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Developmental capacity of mouse oocytes matured *in vitro*: effects of gonadotrophic stimulation, follicular origin and oocyte size

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Summary. Development of mammalian oocytes is usually correlated with ovarian follicular development. This correlation was tested by determining whether gonadotrophic stimulation of follicular development in immature mice resulted in a coordinated increase in the embryonic developmental capacity of the oocytes. Oocyte cumulus cell complexes were isolated at the germinal vesicle stage from small, medium and large antral follicles of 26-day-old mice and matured and fertilized *in vitro*. The frequency with which embryos from oocytes from small follicles completed the two-cell to blastocyst transition was lower than for embryos from oocytes from large follicles (33% and 79%, respectively). Germinal-vesicle stage oocyte–cumulus cell complexes were isolated from 22–26-day-old mice that were unprimed or primed by injection of equine chorionic gonadotrophin 48 h before isolation. Oocytes were matured in control medium, or in medium containing 1 μg follicle-stimulating hormone (FSH) ml^{-1}, and then fertilized *in vitro*. Priming did not increase the number of embryos completing the two-cell stage to blastocyst transition in the 22-day-old group nor did FSH treatment of maturing oocytes when the oocytes were isolated from unprimed 22-day-old mice. In contrast, priming increased the percentage of embryos completing the two-cell stage to blastocyst transition in the 26-day-old group by 20%. FSH treatment of maturing oocytes from the unprimed, 26-day-old group increased the number of embryos completing the transition to the same level as those in the primed 26-day-old group, but FSH did not increase the frequency of transition in the primed 26-day-old group. The developmental capacity of eggs superovulated by 18- and 22-day-old mice was 34% and 10% less, respectively, than in the 26-day-old group. Germinal vesicle stage oocytes of the same size were grouped after isolation from primed 20- and 26-day-old mice and were matured and fertilized *in vitro*. Two-cell stage embryos derived from oocytes isolated from 26-day-old mice developed to the blastocyst stage more frequently than embryos derived from 20-day-old mice, despite the fact that the oocytes were the same size. It is concluded that oocytes of the same size can be qualitatively different in a way that is manifested in their capacity to complete the two-cell stage to blastocyst transition. Furthermore, the preimplantation developmental capacity of mouse oocytes is affected by (i) the size of the follicle and (ii) stimulation of follicular development *in vivo* by exogenous gonadotrophins. The ability of FSH to increase the developmental capacity of oocytes maturing *in vitro* varied depending on the age of the mice and prior gonadotrophin priming *in vivo*. The processes of gonadotrophin-stimulated follicular and oocyte development are, therefore, separable.

Keywords: oocyte maturation; preimplantation development; follicular development; mouse

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Introduction

The development of mammalian oocytes is generally correlated with the development of follicular somatic cells. The initiation of oocyte growth usually coincides with a thickening of the granulosa cell layer, and continued oocyte growth occurs concurrently with proliferation of granulosa cells in preantral follicles (Brambell, 1928). Follicular antrum formation occurs about the time that oocytes near completion of their growth and acquire the capacity to resume the first meiotic division (Brambell, 1928; Szybec, 1972; Erickson & Sorensen, 1974; Sorensen & Wassarman, 1976). Oocytes can achieve advanced phases of maturation sequentially, and this development is also correlated, at least temporally, with the progression of follicular development (Sorensen & Wassarman, 1976; Eppig & Schroeder, 1989). Oocytes of many mammalian species undergo gonadotrophin-induced maturation just before ovulation.

Development of ovarian follicles in mice begins shortly after birth. A large group of follicles develops almost synchronously in neonatal and juvenile mice (Peters, 1969), so oocytes and follicles at increasing stages of development can be isolated from the ovaries of mice until they are about 1 month old (Szybec, 1972; Mangia & Epstein, 1975; Sorensen & Wassarman, 1976). Follicular antrum formation occurs in mice when they are approximately 15 days old and this coincides with the acquisition of the capacity of oocytes to undergo germinal vesicle breakdown upon liberation from the follicles and culture in a supportive medium (Szybec, 1972; Sorensen & Wassarman, 1976; Eppig & Schroeder, 1989). Many oocytes isolated at this stage, however, failed to progress through metaphase I and produce a polar body. An increased frequency of polar body production was observed when the oocytes were isolated from mice that were a few days older (Sorensen & Wassarman, 1976; Eppig & Schroeder, 1989). Acquisition of capacities to undergo germinal vesicle breakdown and subsequently complete meiosis I are, therefore, experimentally separable and sequential events in oocytes.

Oocytes from unprimed immature mice that underwent spontaneous maturation *in vitro* were fertilized and underwent embryonic development with frequencies that depended on the age of the mice (Eppig & Schroeder, 1989). There was no difference in the frequency of germinal vesicle breakdown or polar body production when oocytes were cultured after isolation from 20–26-day-old mice, nor was there a difference in the frequency of cleavage to the two-cell stage after insemination. However, there was a difference in the frequency at which the embryos completed the two-cell stage to blastocyst transition; only 30% of the two-cell stage embryos derived from oocytes isolated from 22-day-old mice developed to blastocysts in contrast with 55% of the two-cell stage embryos derived from oocytes isolated from 26-day-old mice (Eppig & Schroeder, 1989). The capacity of oocytes to complete preimplantation development after maturation and insemination *in vitro* is, therefore, acquired after they develop the capacity to complete meiosis I and to cleave to the two-cell stage.

Administration of exogenous gonadotrophins promotes rapid ovarian follicular development and superovulation in both adult and juvenile mammals. This protocol has been used in clinical, agricultural and experimental applications to increase the number of ova available for fertilization. Experiments were designed to determine whether exogenous gonadotrophin treatment can differentially affect follicular and oocyte development. Oocytes were isolated from the antral follicles of immature mice of various ages, with, or without, prior stimulation by exogenous gonadotrophin, matured and inseminated *in vitro*, and their capacity to complete preimplantation development *in vitro* was assessed. In addition, the effect of FSH treatment of the maturing oocytes isolated from primed and unprimed mice of the two ages was determined. The results show that gonadotrophin priming of mice and FSH treatment of maturing oocytes have differential effects on the preimplantation developmental capacity of the oocytes that depend on the age of the mice. Follicular and oocyte development are, therefore, separable processes. In addition, it is demonstrated that the developmental capacity of oocytes depends upon the size of the follicle from which the oocytes were isolated but not strictly upon the size of the oocyte.

Materials and Methods

Ovaries were removed from immature (C57BL/6J × SJL/J)F_1 20–26-day-old mice, some which were injected 48 h earlier with 5 iu of equine chorionic gonadotrophin (eCG; Diosynth, Chicago, IL 60618, USA). Mice injected with eCG will be referred to as primed and those not injected as unprimed. Oocyte–cumulus-cell complexes from all groups were simultaneously isolated, matured and fertilized, and embryos developed *in vitro* as described by Eppig *et al.* (1990). Groups of randomly selected oocytes were matured in medium containing 1 μg ml FSH ml^{-1} (ovine FSH-17). Some primed animals were given an i.p. injection of 5 iu of human chorionic gonadotrophin (hCG; Sigma Chemical Co., PO Box 14508, St Louis, MO 63178, USA) to induce ovulation and eggs were removed from the oviducts 15 h later. After insemination, the zygotes were washed and cultured in Whitten's medium (Whitten, 1971) as described previously (Schroeder & Eppig, 1984) and the number of eggs that cleaved to the two-cell stage was determined after 24 h culture. The two-cell stage embryos were cultured for a further 96 h, and the number of expanded blastocyst stage embryos was determined. Groups were compared by χ^2 analysis with Yates' correction. A P value equal to, or less than, 0·05 was considered significant.

To determine the proportion of antral follicles in various size groups, we dissected ovaries so that the diameter of individual antral follicles in ovarian clumps could be measured. All of the antral follicles in randomly selected clumps from three mice were measured using a Wild M5A stereomicroscope equipped with an ocular micrometer. A total of 80 follicles was measured for each group. Because the diameters of all the antral follicles in the ovaries were not measured, this method may not exactly describe the profile of the follicle size distribution in whole ovaries. Nevertheless, the method does allow an approximation of the profiles in the four groups of ovaries.

To obtain oocytes from follicles of various sizes, we dissected follicles individually from the ovaries of unprimed 26-day-old mice and divided them into small (299 ± 25 μm (mean ± SD); range 240–320 μm), medium (375 ± 20 μm; 360–400 μm) and large (445 ± 14 μm; 440–480 μm) sizes. The diameter of the follicles was then measured with the aid of an ocular micrometer and the oocytes were isolated, matured and fertilized as described previously (Eppig *et al.*, 1990).

To obtain oocytes of specific sizes, we isolated oocyte–cumulus-cell complexes from the ovaries of 20- and 26-day-old mice that had been primed with eCG 48 h earlier. Individual oocyte–cumulus-cell complexes were placed in 25 μl drops of medium under washed paraffin oil (Schroeder & Eppig, 1984). The diameter of each oocyte was then measured with an ocular micrometer. The oocytes underwent maturation in medium not containing FSH and the ova were collected and grouped according to size for fertilization and embryonic development using the previously described protocols (Eppig *et al.*, 1990).

Results

Effect of eCG-priming on the developmental capacity of oocytes matured *in vitro*

Priming stimulated follicular development in mice of both ages as shown by (i) the presence of follicles in the 500–600 μm category (proportionally, 15% of total; Fig. 1) and (ii) a two-fold increase in ovarian weight at both ages (not shown), and (iii) the recovery of approximately three times as many oocyte–cumulus-cell complexes from primed mice as from unprimed mice of both ages, as described by Schroeder & Eppig (1989).

The frequency of cleavage of ova to the two-cell stage was high in all groups, approximately 80 to 90%, after maturation and fertilization *in vitro* (Fig. 2a). Priming had no effect on the capacity of two-cell stage embryos derived from oocytes isolated from 22-day-old mice to develop to blastocysts; development to the blastocyst stage was approximately 40% in both cases. In contrast, the frequency of development of two-cell stage embryos to the blastocyst stage was 21% higher ($P < 0·01$) when the oocytes were isolated from primed 26-day-old mice compared with the unprimed group of the same age. Most of the embryos that failed to complete the two-cell stage to blastocyst transition failed to develop significantly beyond the two- to four-cell stage (data not shown).

Effect of FSH on the developmental capacity of oocytes undergoing maturation *in vitro*

Treatment of maturing oocytes with FSH slightly increased the frequency ($P < 0·05$) of two-cell stage to blastocyst development in the primed, but not the unprimed, 22-day-old group. In

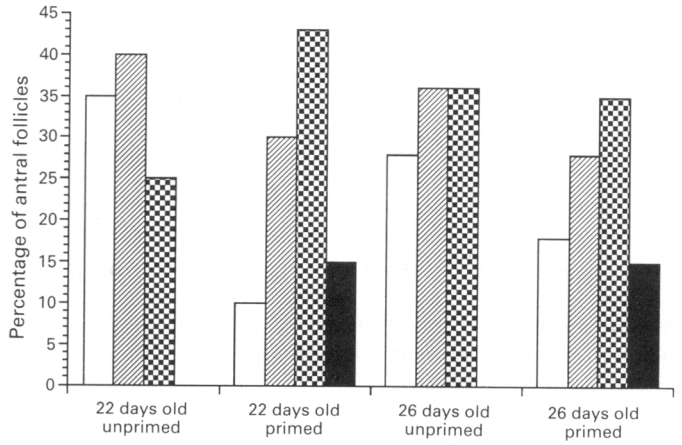

Fig. 1. Effect of equine chorionic gonadotrophin (eCG)-priming on the size distribution of antral follicles in 22- and 26-day-old mice. Mice were primed with eCG on days 20 and 24, respectively. The percentage of antral follicles in four size categories, (□) 200–300 μm, (▨) 300–400 μm, (▩) 400–500 μm and (■) 500–600 μm is presented.

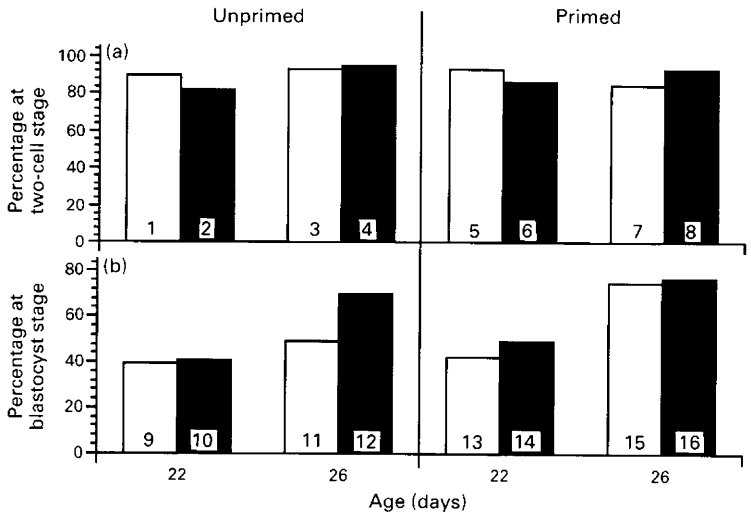

Fig. 2. Developmental capacity of oocytes isolated at the germinal-vesicle stage and matured *in vitro*. Cumulus-cell-enclosed oocytes were isolated from either unprimed or eCG-primed mice at 22 and 26 days of age. They were matured in medium containing 1 μg ml^{-1} FSH (■) or in control (□) medium, inseminated, and the embryos were cultured *in vitro*: (a) percentage of ova that cleaved to the two-cell stage and (b) percentage of the two-cell stage embryos that developed to blastocysts. Data presented on the histogram were pooled from four experiments each with about 75 oocytes per group. The numbers at the base of the bars in the histogram correspond to the numbers that follow to indicate the percentages for each individual experiment. (1) 89,97,89,86; (2) 76,84,84,85; (3) 93,97,83,98; (4) 97,87,97,97; (5) 95,90,92,94; (6) 95,96,86,75; (7) 94,67,97,90; (8) 98,90,92,92; (9) 38,43,44,35; (10) 45,43,39,39; (11) 45,47,54,50; (12) 66,77,65,77; (13) 22,50,42,42; (14) 29,57,58,54; (15) 65,77,76,73; (16) 66,78,90,77.

contrast, FSH treatment of maturing oocytes greatly increased the frequency ($P < 0{\cdot}01$) of two-cell stage to blastocyst development in the unprimed 26-day-old mice (50–70%), but not at all in the primed 26-day-old group (Fig. 2b). Treatment of maturing oocytes isolated from 22-day-old primed mice increased the frequency of two-cell stage to blastocyst development so that it was the same as that for the 26-day-old unprimed group, but this was 25% lower ($P < 0{\cdot}01$) than the FSH-treated 26-day-old unprimed group, and 19% and 29% less ($P < 0{\cdot}05$ and 0·01 respectively) than control and FSH-treated 26-day-old groups respectively (Fig. 2b).

Comparison of the developmental capacity of ovulated eggs in 18-, 22- and 26-day-old mice

The developmental competence of *in vitro* matured mouse oocytes therefore appears to vary depending upon the age and gonadotrophic stimulation of the donor of the oocytes. We then determined whether this result was germane only to mechanically isolated and *in vitro* matured oocytes or whether a similar result would be observed for oocytes selected by hormonal stimulation and induced to mature *in vivo*. Eggs from 18-, 22- and 26-day-old eCG-primed mice that were injected with hCG were removed from the ampulla of the oviduct and, after insemination *in vitro*, 85–90% of the ova cleaved to the two-cell stage in all three groups. In contrast, development of the two-cell stage embryos to blastocysts was 15% higher ($P < 0{\cdot}05$) in the 26-day-old group than in the 22-day-old group, and 34% higher ($P < 0{\cdot}01$) than in the 18-day-old group (Fig. 3).

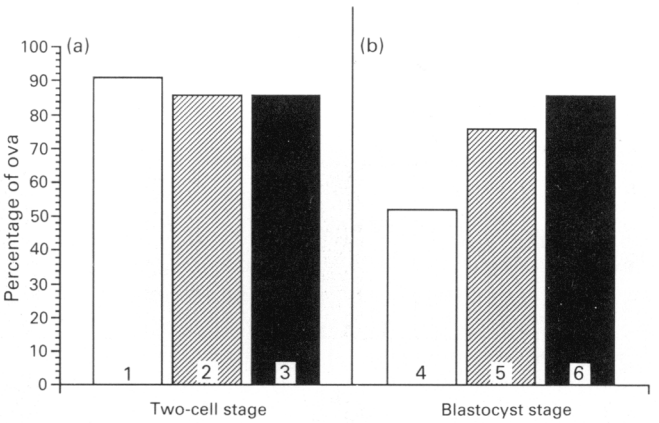

Fig. 3. Developmental capacity of ova recovered from the oviducts of 18- (□), 22- (▨), and 26-day-old (■) mice after superovulation. The ova were inseminated and embryos cultured *in vitro*. (a) Percentage of ova that cleaved to the two-cell stage and (b) percentage of the two-cell stage embryos that developed to blastocysts. Data presented on the histogram were pooled from three experiments each with about 75 oocytes per group. The numbers at the base of the bars in the histogram correspond to the numbers that follow to indicate the percentages for each individual experiment. (1) 95,90,83; (2) 93,93,74; (3) 88,86,84; (4) 48,54,54; (5) 76,72,78; (6) 87,89,84.

Developmental capacity of *in vitro* matured oocytes isolated from follicles of different sizes

The oocyte–cumulus-cell complexes were isolated from small, medium and large follicles, and matured and fertilized *in vitro* as described above. The developmental capacity of the *in vitro* matured ova differed depending on the size of the follicles (Fig. 4). The ability of the ova to cleave to the two-cell stage increased with increasing size of the follicle. The frequency of two-cell stage

embryo development to blastocysts was much lower ($P < 0{\cdot}01$) for the small follicle group than for the medium and large follicle groups.

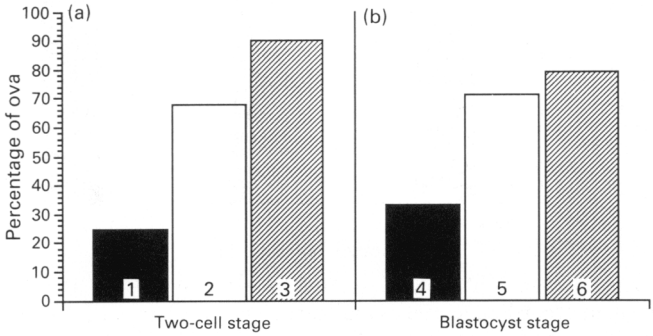

Fig. 4. Developmental capacity of oocytes isolated at the germinal vesicle stage and matured *in vitro*. Cumulus-cell-enclosed oocytes were isolated from small (■), medium (□), and large (▨) follicles of 26-day-old mice. They were matured, inseminated, and embryos cultured *in vitro*: (a) percentage of ova that cleaved to the two-cell stage and (b) percentage of the two-cell stage embryos that developed to blastocysts. The experiment was conducted four times; the total number of oocytes in each group was 36, 28, and 50 from the small, medium and large follicles, respectively and the pooled data are shown. The numbers at the base of the bars in the histogram correspond to the numbers that follow to indicate the percentages for each individual experiment. (1) 25,50,0,22; (2) 33,68,56,80; (3) 100,100,69,95; (4) 25,50,0,0; (5) 75,100,0,63; (6) 86,67,89,74.

Developmental capacity of *in vitro* matured oocytes of the same size isolated from 20- and 26-day-old mice

Oocytes in the same size groups (72·5–77·5 μm, 77·5–82·5 μm and 82·5–87·5 μm) were isolated from medium to large size follicles of 20- and 26-day-old mice, matured, fertilized, and the embryos were cultured *in vitro*. There was no statistically significant difference ($P > 0{\cdot}05$) in the percentage of embryos from oocytes in the 72·5–77·5 μm group from 20- and 26-day-old mice that completed the two-cell stage to blastocyst transition (Fig. 5). In contrast, more embryos from the 26-day-old group of 77·5–82·5 and 82·5–87·5 μm oocytes ($P < 0{\cdot}05$ and 0·01, respectively) completed the two-cell stage to blastocyst transition than embryos from oocytes of the same respective size groups from 20-day-old mice (Fig. 5). In embryos in the 20-day-old group, there was no difference in the frequency of two-cell stage to blastocyst development among any of the size groups. In contrast, embryos from oocytes of the largest size group isolated from 26-day-old mice completed the two-cell to blastocyst transition at a higher frequency (100%) compared with embryos from the smaller oocytes isolated at this age ($P < 0{\cdot}01$).

Discussion

The immature mouse ovary was used as a model system for assessing the effects of (i) gonadotrophic stimulation *in vivo* and *in vitro*, and (ii) follicle and oocyte size on the developmental capacity of oocytes. It was shown previously (Eppig & Schroeder, 1989) that oocytes isolated from the antral follicles of unprimed 16–28-day-old mice differ in their developmental capacity. Although *in vitro* matured oocytes isolated from 20–28-day-old mice cleaved to the two-cell stage at the same frequency after insemination *in vitro*, the frequency with which the embryos completed the two-cell stage to blastocyst transition was much greater in embryos derived from oocytes

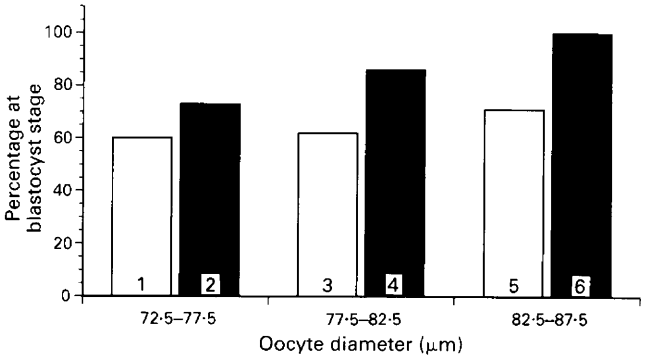

Fig. 5. Developmental capacity of oocytes isolated from the ovaries of 20- (□) and 26-day-old (■) mice and grouped according to size as indicated. The percentage of two-cell stage embryos that developed to blastocysts is presented. The experiment was conducted four times and the results were pooled for presentation. The total number of two-cell embryos derived from oocytes isolated from 20- and 26-day-old mice was 52 and 45 (72·5–77·75 μm), 34 and 49 (77·5–82·5 μm), and 49 and 29 (82·5–87·5 μm) respectively. The numbers at the base of the bars in the histogram correspond to the numbers that follow to indicate the percentages for each individual experiment. (1) 54,67,50,65; (2) 67,100,71,45; (3) 29,71,73,67; (4) 72,78,100,100; (5) 55,64,80,100; (6) 100,100,100,100.

isolated from the older mice (Eppig & Schroeder, 1989). This observation indicated that oocytes sequentially acquire the capacity to develop to more advanced stages of preimplantation embryo development and that this acquisition is correlated with advancing follicular development. The antral follicles present in the ovaries of immature mice advance in size and development with age (Peters, 1969). Similarly, the average size of oocytes isolated from the antral follicles increases with age (Eppig & Schroeder, 1989). Results presented here indicate that the frequency with which embryos derived from oocytes that mature *in vitro* complete the two-cell stage to blastocyst transition is differentially affected by (i) stimulation of follicular development *in vivo* by exogenous gonadotrophins, and (ii) stimulation of the maturing oocytes *in vitro* by FSH, depending on the age of the oocyte donor. In addition, the developmental capacity of oocytes was shown to depend upon the size of the follicle from which the oocytes were isolated, but not strictly upon the size of the oocyte.

When mice were primed with eCG 48 h before oocyte isolation from 22- and 26-day-old mice, a dramatic increase in follicular growth was stimulated in both groups. An increased number of embryos derived from the 26-day-old group completed the two-cell stage to blastocyst transition. Thus, even though follicular development was accelerated by priming in the 22-day-old group so that the frequency distribution of various size groups of antral follicles was the same as in primed 26-day-old mice, oocyte development in the younger group was not coordinately accelerated. The developmental programmes of oocytes and follicles are, therefore, experimentally separable. Gonadotrophin-priming of immature rats also increased the frequency of *in vitro* fertilization after *in vitro* maturation (Zhang & Armstrong, 1989). These results suggest that gonadotrophins can accelerate oocyte development, but only in the more developed follicles.

FSH treatment of maturing oocytes has been reported to increase the frequency of embryos completing the two-cell stage to blastocyst transition (Downs *et al.*, 1986; Schroeder *et al.*, 1988). These studies used oocytes isolated from primed 22–24-day-old mice. This was confirmed in our experiments using oocytes from primed 22-day-old mice and unprimed 26-day-old mice. In contrast, FSH did not have this effect on oocytes isolated from unprimed 22-day-old mice or from primed 26-day-old mice. Thus the action of FSH on the developmental capacity of maturing oocytes probably depends both on the size of the follicle donating the oocyte and on prior gonadotrophin priming *in vivo*.

FSH increased the developmental capacity of maturing oocytes isolated from primed 22-day-old mice to the same level as that of oocytes isolated from unprimed 26-day-old mice, but not to the same extent as that of oocytes from primed 26-day-old mice or FSH-treated oocytes from unprimed 26-day-old mice. FSH treatment of maturing oocytes did not increase the frequency of the two-cell stage to blastocyst transition above that of the primed 26-day-old group, but FSH treatment increased the developmental capacity of maturing oocytes isolated from unprimed 26-day-old mice to the same level as oocytes from the primed 26-day-old group. Thus FSH treatment of maturing oocytes isolated from unprimed 26-day-old mice appeared to accelerate the acquisition of capacity to complete the two-cell stage to blastocyst transition of oocytes to a level equivalent to the 26-day-old primed group. Although both priming of 26-day-old mice and FSH treatment of maturing oocytes from unprimed mice of this group appeared to have the same effect, it is not known whether these effects are mediated by the same mechanisms. It is important to note that action in the case of priming is on germinal-vesicle stage oocytes, whereas, in the case of FSH treatment *in vitro*, the action is on maturing oocytes.

FSH had no effect on the developmental capacity of oocytes isolated from unprimed 22-day-old mice. Nevertheless, FSH stimulates an increase of cAMP levels and cumulus expansion by these cumuli oophori thus showing that they can respond to FSH (data not shown). However, it remains to be determined whether the action of gonadotrophins on oocyte developmental capacity is based on the ability of the somatic cells to signal the oocyte or oocyte response to that signal, or both. The oocyte may even be the direct target of FSH since FSH receptors have been reported in hamster oocytes (Oxberry & Greenwald, 1982).

The developmental capacity of *in vitro* matured mouse oocytes varied depending upon the age of the donor of the oocytes. This difference in developmental capacity was also found when the oocytes were matured *in vivo* and recovered from oviducts after superovulation. This observation supports the hypothesis that hormonal stimulation of accelerated follicular development does not necessarily promote accelerated development of oocytes and supports the idea that follicle and oocyte development can be separated experimentally.

The developmental capacity of oocytes increased with increasing follicular size and similar results have been reported using *in vitro* matured bovine oocytes (Tan & Lu, 1990). There was a follicle-size-dependent difference both in the frequency of cleavage to the two-cell stage and in completion of the two-cell to blastocyst transition. Thus the developmental capacity of the oocytes from the group of small follicles of 26-day-old mice was similar to that of oocytes from the largest follicles of 18-day-old mice (Eppig & Schroeder, 1989).

The oocytes isolated from the antral follicles of 26-day-old mice were larger than those isolated from the antral follicles of 20-day-old mice (Eppig & Schroeder, 1989). It is possible, therefore, that the increased developmental capacity of oocytes isolated from the 26-day-old mice compared with those isolated from younger mice may simply be due to the larger size of the oocytes. Nevertheless, oocytes isolated from 20-day-old mice completed the two-cell stage to blastocyst transition much less frequently than oocytes of the same size isolated from 26-day-old mice. Oocytes of the same size can therefore be qualitatively different in a way that is manifested in their capacity to complete the two-cell stage to blastocyst transition. These qualitative differences in the oocytes of the same size appear related to the physiological and developmental status of the oocyte donor. Nevertheless, the largest oocytes obtained from 26-day-old mice showed the highest frequency of completion of the two-cell stage to blastocyst transition. Thus, even though oocyte size is not the sole correlate of the best developmental capacity, larger oocytes do exhibit a greater developmental capacity when they are isolated from the most advanced follicles.

The most dramatic effects seen were not in the ability of the ova to become fertilized and cleave to the two-cell stage after insemination, but in the ability of the two-cell stage embryos to develop to the blastocyst stage. Thus processes involved in oocyte development, both before and during oocyte maturation, are directly related to their capacity to complete the two-cell stage to blastocyst transition. The ability of gonadotrophins to affect this developmental process in oocytes depends

on the stage of follicular development. The nature of the follicular response to gonadotrophins that affects the developmental programme of oocytes and the specific changes that they bring about in the oocytes must be resolved to identify and characterize the maternal factors essential for successful early embryonic development.

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