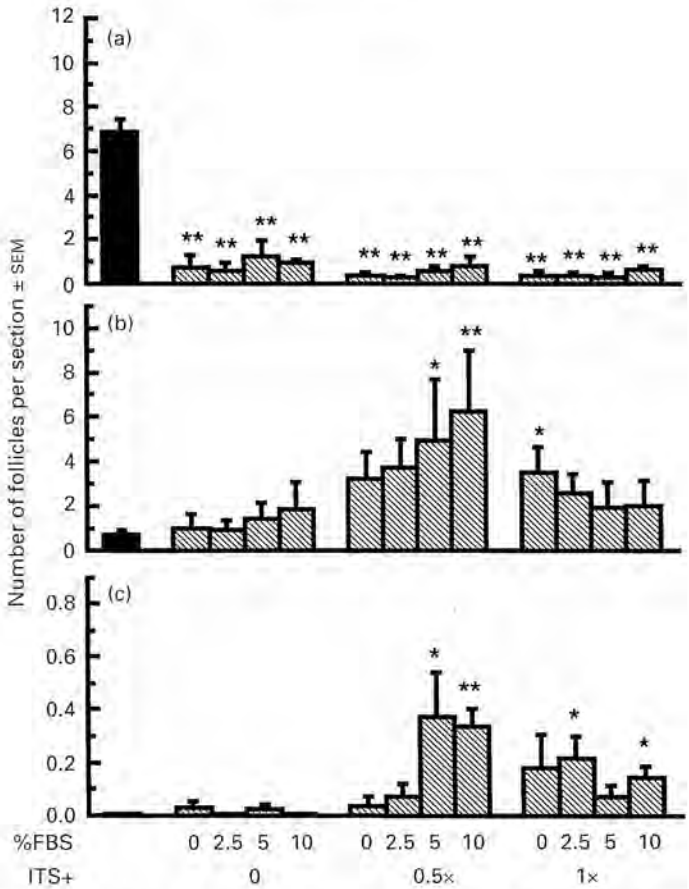

Fig. 3. Effects of culture in different media on numbers of primordial (a), primary (b) and secondary (c) follicles in pieces of fetal bovine ovarian cortex ($n = 4$ fetuses). Cortical pieces were fixed immediately after isolation (day 0 control; ■) or after 10 days of culture (▨) in 0, 2.5, 5, or 10% fetal bovine serum (FBS) in the presence or absence of full strength (1 ×) or half-strength (0.5 ×) ITS+ (insulin, transferrin, selenium, BSA and linoleic acid). Asterisks indicate significant differences from the day 0 control (*, $P < 0.05$; **, $P < 0.01$).

secondary follicles after activation of primordial follicles. Cortical pieces from four bovine fetuses were cultured for 10 days in Waymouth MB 752/1 medium containing 0, 2, 5 or 10% FBS in the presence or absence of half-strength ITS+ (0.5 × the normal concentration) or full-strength ITS+ (1×) and then subjected to histological morphometry and statistical analysis by methods described by Wandji *et al.* (1996b). The combination of 0.5 × ITS+ and 5% or 10% FBS was most effective, of the media tested, at supporting the growth of follicles to the primary and secondary stages (Fig.3). Surprisingly, Waymouth medium plus 10% FBS, which supports normal follicular development in newborn mouse ovaries (Eppig and O'Brien, 1996) and activates baboon oocytes without activating their granulosal cells (Wandji *et al.*, 1997), provided a very poor environment for the activation and growth of bovine follicles. These results indicate that the type of culture medium can markedly affect the growth of follicles after activation of primordial follicles and that the optimal medium conditions may vary from species to species.

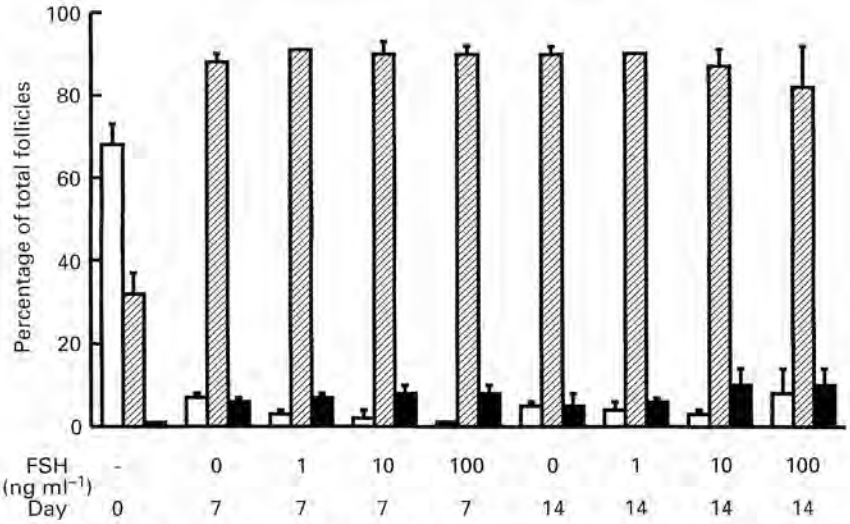

Fig. 4. Lack of effect of FSH on cultures of fetal bovine ovarian cortex. Bars indicate percentages (±SEM) of healthy follicles that were primordial (□), primary (▨), or early secondary (■) stage in freshly isolated tissue or after culture with graded doses of FSH (0, 1, 10 or 100 ng ml^{-1}) for 7 or 14 days (n = 4 cortical pieces, 2 from each of 2 fetuses; with 472–1232 follicles examined per treatment). (Reprinted from Fortune *et al.*, 1998 with permission.)

FSH

The development of preantral follicles in hypophysectomized mammals (Dufour *et al.*, 1979; Hirshfield, 1985) indicates that gonadotrophins are not absolutely required for follicular development until the antral stage. However, since hypophysectomy reduces the number of growing preantral follicles in sheep (Dufour *et al.*, 1979) and since bovine ovarian follicles bind FSH (Wandji *et al.*, 1992a) and ovine follicles express messenger RNA for the FSH receptor (Tisdall *et al.*, 1995) beginning with the primary stage, it is possible that FSH could facilitate the growth of bovine follicles *in vitro*, after the activation of primordial follicles. Braw-Tal and Yossefi (1997) cultured bovine cortical pieces from adult ovaries for 2 days in medium containing FSH (100 ng ml^{-1}, NIDDK oFSH-17) and found no effect on the distribution of follicles among the primordial, primary and preantral size classes (Table 1). To determine whether a longer period of exposure to FSH would induce newly activated follicles in cortical pieces from fetal bovine ovaries to develop to the secondary stage, we cultured pieces for 7 or 14 days with graded doses of FSH (0, 1, 10 or 100 ng ml^{-1}; NIDDK oFSH-17). No dose of FSH had a significant effect on the distribution of follicles among the primordial, primary and secondary size classes after either 7 or 14 days of culture (Fig. 4; Fortune *et al.*, 1998). These results are consistent with the suggestion of Wandji *et al.* (1992b) that the FSH receptors in fetal ovaries may not be linked to the adenylate cyclase second messenger system.

Mayerhofer *et al.* (1997) reported that treating neonatal rat ovaries, which contain only primordial follicles, with vasoactive intestinal peptide (VIP) or other agents that increase cAMP induced messenger RNA (mRNA) for FSH receptors. In addition, a short pretreatment with these agents induced the ovaries to become responsive to subsequent treatment with exogenous FSH in terms of cAMP secretion and in terms of follicular growth, which proceeded rapidly to the secondary stage in some follicles. To determine whether the lack of response of bovine cortical pieces to FSH that we (Fortune *et al.*, 1998) and Braw-Tal and Yossefi (1997) had observed could be reversed by previous exposure of cortical pieces to agents that increase cyclic AMP, we replicated the

Table 2. Cyclic AMP secretion (pg h^{-1} per culture ±SEM) by pieces of bovine ovarian cortex cultured for 32 h ($n = 6$ cultures, 2 from each of 3 fetuses)[a]

A. Effects of VIP or forskolin 0–8 h of culture		B. Effects of FSH (300 ng ml^{-1}) 8–32 h of culture	
Treatment	cAMP secretion	Treatment	cAMP secretion
Control	32 ± 15[b]	Control	22 ± 4[b]
	31 ± 9[b]	+ FSH	34 ± 9[b]
VIP	391 ± 42[c]	Control	180 ± 14[d]
(10 μmol l^{-1})	416 ± 81[c]	+ FSH	195 ± 14[d]
Forskolin	753 ± 71[c,d]	Control	124 ± 15[d]
(40 μmol l^{-1})	792 ± 108[d]	+ FSH	164 ± 38[d]

[a] Each culture contained four pieces of freshly isolated, fetal ovarian cortex (approximately 0.5 mm × 0.5 mm × 0.3 mm) on a membrane insert in 350 μl Waymouth MB containing ITS+ (insulin, transferrin, selenium, and BSA); medium also contained isobutyl methylxanthine, IBMX (0.5 mmol l^{-1}) to inhibit metabolism of cAMP.
[b,c,d] Within columns means with no common superscript are significantly different. b versus c or c versus d, $P < 0.05$; b versus d, $P < 0.01$.

experiment of Mayerhofer *et al.* (1997) by treating bovine cortical pieces with VIP or forskolin for 8 h, followed by treatment with FSH for 24 h (300 ng ml^{-1}; NIDDK oFSH-17). Media were collected and measured for cAMP by radioimmunoassay. Both VIP and forskolin increased the secretion of cAMP during the first 8 h of culture (Table 2). In addition, they continued to exert a 'carry-over' stimulatory effect during the next 24 h. However, FSH did not increase the secretion of cAMP whether or not tissue had been pretreated with VIP or forskolin. These results suggest that, at least under the conditions used, a short exposure to increased cAMP is not sufficient to induce functional FSH receptors and a cAMP response to FSH in bovine ovarian cortex, in contrast to the results for neonatal rat ovaries (Mayerhofer *et al.*, 1997). Therefore, it appears that the addition of FSH to cultures of bovine primary follicles grown *in vitro* is not useful. However, FSH does have effects on cultures of larger preantral follicles from mice (Eppig and O'Brien, 1996; Cortvrindt *et al.*, 1997). Therefore, if methods can be devised for growing bovine follicles to later preantral stages, FSH could then be tested for facilitation of further growth *in vitro* of larger preantral follicles.

Other factors: growth factors (GDF-9, bFGF), activin, WT1

Several hormones and growth factors have been implicated indirectly in early follicular development. It would be of interest to determine whether one or more of these agonists could stimulate the growth of follicles activated *in vitro* to the early secondary stage and beyond. Growth differentiation factor 9 (GDF-9), a member of the transforming growth factor-β superfamily, is of particular interest since mRNA for GDF-9 is found only in oocytes from the primary follicle stage through ovulation. In mice homozygous for a GDF-9 'knockout', follicles were activated but did not proceed beyond the primary stage (Dong *et al.*, 1996), indicating that production of GDF-9 by the oocyte is critical for follicular development after the primary stage. Basic fibroblast growth factor (bFGF) is also of interest. Van Wezel *et al.* (1995) immunolocalized bFGF to bovine primordial and primary oocytes and suggested a role for this growth factor in stimulating granulosal cell proliferation. This hypothesis is consistent with experiments that showed that the binding of radiolabelled bFGF to bovine follicle cells is highest in the preantral stages, including primary follicles (Wandji *et al.*, 1992c) and that bFGF stimulates thymidine incorporation into bovine granulosal cells of preantral follicles *in vitro* (Wandji *et al.*, 1996a).

Activin A markedly stimulated the growth of preantral follicles (100–120 μm in diameter) and synergized with FSH when follicles were obtained from immature mice, but not adult mice (Yokota *et al.*, 1997). Tisdall *et al.* (1995) detected mRNA for β_B inhibin (needed for synthesis of activin B) as

early as the primary stage of ovine follicular development. Finally Wilms' tumour gene, WT1, is a gene deleted in some Wilms' tumours that codes for a transcription factor; its mRNA is expressed strongly in the early stages of rat preantral follicular development (Hsu *et al.*, 1995). What role(s) activin or WT1 may play in early follicular development remains to be elucidated.

Conclusions

Primordial follicles of rodents, cattle and primates can be activated *in vitro* to begin growth. In the experiments conducted thus far, the culture of intact whole ovaries results in the activation of an approximately normal number of primordial follicles and some of them grow to the multilayered secondary stage within a few days to a week of culture (Eppig and O'Brien, 1996; Mayerhofer *et al.*, 1997). In contrast, in isolated pieces of ovarian cortex from cattle and primates most primordial follicles initiate growth, but few follicles proceed to the secondary stage (Wandji *et al.*, 1996b, 1997; Fortune *et al.*, 1998). These findings raise interesting questions about what regulates the activation of primordial follicles *in vivo*. Although preliminary evidence implicates kit ligand as a stimulator of primordial follicle activation (Parrott and Skinner, 1997), the results also suggest that an inhibitor(s) is involved. The development of conditions *in vitro* that will allow the development of ruminant and primate follicles activated *in vitro* to a size where they might be isolated for further culture is essential to achieve the goal of producing embryos from the reservoir of primordial follicles. A number of laboratories have cultured larger preantral follicles (for review see van den Hurk *et al.*, 1997) and their experience will be helpful in developing culture strategies for large preantral follicles grown *in vitro*, if methods can be devised for getting them to that stage. Thus far one live mouse has been produced from a primordial follicle (Eppig and O'Brien, 1996). This shows that it is possible to use the primordial pool as a source of oocytes to produce embryos, but the difficulties of doing so will be much greater in ruminants and primates because of the larger size and longer developmental period of their oocytes. Some progress has been made towards the goal, but much more remains to be done. However, recent reports of follicle growth *in vitro* or production of live offspring after cryopreservation of rodent or human ovarian tissue (Carroll and Gosden, 1993; Hovatta *et al.*, 1997; Sztein *et al.*, 1998) indicate that the 'banking' of frozen ovarian tissue from valuable domestic animals, endangered species or women scheduled for radiation or chemotherapy, coupled with the ability to produce embryos from primordial follicles, would provide a powerful method for enhancing fertility in these groups.

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References

Blandau RJ, Warrick E and Rumery RE (1965) *In vitro* cultivation of fetal mouse ovaries *Fertility and Sterility* **16** 705–715

Braw-Tal R and Yossefi S (1997) Studies *in vivo* and *in vitro* on the initiation of follicle growth in the bovine ovary *Journal of Reproduction and Fertility* **109** 165–171

Carroll J and Gosden RG (1993) Transplantation of frozen–thawed mouse primordial follicles *Human Reproduction* **8** 1163–1167

Cortvrindt R, Smitz J and Van Steirteghem AC (1997) Assessment of the need for follicle stimulating hormone in early preantral mouse follicle culture *in vitro*. *Human Reproduction* **12** 759–768

Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N and Matzuk MM (1996) Growth differentiation factor-9 is required during early ovarian folliculogenesis *Nature* **383** 531–535

Dufour J, Cahill LP and Mauleon P (1979) Short- and long-term effects of hypophysectomy and unilateral ovariectomy on ovarian follicular populations in sheep *Journal of Reproduction and Fertility* **57** 301–309

Eppig JJ and O'Brien MJ (1996) Development *in vitro* of mouse oocytes from primordial follicles *Biology of Reproduction* **54** 197–207

Fortune JE, Kito S, Wandji S-A and Srsen V (1998) Activation of bovine and baboon primordial follicles *in vitro*. *Theriogenology* **49** 441–449

Guraya SS (1985) Primordial follicle. In *Biology of Ovarian Follicles in Mammals* pp 3–14 Springer-Verlag, New York

Henricson B and Rajakoski E (1959) Studies of oocytogenesis in cattle *Cornell Veterinarian* **49** 494–503

Hirshfield AN (1985) Comparison of granulosa cell proliferation in small follicles of hypophysectomized, prepubertal, and mature rats *Biology of Reproduction* **32** 979–987

Hirshfield AN (1991) Development of follicles in the mammalian ovary *International Review of Cytology* **124** 43–101

Hirshfield AN (1994) Relationship between the supply of primordial follicles and the onset of follicular growth in rats *Biology of Reproduction* **50** 421–428

Hovatta O, Silye R, Abir R, Krausz T and Winston RML (1997) Extracellular matrix improves survival of both stored and fresh human primordial and primary ovarian follicles in long-term culture *Human Reproduction* **12** 1032–1036

Hsu SY, Kubo M, Chun S-Y, Haluska FG, Housman DE and Hsueh AJW (1995) Wilms' tumor protein WT1 as an ovarian transcription factor: decreases in expression during follicle development and repression of inhibin-α gene promoter *Molecular Endocrinology* **9** 1356–1366

Krarup T, Pedersen T and Faber M (1969) Regulation of oocyte growth in the mouse ovary *Nature* **224** 187–188

Manova K, Huang EJ, Angeles M, De Leon V, Sanchez S, Pronovost SM, Besmer P and Bachvarova RF (1993) The expression pattern of the *c-kit* ligand in gonads of mice supports a role for the *c-kit* receptor in oocyte growth and in proliferation of spermatogonia *Developmental Biology* **157** 85–99

Mayerhofer A, Dissen GA, Costa ME and Ojeda SR (1997) A role for neurotransmitters in early follicular development: induction of functional follicle-stimulating hormone receptors in newly formed follicles of the rat ovary *Endocrinology* **138** 3320–3329

Motro B and Bernstein A (1993) Dynamic changes in ovarian *c-kit* and *Steel* expression during the estrous reproductive cycle *Developmental Dynamics* **197** 69–79

Parrott JA and Skinner MK (1997) Theca cell–granulosa cell interactions that induce primordial follicle development and promote folliculogenesis *Biology of Reproduction* **56**, Supplement 1 125

Russe I (1983) Oogenesis in cattle and sheep *Bibliotheca Anatomica* **24** 77–92

Sztein J, Sweet H, Farley J and Mobraaten L (1998) Cryopreservation and orthotopic transplantation of mouse ovaries: new approach in gamete banking *Biology of Reproduction* **58** 1071–1074

Tisdall DJ, Smith P, Leeuwenberg B and McNatty KP (1995) FSH-receptor, b_B inhibin subunit, follistatin, β_A and α inhibin subunits and IGF-I genes are expressed sequentially in ovine granulosa cells during early follicular development *Journal of Reproduction and Fertility Abstract Series* **15** Abstract 28

van den Hurk R, Bevers MM and Beckers JF (1997) *In-vivo* and *in-vitro* development of preantral follicles *Theriogenology* **47** 73–82

van Wagenen G and Simpson ME (1965) *Embryology of the Ovary and Testis: Homo sapiens and Macaca mulatta* Yale University Press, New Haven

van Wezel IL and Rodgers RJ (1996) Morphological characterization of bovine primordial follicles and their environment *in vivo*. *Biology of Reproduction* **55** 1003–1011

van Wezel IL, Umapathysivam K, Tilley WD and Rodgers RJ (1995) Immunohistochemical localization of basic fibroblast growth factor in bovine ovarian follicles *Molecular and Cellular Endocrinology* **115** 133–140

Wandji S-A, Pelletier G and Sirard M-A (1992a) Ontogeny and cellular localization of ^{125}I-labeled insulin-like growth factor-I, ^{125}I-labeled follicle-stimulating hormone, and ^{125}I-labeled human chorionic gonadotropin binding sites in ovaries from bovine fetuses and neonatal calves *Biology of Reproduction* **47** 814–822

Wandji S-A, Fortier MA and Sirard M-A (1992b) Differential response to gonadotropins and prostaglandin E_2 in ovarian tissue during prenatal and postnatal development in cattle *Biology of Reproduction* **46** 1034–1041

Wandji S-A, Pelletier G and Sirard M-A (1992c) Ontogeny and cellular localization of ^{125}I-labeled basic fibroblast growth factor and ^{125}I-labeled epidermal growth factor binding sites in ovaries from bovine fetuses and neonatal calves *Biology of Reproduction* **47** 807–813

Wandji S-A, Eppig JJ and Fortune JE (1996a) FSH and growth factors affect the growth and endocrine function *in vitro* of granulosa cells of bovine preantral follicles *Theriogenology* **45** 817–832

Wandji S-A, Srsen V, Voss AK, Eppig JJ and Fortune JE (1996b) Initiation *in vitro* of growth of bovine primordial follicles *Biology of Reproduction* **55** 942–948

Wandji S-A, Srsen V, Nathanielsz PW, Eppig JJ and Fortune JE (1997) Initiation of growth of baboon primordial follicles *in vitro*. *Human Reproduction* **12** 1993–2001

Yokota H, Yamada K, Liu X, Kobayashi J, Abe Y, Mizunuma H and Ibuki Y (1997) Paradoxical action of activin A on folliculogenesis in immature and adult mice *Endocrinology* **138** 4572–4576

Yoshida H, Takakura N, Kataoka H, Kunisada T, Okamura H and Nishikawa S-I (1997) Stepwise requirement of c-kit tyrosine kinase in mouse ovarian follicle development *Developmental Biology* **184** 122–137