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Initiation of growth of baboon primordial follicles *in vitro*

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Factors that cause some primordial follicles to enter the growth phase while the others remain quiescent are unknown. The hypothesis was tested that primate primordial follicles can survive and initiate growth *in vitro* in serum-free medium. Superficial pieces of ovarian cortex, containing mostly primordial follicles, were obtained from baboon fetuses during late gestation and cultured for 0, 2, 4, 7, 10 or 20 days in Waymouth MB 752/1 medium supplemented with insulin, transferrin, selenium, linoleic acid, and bovine serum albumin (ITS+). Histological examination of cortical pieces revealed that after 2 and 4 days in culture, the total number of primordial follicles had decreased by 55 and 76% ($P < 0.01$) respectively, relative to day 0 of culture. This was associated with a sustained, 5- to 8-fold increase in total primary follicles ($P < 0.01$) beginning on day 2 of culture. There was also a gradual increase in the total number of early secondary and secondary follicles. The average diameter of follicles and oocytes increased gradually throughout culture for all follicular categories ($P < 0.01$), except secondary follicles and oocytes. Immunohistochemical localization of proliferating cell nuclear antigen (PCNA), a marker for cell proliferation and growth, showed that PCNA was generally absent in primordial follicles on day 0, but was observed after 2 or 4 days in culture in both granulosa cells and oocytes of most growing follicles. Comparison of cortical pieces cultured for 10 or 20 days with ITS+ versus 10% fetal bovine serum (FBS) showed a more pronounced decrease in the numbers of primordial follicles and more primary, early secondary and secondary follicles in ITS+ compared to FBS-treated cortical pieces ($P < 0.01$ at 20 days). These results show that primordial follicles from non-human primates can survive and develop to the secondary stage *in vitro* in serum-free conditions.

Key words: baboon/follicle/oocytes/ovary/primordial follicles

Introduction

Mammalian ovaries contain thousands of primordial follicles which will be the only source of gametes during the entire reproductive life of the female. In primates, primordial follicles, consisting of an oocyte surrounded by a single layer of flattened, pre-granulosa cells, begin to form during the last half of gestation (van Wagenen and Simpson, 1965). Beginning in fetal life, primordial follicles progressively leave the resting stage to enter the growth phase. The architecture of the ovary shows that primordial follicles are located in the outermost part of the ovarian cortex, an almost avascular area, whereas growing follicles occupy the innermost part of the cortex which has a rich vascular supply (Guraya, 1985; Koering *et al.*, 1991). Primordial follicles are a potentially valuable source of oocytes for assisted reproduction in humans, domestic animals and endangered species.

Although nothing is known at this point about the mechanisms that regulate the onset of primordial follicle growth, activation of primordial follicles *in vitro* has been achieved in mice (Blandau *et al.*, 1965; Eppig and O'Brien, 1996) and cattle (Wandji *et al.*, 1996; Braw-Tal and Yossefi, 1997). The objective of the present study was to induce primate primordial follicles to initiate growth *in vitro*; results from such experiments might ultimately be extrapolated to the culture of primordial follicles from human adult ovaries for oocyte donor programmes. Ovaries from late gestational baboon fetuses were used since they contain large numbers of primordial follicles. Isolated pieces of ovarian cortex, containing mostly primordial follicles, were cultured in either serum-free or serum-containing media in the absence of gonadotrophins, since there is no evidence that primordial follicles have gonadotrophin receptors. Histological morphometry and immunohistochemical localization of proliferating cell nuclear antigen (PCNA), a marker of cell proliferation and growth (Liu *et al.*, 1989), were used to assess follicular activation and growth *in vitro*.

Materials and methods

Animals and culture methods

Baboon fetuses were obtained by Caesarean section near term (around day 160 of gestation). Fetal ovaries were collected within 5 min of death and placed in Leibovitz' L-15 medium (Gibco BRL Life Technologies, Inc., Grand Island, NY, USA) supplemented with 1% fetal bovine serum (FBS), 50 units penicillin/ml and 50 μg streptomycin/ml (Gibco). Ligaments and hili were dissected out and the ovaries were cut transversely into thin slices ~1 mm thick. The slices were transferred to Waymouth MB 752/1 medium [(Way); Sigma Chemical Co., St Louis, MO, USA] supplemented with

25 μg/ml pyruvic acid, sodium salt (Sigma), ITS+ [6.25 μg insulin, 6.25 μg transferrin, 6.25 ng selenous acid, 1.25 mg bovine serum albumin (BSA) and 5.35 μg linoleic acid/ml; Collaborative Biomedical Products, Becton Dickinson Labware, Bedford, MA], 50 μg/ml streptomycin sulphate, 75 μg/ml penicillin-G (both from Sigma), and 25 ng/ml fungizone (Gibco) for dissection of superficial pieces of ovarian cortex, which began within 30 min after the ovaries were obtained. Ovarian cortical pieces ~1.0×0.5×0.5 mm (length× width×thickness), containing mostly primordial follicles, were dissected away from the stroma in the ovarian slices with fine forceps under a dissecting microscope and transferred onto uncoated culture plate inserts (four pieces per insert; Millicell-CM, 0.4 μm pore size; Millipore Co., Bedford, MA, USA) in individual wells of a 24-well culture dish. The culture inserts had been equilibrated previously for 2 h with 300 μl of culture medium in a humidified incubator gassed with 5% CO_2 in air, at 37°C. The volume of the culture medium was adjusted so that the pieces of ovarian cortex were covered by a very thin film of culture medium generated by surface tension (Eppig and O'Brien, 1996). Every other day, 200 μl of culture medium were replaced with fresh medium.

In a preliminary experiment, pieces of ovarian cortex from one fetus were cultured in the medium described above (Way/ITS+) for 0 or 10 days and subjected to histological analysis as described below. Since primordial follicles had initiated growth during 10 days of culture, three additional fetuses were used to analyse follicular development after 0, 2, 4, 7, 10 or 20 days of culture in Way/ITS+. To determine if serum affects the initiation of follicle growth, cortical pieces from these three fetuses were also cultured for 10 or 20 days in the medium described above, but supplemented with 10% FBS instead of ITS+.

Assessment of follicular and oocyte survival and growth

On day 0 of culture, representative pieces of ovarian cortex were fixed and embedded in LR White plastic (EMS, Fort Washington, PA, USA) and 2 μm thick sections were cut as described previously (Wandji *et al.*, 1996) to serve as uncultured controls. After 10 days ($n = 4$ fetuses) or 2, 4, 7 or 20 days of culture ($n = 3$ fetuses), pieces of ovarian cortex from one culture (four pieces/fetus/day of culture) were fixed and processed in LR White plastic. Alternate sets of ten consecutive sections were mounted on gelatin-treated slides and stained with toluidine blue. Under the microscope, the largest cross-section, i.e. the one that showed the largest number of oocytes with a visible nucleolus, was identified. In the largest cross-section, only follicles with a visible oocyte nucleus or condensed chromatin (atretic follicles) were counted. Follicular and oocyte diameters were measured only for non-atretic follicles with a visible oocyte nucleolus. Follicular and oocyte diameters were measured using the NIH Image analysis program (NIH Image) as described earlier (Wandji *et al.*, 1996). The basement membrane surrounding the granulosa cell layer was considered to be the outer boundary of the follicle and, since most follicles were not perfectly spherical, both the largest and smallest diameters of each follicle were measured and these measurements were averaged.

Follicles were classified according to stage of development as either primordial (one layer of flattened somatic cells around the oocyte), early primary (unilaminar, with both flattened and cuboidal cells), primary (one layer of completely cuboidal cells), early secondary (incomplete second layer of granulosa cells around the oocyte) or secondary (two complete layers of granulosa cells around the oocyte). Follicles were also classified as either healthy (intact basal lamina, oocyte with no more than three cytoplasmic vacuoles, intact germinal vesicle and nucleolus) or atretic. Follicles considered to be in early stages of atresia had oocytes with more than three cytoplasmic vacuoles and slight chromatin condensation. Later stages of atresia were characterized by pronounced condensation of the oocyte chromatin or fragmentation of the oocyte cytoplasm and nucleus. Many primordial and primary follicles with a degenerating oocyte had no (or only a few) pyknotic granulosa cells, and therefore the condition of granulosa cells was considered of secondary relevance in assessing atresia.

Immunohistochemistry

Some ovarian cortical pieces (four pieces/fetus/day of culture) from three fetuses were fixed in Bouin's solution (45 min) on day 0, 2 or 4 of culture, and 4 μm paraffin sections were stained for PCNA immunohistochemistry as described previously (Wandji *et al.*, 1996).

Statistical analysis

Mean numbers of healthy and atretic primordial, early primary, primary, early secondary, and secondary follicles and mean diameters of healthy follicles and oocytes were calculated for each fetus at each time *in vitro*. The averages of these means were calculated for each time of culture and medium type (ITS+ versus FBS) and are presented in the figures. The data (means for individual fetuses) for each class of follicle (primordial, etc.) were subjected to separate analyses of variance (ANOVA) using a randomized complete block design to test the effects of treatments (i.e. time in culture and/or type of culture medium). If Hartley's test indicated heterogeneity of variance, data were log-transformed before ANOVA. Following ANOVA, differences among individual means were tested using Duncan's multiple range test.

Results

Effects of culture on the survival and distribution of follicles in the growing and resting pools

On day 0, pieces of baboon ovarian cortex contained mostly primordial and only a few primary follicles (Figure 1A), whereas on day 10 of culture the pieces contained dramatically fewer primordial follicles but many more primary follicles (Figure 1C). After 10 days of culture, there was an 82% decrease in the total number of primordial follicles ($P < 0.01$) and a 7-fold increase in the total number of primary follicles ($P < 0.05$) relative to the values on day 0 of culture (Figure 2). During the same time period, there was also an 83% decrease and a 9.5-fold increase in the number of healthy primordial and primary follicles, respectively, relative to day 0 ($P < 0.01$ and $P < 0.025$, respectively, data not shown). The number of early secondary follicles also increased between day 0 and day 10 of culture ($P < 0.01$, Figure 2).

Analysis of more frequent time points during culture showed that after 2 or 4 days in culture, the total number of primordial follicles had decreased by 55 and 76% ($P < 0.01$), respectively, relative to day 0 of culture (Figures 1A,B and 3). This was correlated with a transient 3.3-fold increase in the total number of early primary follicles on day 2 ($P < 0.01$) and a sustained 5- to 8-fold increase in total primary follicles ($P < 0.01$) beginning on day 2 (Figure 3). There was a gradual increase in early secondary ($P < 0.01$) and secondary follicles ($P < 0.05$) during the culture period (Figures 1 and 3). Changes in the numbers of healthy and atretic follicles with time in culture generally mirrored the changes in total follicle number (Figure 4).

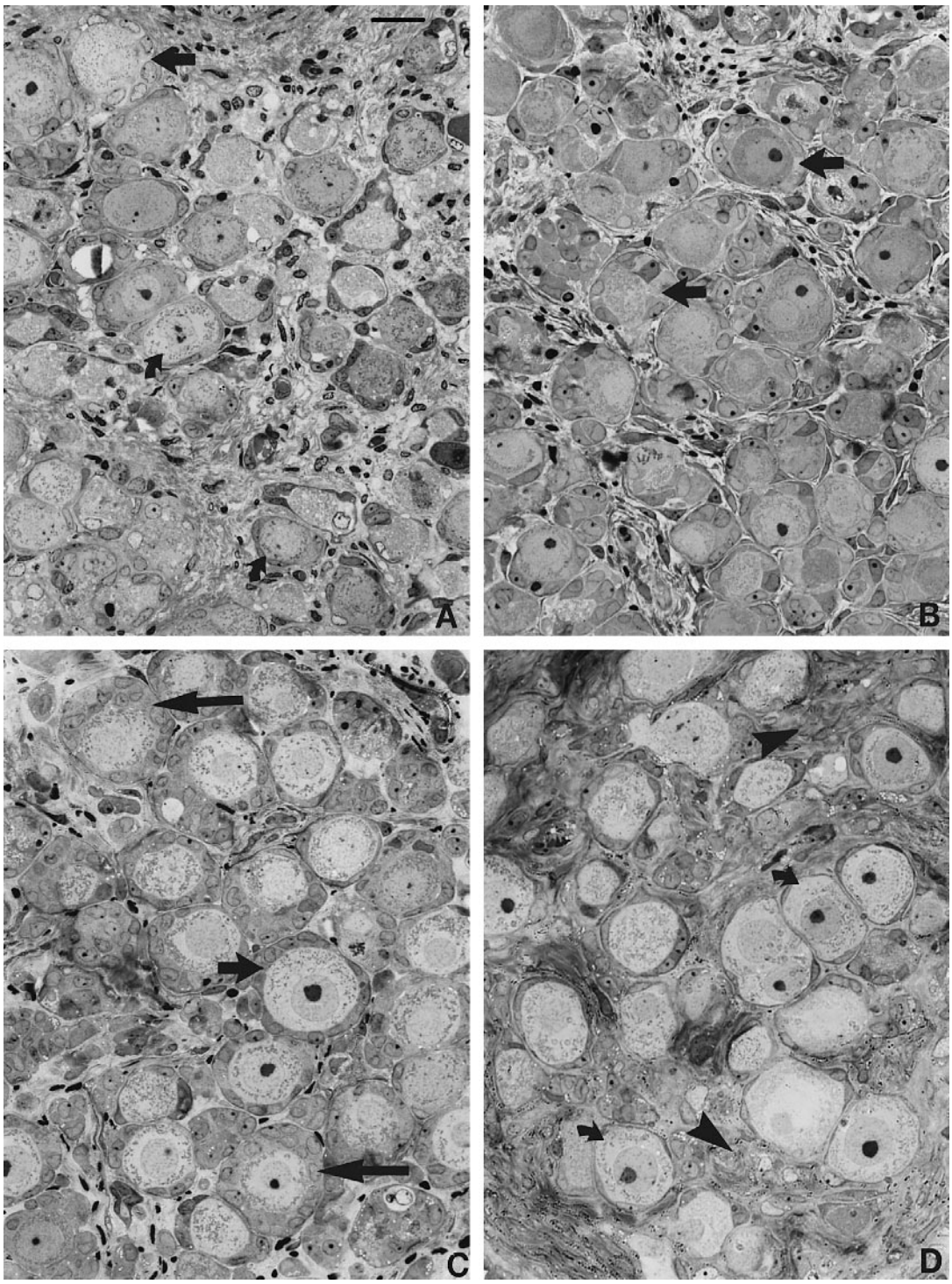

Figure 1. Effects of organ culture on the morphology of pieces of ovarian cortex from fetal baboons. (**A**) Control, uncultured, ovarian cortical pieces containing predominantly primordial follicles, such as those indicated by the curved arrows. Occasionally, primary follicles, such as that indicated by the arrow, were also observed. (**B**) Ovarian cortex cultured for 2 days in medium supplemented with ITS+ (for composition see text). Almost all follicles appear to have been activated to become primary follicles, such as those indicated by the arrows. (**C**) Ovarian cortex cultured for 10 days in medium supplemented with ITS+. In addition to the many primary follicles, such as those indicated by the smaller arrow, some early secondary follicles (large arrows) were also observed. (**D**) Ovarian cortex cultured for 10 days in medium supplemented with 10% fetal bovine serum (FBS). Most of the follicles appear to have remained at the primordial stage (curved arrows), although oocyte growth occurred. Moreover, it appears that FBS promoted the hyperplasia of fibroblastic extrafollicular cells (arrow heads). The scale bar in the upper right corner of panel **A** represents 25 μm; all photomicrographs are the same magnification.

The average percentage of atretic follicles in freshly isolated and cultured pieces was 14 and 21% for primordial follicles, 36 and 27% for early primary follicles and 37 and 14% for primary follicles, respectively. A very low percentage of early secondary and secondary follicles was atretic (5 and 0.04% respectively).

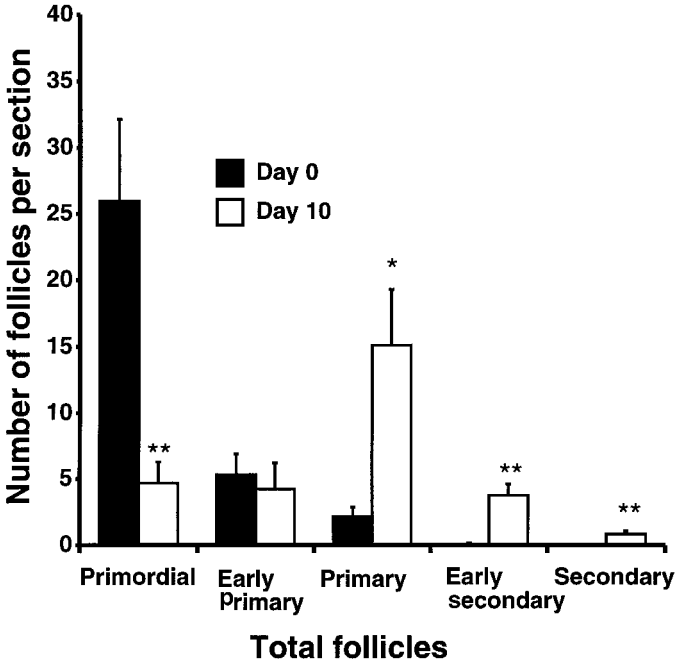

Figure 2. Distribution of the total number of follicles (mean per histological section ± SEM, $n = 4$ fetuses, with 49–72 sections examined per day of culture) in pieces of baboon ovarian cortex after 0 or 10 days of culture in the presence of ITS+ (for composition see text). Within each follicular stage, bars with asterisks are different from day 0 (*$P < 0.05$; **$P < 0.01$).

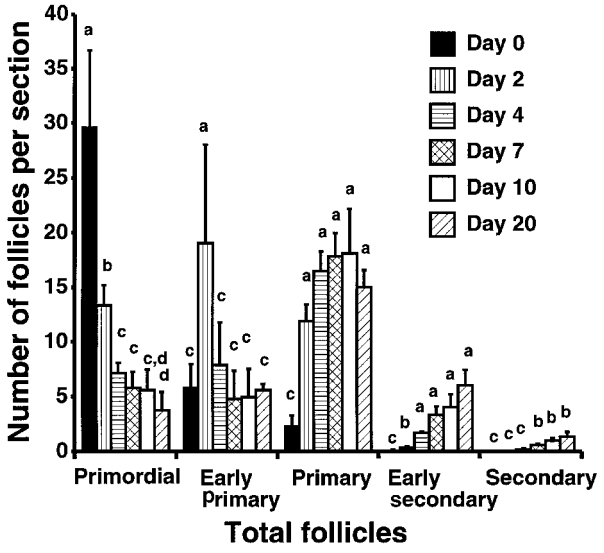

Figure 3. Distribution of the total number of follicles (mean per histological section ± SEM, $n = 3$ fetuses, with 36–54 sections examined per day of culture) in pieces of baboon ovarian cortex after 0, 2, 4, 7, 10 or 20 days of culture in the presence of ITS+ (for composition see text). Within each follicular stage, bars with no common superscript are different: a>b, b>c, c>d, $P < 0.05$; a>c, a>d, b>d, $P < 0.01$.

Effects of culture on the diameter of follicles and oocytes in the growing and resting pools

In medium supplemented with ITS+, there were significant increases ($P < 0.01$) in the diameters of primordial, early primary and primary follicles compared to day 0 values (Figure 5). The increase in diameter of primordial follicles was first

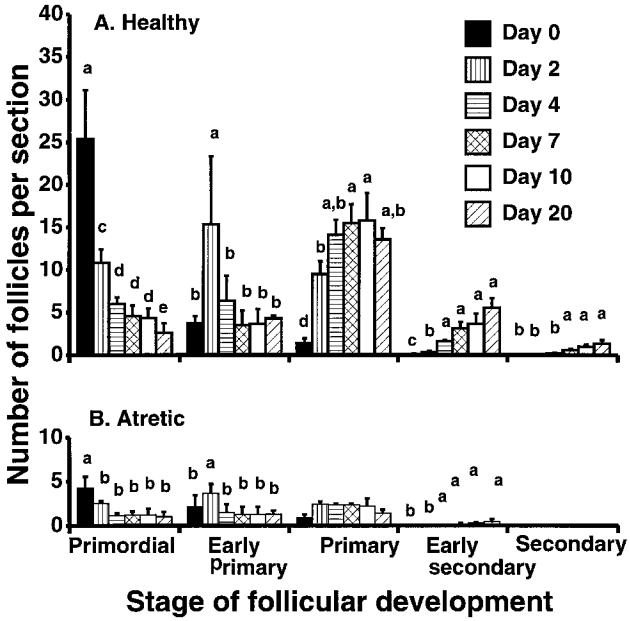

Figure 4. Numbers of healthy (**A**) and atretic (**B**) follicles (mean per histological section ± SEM, $n = 3$ fetuses, with 36–54 sections examined per day of culture) in pieces of baboon ovarian cortex after 0, 2, 4, 7, 10 or 20 days in culture in the presence of ITS+ (for composition see text). Within each follicular stage, bars with no common superscript are different: a>b, b>c, c>d, $P < 0.05$; a>c, a>d, a>e, c>e, $P < 0.01$.

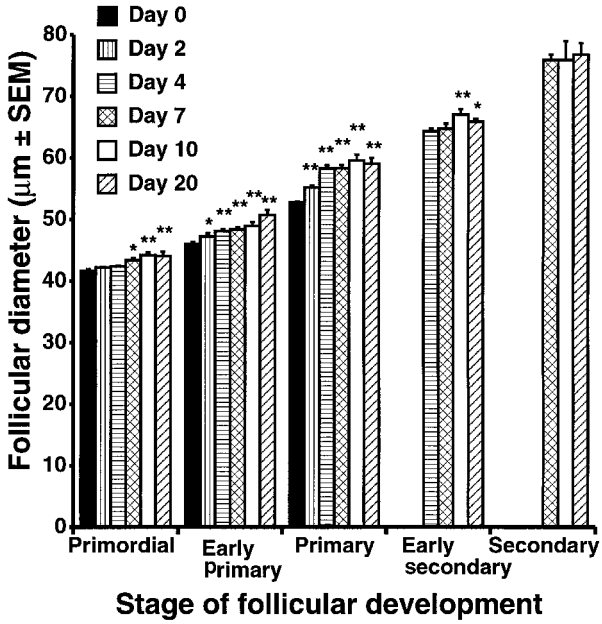

Figure 5. Diameter (μm; mean ± SEM) of healthy follicles in pieces of baboon ovarian cortex after 0, 2, 4, 7, 10 or 20 days in culture in the presence of ITS+ (for composition see text) ($n = 3$ fetuses, with 200–252 primordial, 191–264 early primary, 209–236 primary, 84–94 early secondary and 29–40 secondary follicles/oocytes measured per fetus). Within each follicular stage, bars with asterisks are different from day 0 (*$P < 0.05$; **$P < 0.01$).

significant on day 7 of culture ($P < 0.05$; days 10 and 20, $P < 0.01$, Figure 5). Follicular diameter increased as early as day 2 of culture in early primary follicles ($P < 0.05$, day 2; $P < 0.01$, days 4, 7, 10 and 20) and primary follicles ($P < 0.01$,

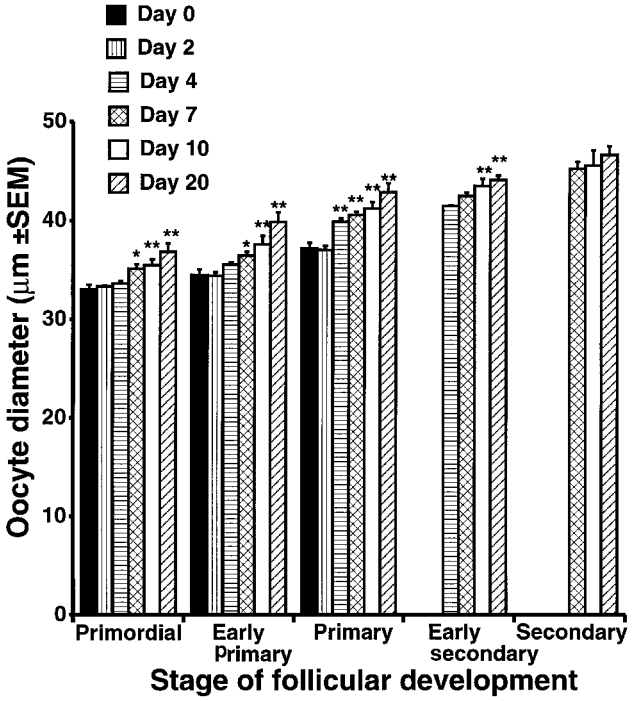

Figure 6. Diameter of oocytes (μm; mean ± SEM) in healthy follicles in pieces of baboon ovarian cortex after 0, 2, 4, 7, 10 or 20 days in culture in the presence of ITS+ (for composition see text) (n = 3 fetuses, with 200–252 primordial, 191–264 early primary, 209–236 primary, 84–94 early secondary and 29–40 secondary follicles/oocytes measured per fetus). Within each follicular stage, bars with asterisks are different from day 0 (*$P < 0.05$; **$P < 0.01$).

Figure 5). The diameter of early secondary follicles, which appeared by day 4 of culture, also increased by day 10 of culture ($P < 0.01$), whereas there was no substantial increase in the diameter of secondary follicles (Figure 5).

In the presence of ITS+, there was an increase in the diameter of oocytes in primordial and early primary follicles beginning on day 7 of culture ($P < 0.05$, day 7; $P < 0.01$, days 10 and 20), whereas the diameter of oocytes in primary follicles increased as early as day 4 ($P < 0.01$, Figure 6). There was also an increase in the diameter of oocytes in early secondary follicles ($P < 0.01$), but not secondary follicles (Figure 6).

Effects of culture on immunohistochemical localization of PCNA in ovarian cortical pieces

Moderate PCNA staining was present in granulosa cells and oocytes of the few growing follicles present on day 0 of culture, whereas primordial follicles were generally PCNA-negative (Figure 7A). In a few primordial follicles, positive PCNA staining could be seen in both the flattened pre-granulosa cells and the oocyte (Figure 7A). After 2 or 4 days of culture in ITS+, intense PCNA staining was localized to granulosa cells and oocytes of most growing follicles (Figure 7B).

Effects of serum-free (ITS+) versus serum-containing medium on follicle and oocyte growth

After 20 days in culture, there was a more pronounced decrease in the mean number of primordial follicles in ITS+ compared to FBS-treated pieces of ovarian cortex ($P < 0.05$, Figures 1 and 8). Moreover, the numbers of primary, early secondary and secondary follicles were greater in ITS+ than in FBS-treated cultures ($P < 0.01$) after 10 and 20 days (Figures 1C,1D and 8). The differences in the total follicle populations (Figure 8) were also observed when only healthy follicles were considered (Figure 9). In addition, more interstitial tissue surrounded follicles in cortical pieces cultured with FBS compared to ITS+ (Figure 1C,D). Although this difference was not quantified morphometrically, it was observed consistently.

FBS caused a greater increase than ITS+ in the diameter of primordial follicles and their oocytes after 10 or 20 days in culture ($P < 0.05$), but the difference between the two treatments was less pronounced in early primary and absent in primary follicles (Figures 1C,1D, 10 and 11). FBS appeared to stimulate the growth of oocytes in many primordial follicles without promoting the growth of surrounding pre-granulosa cells (Figure 1D).

Discussion

Results presented here indicate that primate primordial follicles can be induced to initiate growth *in vitro*. First, activation of primordial follicles, as indicated by the precipitous decrease in the number of primordial follicles after only 2 days in culture, occurred concomitantly with a transient increase in the number of early primary follicles. Second, the observation that there was a sustained increase in the number of primary and early secondary follicles beginning on days 2 and 4, respectively, demonstrates the ability of these activated follicles to continue growing. Third, the expression of a marker of cell proliferation, PCNA, increased in oocytes and granulosa cells coincident with follicular activation.

The ability of the activated follicles to grow in cultures treated with ITS+ was demonstrated by increased follicular diameters in all follicular categories, except in secondary follicles. The increase in the diameter of primordial follicles may be accounted for by increases in the size of the oocyte, whereas increases in the diameters of early primary, primary and early secondary follicles were due to both oocyte and follicular growth. That proliferation of granulosa cells contributed to the increase in follicular diameter is demonstrated by their intense PCNA immunoreactivity. PCNA expression was shown to be associated with early follicular growth in rats (Oktay *et al.*, 1995) and cattle (Wandji *et al.*, 1996). The localization of immunoreactive PCNA to the oocyte of growing follicles in the current study is also in accordance with other reports (Oktay *et al.*, 1995; Wandji *et al.*, 1996). The increase in PCNA staining in the oocyte could be due to DNA repair (Downey *et al.*, 1990) during the intense RNA transcription that occurs in growing oocytes (Lintern-Moore and Moore, 1979).

In the ovary, the quiescent primordial follicles are located in the outermost part of the cortex, a poorly vascularized area, whereas the primordial follicles that show signs of growth are always found in the cortico-medullary border which is richly vascularized (Guraya, 1985; Koering *et al.*, 1991; van Wezel and Rodgers, 1996). It would appear reasonable, therefore, to suggest that primordial follicles depend on some nutrients,

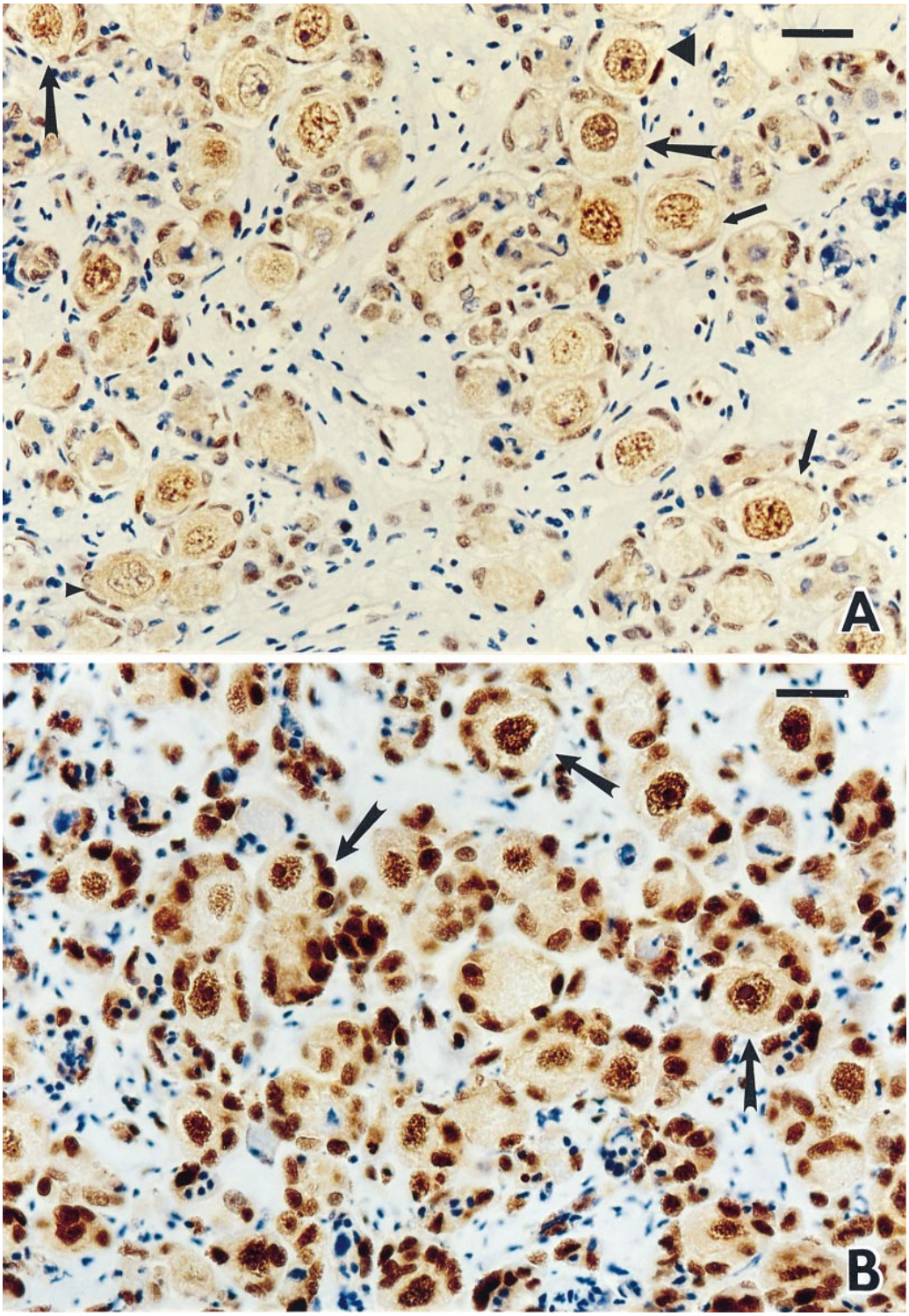

Figure 7. Immunohistochemical localization of proliferating cell nuclear antigen (PCNA) in pieces of ovarian cortex. Ovarian cortical pieces from three fetuses were fixed in Bouin's solution at time 0 or after 4 days of culture with ITS+ (for composition see text), dehydrated, embedded in paraffin, sectioned at 4 μm and processed for immunohistochemistry. Sections were stained with haematoxylin. (**A**) Day 0 of culture; moderate PCNA staining is present in granulosa cells and oocytes of the few growing follicles present (large arrows). PCNA immunoreactivity in pre-granulosa cells of most primordial follicles is weak or absent (small arrows). Occasionally, a moderate PCNA signal is present in pre-granulosa cells and oocytes of primordial follicles (arrow heads). (**B**) Day 4 of culture; most follicles have initiated growth as indicated by the cuboidal shape of granulosa cells; this coincides with strong PCNA staining in granulosa cells and oocytes of most growing follicles (arrows). The bars represent 25 μm.

hormones and/or growth factors for their activation to grow. In the current study, the culture of isolated pieces of ovarian cortex could have increased access to nutrients in the medium of primordial follicles relative to their access *in vivo*. The observation that the vast majority of these primordial follicles grew in culture may suggest that the addition of ITS+ to our

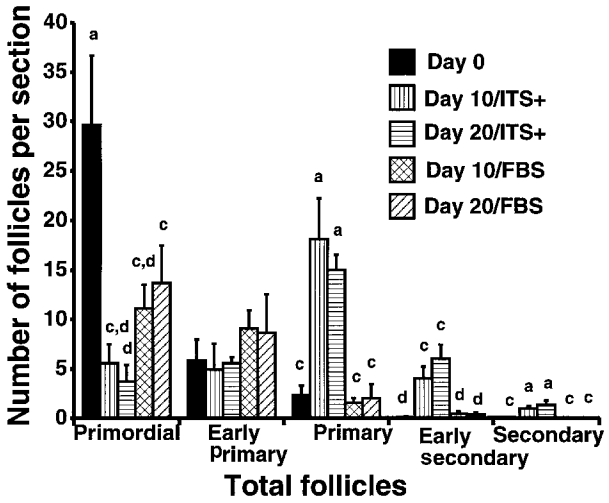

Figure 8. Distribution of the total number of follicles (mean per histological section ± SEM, $n = 3$ fetuses, with 36–54 sections examined per day of culture) in pieces of baboon ovarian cortex after 0, 10 or 20 days in culture in the presence of ITS+ (for composition see text) or 10% fetal bovine serum (FBS). Within each follicular stage, bars with no common superscript are different: c>d, $P < 0.05$; a>c, a>d, $P < 0.01$.

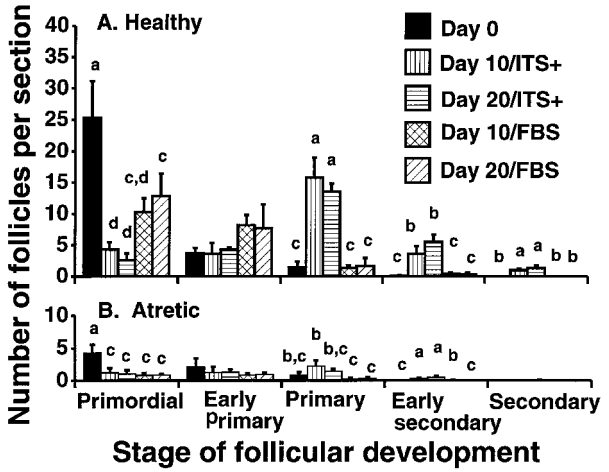

Figure 9. Numbers of healthy (**A**) and atretic (**B**) follicles (mean per histological section ± SEM, $n = 3$ fetuses, with 36–54 sections examined per day of culture) in pieces of baboon ovarian cortex after 0, 10 or 20 days in culture in the presence of ITS+ (for composition see text) or 10% fetal bovine serum (FBS). Within each follicular stage, bars with no common superscript are different: a>b, b>c, c>d, $P < 0.05$; a>c, a>d, $P < 0.01$.

cultures provided these primordial follicles with one or several factors needed for their development. We and others have recently reported that a high percentage of primordial follicles in cortical pieces isolated from fetal or adult bovine ovaries also initiate development *in vitro* in serum-free medium (Wandji *et al.*, 1996; Braw-Tal and Yossefi, 1997). *In vivo*, subtle differences in the vascular supply within the cortex may account at least in part for the selectivity of initiation of primordial follicle growth. Alternatively, the high rate of activation of primordial follicles in our cultured cortical pieces could be due to the removal of the stromal and medullary components of the ovaries; if so, this would suggest that *in vivo*, an inhibitor of primordial follicle growth is produced

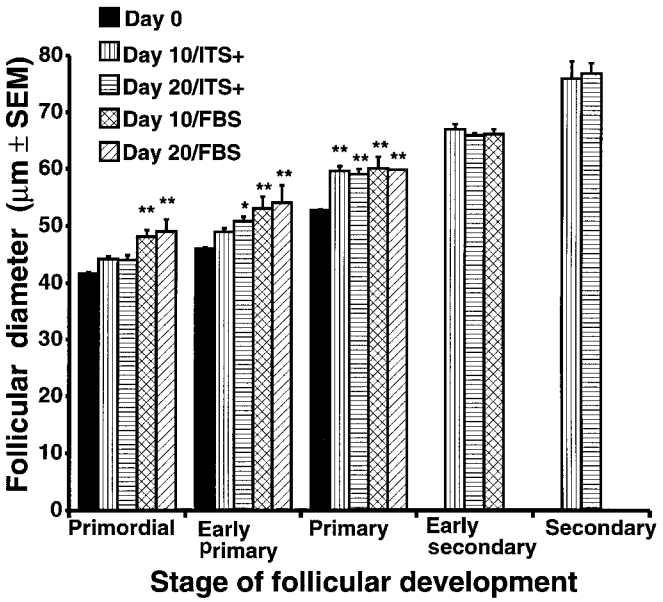

Figure 10. Diameter (μm; mean ± SEM) of healthy follicles in pieces of baboon ovarian cortex after 0, 10 or 20 days of culture in the presence of either ITS+ (for composition see text) or fetal bovine serum (FBS) ($n = 3$ fetuses, with 180–242 primordial, 127–190 early primary, 93–113 primary, 52–70 early secondary and 18–22 secondary follicles/oocytes measured per fetus). Within each follicular stage, bars with asterisks are different from day 0 (*$P <$ 0.05; **$P < 0.01$).

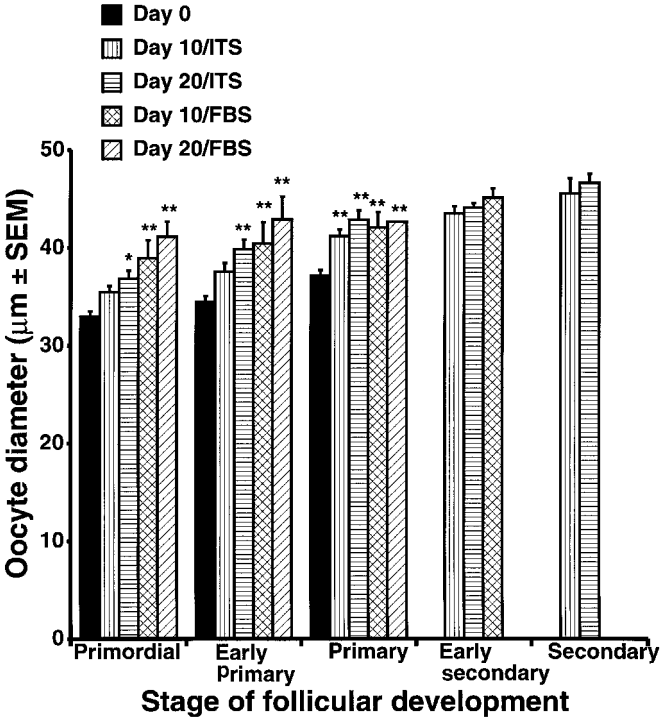

Figure 11. Diameter (μm; mean ± SEM) of oocytes in healthy follicles in pieces of baboon ovarian cortex after 0, 10 or 20 days of culture in the presence of either ITS+ (for composition see text) or fetal bovine serum (FBS) ($n = 3$ fetuses, with 180–242 primordial, 127–190 early primary, 93–113 primary, 52–70 early secondary and 18–22 secondary follicles/oocytes measured per fetus). Within each follicular stage, bars with asterisks are different from day 0 (*$P < 0.05$; **$P < 0.01$).

by the central part of the ovary. Finally, it has been reported that the fraction of follicles in the growing phase at any given time is inversely related to the number of follicles that remain in the resting pool (Krarup *et al.*, 1969; Hirshfield, 1994). Therefore, it is possible that a very high percentage of follicles initiated growth in the current experiments and in previous studies (Wandji *et al.*, 1996; Braw-Tal and Yossefi, 1997) in which small pieces of fetal or adult ovarian cortex were cultured because the initial number of primordial follicles in the ovary was artificially reduced by its division into small pieces. However, this hypothesis fails to explain why more follicles were activated in medium containing ITS+ than in medium with 10% FBS.

The addition of 10% FBS to ovarian cortical cultures inhibited the transition from primordial to primary follicles, relative to cultures with ITS+. Specifically, FBS maintained the pre-granulosa cells of primordial follicles in their flattened conformation, but supported growth of the oocyte. It appears that some factors in the serum are selectively inhibitory to the somatic cells of primordial follicles. Therefore, the growth of the germinal and somatic compartments of these follicles may be regulated by different factors.

Another interesting observation is that FBS appeared to increase, and ITS+ to decrease, the amount of interstitial tissue in cultured cortical pieces, relative to uncultured tissue. Eppig and O'Brien (1996) have observed that in FBS-treated cultures of whole neonatal mouse ovaries, primordial follicles entered the growth phase in a pattern roughly similar to the rate of primordial follicle activation *in vivo*. These observations, in conjunction with our current results showing that FBS is inhibitory to the activation of the somatic (pre-granulosa) cells of primordial follicles, but apparently stimulatory to the growth of interstitial/stromal cells, suggest that the initiation of growth of somatic cells of primordial follicles may be inhibited by non-follicular/stromal elements. In the presence of ITS+, such elements are much less prominent and more primordial follicles are activated. The role of such interstitial/stromal elements and their hypothetical inhibitory products warrants more investigation. Alternatively, FBS may exert independent effects on the interstitial cells and pre-granulosa cells of cortical pieces.

Although FSH binding and mRNA for FSH receptor have been detected in granulosa cells of pre-antral follicles (Wandji *et al.*, 1992; Tisdall *et al.* 1995), our current and previous observations (Eppig and O'Brien, 1996; Wandji *et al.*, 1996) clearly indicate that primordial follicle activation and the earliest stages of follicular growth can occur in the absence of gonadotrophins. However, the absence of substantial growth in secondary follicles in the current study may be due to a requirement for specific hormones and/or growth factors for the continuation of follicular development beyond that stage. One of these factors could be FSH. Although pre-antral follicles can develop in the absence of FSH, it has been shown that once follicles reach a diameter of 1 mm in the rhesus monkey, they degenerate if not adequately supported by FSH (Koering, 1969).

Before this stage of 'FSH-dependency', it is possible that FSH plays a more subtle role in follicular development. For example, FSH stimulates the production by granulosa cells from rat pre-antral follicles of a factor which stimulates thecal cell differentiation (Magoffin and Magarelli, 1995). The thecal compartment, which develops around the follicle during the secondary stage, is a source of growth factors that could be required to support follicular growth beyond the secondary stage. One such factor could be transforming growth factor-alpha (TGFα; Skinner and Coffey, 1988; Lobb *et al.*, 1989) which is a potent mitogen for granulosa cells (Skinner and Coffey, 1988). In the current study, growth factors of thecal origin may not have been available because of the reduction of stromal/interstitial elements in ITS+ cultures. Furthermore, some stromal elements, including macrophages and vascular pericytes, have been found to migrate among proliferating granulosa cells during the earliest stages of pre-antral follicle development in human ovaries (Bukovsky *et al.*, 1993). Macrophages, vascular pericytes and other mesenchymal cells such as platelets and endothelial cells are important sources of mitogenic growth factors and cytokines, e.g. basic fibroblast growth factor, epidermal growth factor and TGFα (for a review, see Klagsbrun and D'Amore, 1991), all of which are mitogenic for granulosa cells (Gospodarowicz and Bialecki, 1978, 1979; Skinner and Coffey, 1988). Consequently, the reduction in interstitial cells in cultured pieces treated with ITS+ in the current study may also account for the absence of growth in secondary follicles.

In summary, we have shown that primordial follicles from non-human primates can survive and develop to the secondary stage *in vitro* in serum-free conditions. Culture in medium containing 10% serum inhibited the growth of flattened pre-granulosa cells, but stimulated the enlargement of oocytes of primordial follicles and the development of interstitial cells. The current study demonstrates that initiation of growth of primate primordial follicles and subsequent development to the secondary stage can occur in the absence of gonadotrophins. Our results provide a basic model for further characterization of this least understood period of folliculogenesis and could have far-reaching impact in the treatment of some types of human infertility and the development of culture systems for oocyte donation.

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