Basic fibroblast growth factor promotes macaque follicle development *in vitro*

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Abstract

Fertility preservation is an important type of frontier scientific research in the field of reproductive health. The culture of ovarian cortices to i) initiate primordial follicle growth and ii) procure developing follicles for later oocyte maturation is a promising fertility preservation strategy, especially for older women or cancer patients. At present, this goal remains largely unsubstantiated in primates because of the difficulty in attaining relatively large follicles via ovarian cortex culture. To overcome this hurdle, we cultured macaque monkey ovarian cortices with FSH, kit ligand (KL), basic fibroblast growth factor (bFGF), and/or epidermal growth factor (EGF). The various factors and factor combinations promoted primordial follicle development to different extents. Notably, both bFF (bFGF, 100 ng/ml and FSH, 50 ng/ml) and KF (KL, 100 ng/ml and FSH, 50 ng/ml) contributed to the activation of primordial follicles at day 12 (D12) of culture, whereas at D18, the proportions of developing follicles were significantly higher in the bFF and KF groups relative to the other treatment groups, particularly in the bFF group. Estradiol and progesterone production were also highest in the bFF group, and primary follicle diameters were the largest. Up until D24, the bFF group still exhibited the highest proportion of developing follicles. In conclusion, the bFGF–FSH combination promotes nonhuman primate primordial follicle development *in vitro*, with the optimal experimental window within 18 days. These results provide evidence for the future success of human ovarian cortex culture and the eventual acquisition of mature human follicles or oocytes for fertility restoration.

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Introduction

Fertility preservation is a critical frontier research area in the field of reproductive health. Significant advances have recently been made in fertility management, especially for older female subjects who desire later fertility and for young cancer patients. In the latter case, cryopreservation of ovarian tissues before chemoradiotherapy or treatment of estrogen-sensitive tumors, followed by orthotopic or heterotopic implantation after symptom remission, can restore the ovaries and protect reproductive function. Worldwide, more than 30 live births have been reported in cancer patients after transplantation of frozen/thawed ovarian tissues (Gamzatova *et al.* 2014). However, in instances of leukemia or other cancers, grafting is associated with a risk of disease recurrence because reintroduction of malignant cells into the cryopreserved ovarian tissue can take place in patients with leukemia (Donnez *et al.* 2006, Dolmans *et al.* 2010, Rosendahl *et al.* 2010). Therefore,

to avoid the transfer of malignant cells, ovarian cortex culture ensuing *in vitro* differentiation of ovarian follicles or immature cumulus–oocyte complexes into mature oocytes is the best and perhaps only realistic option for fertility preservation (Picton *et al.* 2008).

We and numerous other research groups have attempted to culture isolated, preantral follicles for several days, with some preliminary success (Telfer *et al.* 2008, Amorim *et al.* 2009, Xu *et al.* 2009, 2013, Desai *et al.* 2010). Nonetheless, preantral follicles and oocytes are scarce, and preantral follicles can only rarely be isolated in clinical settings, particularly where estrogen-sensitive cancer patients and older subjects are concerned. Thus, ovarian cortex culture and primordial follicle activation are especially necessary to meet the demand for fertility preservation.

Initial attempts to promote primordial follicle development *in vitro* focused on the growth of isolated follicles. Many investigators clearly showed that it is fairly

straightforward to harvest rodent primordial follicles by mechanical means, while in the tougher ovarian cortex of large animals and human beings, primordial follicles must be isolated via mechanical together with enzymatic methods (Greenwald & Moor 1989, Oktay *et al.* 1997, Abir *et al.* 1999, Rice *et al.* 2008). However, the lack of influence from extrafollicular cortical cells leads to the demise of isolated primordial follicles *in vitro*. Therefore, in large animals and humans, the *in vitro* growth and maturation of primordial oocytes are only likely to be initiated *in situ* by culture of the ovarian cortex. Preantral follicles can then be isolated and grown into the antral stage of folliculogenesis. Accordingly, ovarian cortex culture and primordial follicle activation are both technically necessary for primordial follicle growth and development.

Activation of primordial follicles is modulated through an intricate balance between stimulatory and inhibitory factors (Picton 2001, Picton *et al.* 2008). Hsueh AJ. Laboratory has recently demonstrated fragmentation of ovary-disrupted ovarian Hippo signaling, leading to increased expression of downstream growth factors and promotion of follicle growth. Phosphatidylinositol 3 kinase (PI3K)/AKT pathway allows short-term activation of dormant ovarian follicles *in vitro* (Li *et al.* 2010, Kawamura *et al.* 2013). Addition of FSH, epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) are beneficial for the follicular transition (Matos *et al.* 2007a,b, Peng *et al.* 2010). Kit ligand (KL)/stem cell factor was one of the first identified factors in the promotion of primordial follicle development (Parrott & Skinner 1999); importantly, KL also promotes follicle survival and antral cavity formation of caprine preantral follicles *in vitro* in the absence of FSH (Lima *et al.* 2011).

EGF, for its part, is essential for the maintenance of intraovarian primordial follicle viability in the prepubertal cat, albeit without promoting follicle activation (Fujihara *et al.* 2014). Furthermore, EGF and FSH both increase the size of goat primordial follicles by stimulating oocyte growth (Silva *et al.* 2004). Contrarily, bFGF initiates the activation of primordial follicles in the rat (Kezele *et al.* 2002), and interactions between bFGF and KL effectively induce the primordial-to-primary follicle transition in the rat ovary (Nilsson & Skinner 2004). Matos *et al.* (2007a,b,c) showed that bFGF at a concentration of 50 ng/ml not only maintained the morphological integrity of caprine preantral follicles in culture for 5 days, but also stimulated the activation of primordial follicles and the subsequent growth of the activated follicles. FSH at 50 ng/ml functioned in a manner reminiscent of bFGF, as did the combination of FSH and bFGF (i.e., by stimulating the activation as well as the growth of primordial follicles into mature follicles) (Matos *et al.* 2007a,b,c). Other investigators similarly found that after 7-day culture of the goat ovarian cortex, FGF-10 upheld preantral follicle morphological integrity and promoted activated follicle growth *in vitro* (Chaves

et al. 2010). In addition, high doses of bFGF reportedly enhanced follicle development in frozen/thawed ovarian tissues of cancer patients under serum-free conditions, and also in cultured human fetal ovarian tissue (Garor *et al.* 2009).

Nevertheless, few reports are available regarding the impact of growth factors and hormone signals on human or nonhuman primate ovarian tissue culture, and the coordination of various growth factors and hormones required for proper follicle development and oocyte maturation *in vitro* remains challenging. Therefore, this study set out to evaluate the effects of EGF, bFGF, and KL in combination with FSH on the *in vitro* development of primordial follicles derived from the ovarian cortex of the macaque monkey.

Materials and methods

Ethical approval

This study was approved by the Institutional Review Board of Pecking University Third Hospital (Beijing, China). Acquisition of the macaque ovaries was in accordance with the Guide for Care and Use of Laboratory Animals of Peking University (Beijing, China). The protocol was approved by the Institutional Review Board of Animal Experiments of Peking University Third Hospital.

In vitro culture

Immediately after slaughter, ovaries were collected by laparoscopy from 2- to 3-year-old female macaque monkeys with normal body condition. The ovaries were transported in Leibovitz's L-15 Medium (Invitrogen) supplemented with 1% human serum albumin (LifeGlobal, Guilford, CT, USA), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Invitrogen), and placed on ice for transport to the laboratory within 2 h. The ovaries were washed three times with 0.9% saline supplemented with penicillin and streptomycin. Next, in each replicate, the cortices of eight ovaries were dissected into cortical plate fragments (~3 mm long×3 mm wide×1 mm thick), and 100 fragments were selected for the experiments described below. Four fragments were histologically analyzed as uncultured controls, and the remaining 96 fragments were randomly divided into the wells of a 24-well plate, with a total of six different treatments (four fragments per well, yielding a total of 16 fragments per treatment).

Each well contained 250 µl of culture media, consisting of basic medium (alpha-minimum essential medium (α-MEM) plus 1% human serum albumin, 1% insulin/transferrin/selenium (5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium, ITS) (Invitrogen), 50 mg/ml 6% ascorbic acid (Sigma-Aldrich), 2 mM L-glutamine, 75 mg/ml penicillin G, and 50 mg/ml streptomycin. In some cases, the medium was further supplemented with 50 ng/ml FSH or FSH plus EGF (75 ng/ml), bFGF (100 ng/ml), and/or KL (100 ng/ml). The concentrations of the growth factors were based on their stimulatory effects in earlier studies (Muruvi *et al.* 2005, Matos *et al.* 2007a,b, Peng *et al.* 2010). The six treatment groups

finally employed in this study were as follows: i) α -MEM, ii) FSH, iii) bFF (bFGF and FSH), iv) EF (EGF and FSH), v) KF (KL and FSH), and vi) EbKF (EGF, bFGF, KL, and FSH). The medium was changed every 3 days. Noncultured fragments were fixed in 10% formaldehyde on day 0 (D0), while cultured fragments were fixed in 10% formaldehyde on D6, D12, D18, and D24 post-culture for histological analysis.

Morphological classification and histological analysis of growing follicles

The fixed samples were embedded in paraffin wax, and every 12th serial section (5- μ m-thick) was chosen to prevent follicles from being counted twice. Routine hematoxylin and eosin staining was performed for histologic examination under light microscopy. Only normal or healthy follicles (spherical shape, oocyte-bearing, intact theca, regularly distributed granulosa cells) with visible oocyte nuclei were recorded.

Follicular stages were classified into five types based on the descriptions of Fortune (2003), as follows: i) primordial follicles (containing an oocyte surrounded by a single layer of flattened granulosa cells); ii) primary follicles (containing an oocyte surrounded by a single layer of cuboidal granulosa cells); iii) secondary follicles (containing a growing oocyte surrounded by two to five layers of granulosa cells); iv) preantral follicles (containing six or more layers of granulosa cells, or with a diameter of $>120\ \mu\text{m}$, and without any signs of antrum formation); and v) antral follicles (containing an oocyte surrounded by multiple layers of granulosa cells, and with an antrum). In this study, follicles from groups 2, 3, 4, and 5 were

collectively termed developing follicles (Gougeon 1996, Oktay *et al.* 1998, Fortune 2003). All follicles showing no signs of atresia were counted for each treatment group at D6, D12, D18, and D24. Follicle diameters were measured as the mean of two perpendicular diameters. The results of independent triplicate experiments were presented after Statistical analyses (see below).

Hormone assay

Culture media from all treatment groups at each time point (D6, D12, D18, and D24) were collected and stored at $-80\ ^\circ\text{C}$ until use for the hormone assay. The levels of 17β -estradiol (E_2) were measured by using a commercial RIA kit at a commercial laboratory (Chemclin Biotech Co. Ltd, Beijing, China). The sensitivity of the E_2 assay was $<3\ \text{pg/ml}$. Progesterone levels were assessed by using an ELIZA kit (Roche Diagnostics GmbH). The sensitivity of the progesterone assay was $0.03\ \text{ng/ml}$.

Statistical analyses

The percentages of follicles at different developmental stages and the follicle diameters were calculated and analyzed by first assessing the normality and homogeneity of the data variance, and then by conducting a two-way ANOVA. Next, a one-way ANOVA was conducted, followed by appropriate *post hoc* analyses with Tukey's test and the least square difference test. Hormone production levels were analyzed by conducting a repeated measures ANOVA. The values were considered to be

Table 1 Numbers of surviving primordial, primary, secondary, and preantral, and antral follicles in the ovarian cortex on D0, D6, D12, D18, and D24 post-culture.

	Days	Surviving follicles (n)				
		Primordial	Primary	Secondary	Pre-/antral	Total
Uncultured	0	890	33	39	22	984
α -MEM	6	510	52	30	5	597
	12	150	81	34	13	278
	18	35	20	7	0	62
	24	47	14	7	0	68
	6	154	12	12	3	181
FSH	12	237	165	43	3	448
	18	240	72	23	5	340
	24	288	88	9	2	387
	6	414	45	23	9	491
bFF	12	121	72	12	11	216
	18	307	144	56	13	520
	24	87	54	11	0	152
	6	1022	101	74	20	1217
EF	12	77	55	41	11	184
	18	259	36	16	3	314
	24	145	65	6	0	216
	6	253	67	31	16	367
KF	12	196	172	125	11	504
	18	185	50	1	0	236
	24	38	12	4	1	55
	6	417	81	12	3	513
EbKF	12	152	61	72	10	295
	18	203	36	18	5	262
	24	104	33	30	0	167

The six experimental groups were as follows: i) α -MEM, ii) FSH, iii) bFF (bFGF and FSH), iv) EF (EGF and FSH), v) KF (KL and FSH), and vi) EbKF (EGF, bFGF, KL, and FSH). α -MEM, α -minimal essential medium; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; KL, kit ligand.

Figure 1 Fractions of primordial and developing follicles in the six treatment groups (α -MEM, FSH, bFF (bFGF and FSH), EF (EGF and FSH), KF (KL and FSH), and EbKF (EGF, bFGF, KL, and FSH)) at selected time points (D6, D12, D18, and D24). The uncultured control group is denoted by D0. The asterisk (*) indicates that the cultured treatment groups were significantly different from the uncultured control (Ctrl) group (D0) ($P < 0.05$). Different lowercase letters (a, b, and c) indicate significant differences ($P < 0.05$ or $P < 0.01$) between the various treatments on the same day of culture.

statistically significant at $P < 0.05$. SPSS19 Software for Windows (SPSS Science) was used for all calculations.

Results

Development of follicles in the in vitro-cultured ovarian cortex

The numbers of surviving primordial, primary, secondary, preantral, and antral follicles were counted for the uncultured control at D0 and the various treatment conditions at D6, D12, D18, and D24 (Table 1). We then used these numbers to calculate the fractions of primordial and developing follicles at each stage of development.

Figure 1 shows the effects of α -MEM, FSH, bFF, EF, KF, and EbKF on follicle maturation at D6, D12, D18, and D24. In general, the fraction of primordial follicles in the ovarian cortex sections decreased with each time points of culture, while the fraction of developing follicles increased. At 6 days in culture (D6), the fractions of primordial follicles (~ 0.80 – 0.85) and developing follicles (~ 0.15 – 0.20) were similar across treatment groups, whereas at D12, the developing follicles increased in number, with the highest fraction observed in the bFF group. After 18 days in culture (D18), the fraction of developing follicles showed significant increases in both the bFF and KF groups relative to the other groups, but was still highest in the bFF group. Up until D24, bFF treatment continued to enhance follicle

development compared with the other five groups, although all groups showed attenuated follicle development at D24 vs D18. The addition of the factor EbKF (the combination of bFGF, EGF, KL, and FSH) did not significantly increase the percentage of developing follicles at any time point compared with single factors alone (Fig. 1).

Figure 2 shows the time-dependent actions of α -MEM, FSH, bFF, EF, KF, and EbKF on follicle development. The numbers of developing follicles increased gradually with time in culture from D6 to D18 for all treatment groups, with the highest numbers observed at D18, along with the lowest numbers of primordial follicles. However, in all cases, the percentage of developing follicles decreased at D24 to the levels similar to those observed at D12.

Histological observation of the in vitro-cultured ovarian cortex

Figure 3 shows representative histological hematoxylin and eosin-stained images of the ovarian cortex in the α -MEM and bFF groups on D6, D12, and D18 of culture. Every stage of follicle development is illustrated. Normal (healthy) follicles were spherical in shape and contained oocytes surrounded by regular granulosa cells and a circular distribution of theca cells. On the other hand, nearly only primordial follicles were observed in the uncultured control group (i.e., the untreated ovarian cortex at D0). The majority of the follicles in the α -MEM group remained in the primordial stage at D6, with only

Figure 2 Time-dependent effects of α -MEM, FSH, bFF, EF, KF, and EbKF on follicle development at D6, D12, D18, and D24. The uncultured control group is denoted by D0. The asterisk (*) indicates that the cultured treatment groups were significantly different from the uncultured control (Ctrl) group. Different lowercase letters (a, b, and c) indicate significant differences between the same treatment on different days of culture ($P < 0.05$ or $P < 0.01$).

Figure 3 Histological examination of the ovarian cortex. Images are shown at 20 $\times$ magnification for the uncultured control group (Ctrl), the cultured α -MEM group at D6, D12, D18, and D24, and the cultured bFF group at D6, D12, D18, and D24. Different types of follicles were labeled with the following: primordial, primary, secondary, and preantral. Scale bar: 50 μ m.

a few primary follicles discerned. At D12, some of the primordial follicles in the α -MEM group became activated, and developed to the primary or preantral stage. However, developing follicles no longer grow significantly at D18. Contrarily, an obvious tendency toward follicle development was observed in the bFF group, especially from D6 to D18. All groups exhibited decreased numbers of viable follicles and/or increased numbers of degenerated follicles until D24 compared with D18. Degenerated follicles corresponded to follicles with a retracted oocyte or a pyknotic nucleus, and/or those surrounded by disorganized granulosa cells that were detached from the basement membrane (Matos *et al.* 2007a,b).

Assessment of hormone production

We next assessed hormone production in the various treatment groups (Fig. 4). In general, all six treatments

increased progesterone production over time in culture, with the highest overall progesterone levels seen at D18. In detail, progesterone levels increased from D6 to D12, with the most progesterone production at D12 in the bFF and KF groups (bFF vs KF group, 50.6 ng/ml vs 48.6 ng/ml). At D18, progesterone production in the bFF group rose to 233.9 ng/ml; this value was significantly higher than that observed in any of the other treatment groups at the same time point. However, progesterone levels markedly decreased from D18 to D24 in the bFF group, and likewise in the FSH, EF, and EbKF groups. Progesterone production remained fairly constant in the α -MEM and KF groups during this time period (Fig. 4A and B).

Similar results were observed for E_2 production. The E_2 level in the bFF group was relatively high at D12, and significantly higher than in any of the other treatment groups at D18. E_2 levels were also higher at D18 in the FSH and KF groups vs the EF and EbKF groups. At D24,

Figure 4 Hormone profiles of progesterone (A and B) and estradiol (C and D) in the six treatment groups (α -MEM, FSH, bFF, EF, KF, and EbKF) at D6, D12, D18, and D24. Data are given as the means $\pm$ the S.E.M. Different lowercase letters (a, b, and c) indicate significant differences between the various treatments on the same day of culture (A and C) or significant differences between the same treatment on different days of culture (B and D) ($P < 0.05$).

the E_2 levels generally decreased to lower levels, which were similar to those observed at D12, except for in the α -MEM and FSH groups. In these two groups, E_2 levels were significantly lower at D24 than at D12 (Fig. 4C and D).

Measurement of follicle diameters

Lastly, the diameters of all surviving primordial, primary, and secondary follicles were measured at D6, D12, D18, and D24 (Fig. 5). No significant differences were observed for primordial follicle diameters within each treatment group across the various time points in culture. However, the diameters of the primordial follicles were

significantly larger in the bFF group than in the α -MEM, FSH, and KF groups ($P = 0.004$, 0.038 , and 0.013 respectively; not labeled in the figure). For the primary follicles, significant differences were generally seen between the D6, D12, D18, and D24 time points, in that the diameters at D24 were significantly smaller than those at D6, D12, and D18 ($P = 0.001$, < 0.001 , and 0.001 respectively; not labeled in the figure). In addition, the primary follicle diameters were clearly larger in the bFF group than in the KF and EbKF groups ($P = 0.002$ and 0.003 respectively); similar results were obtained for the EF group vs the KF group ($P = 0.004$). However, no significant differences were discerned among the secondary follicle diameters.

Figure 5 Effects of α -MEM, FSH, bFF, EF, KF, and EbKF on the diameters of primordial, primary, and secondary follicles in the ovarian cortex at D6, D12, D18, and D24. Data are given as the means $\pm$ the S.E.M. Different lowercase (a and b) and uppercase (A and B) letters indicate significant differences between the various treatment groups on the same day of culture (a and b: $P < 0.05$; A and B: $P < 0.01$).

Discussion

The use of ovarian cortex culture to obtain preantral follicles is an important strategy for fertility preservation. Several factors and hormones reportedly promote the survival and activation of primordial follicles (Esmailzadeh *et al.* 2013). However, until now, no efficient method was documented for early primordial follicle activation and maturation *in vitro* in primates. To address this shortcoming, the current study employed a macaque monkey ovarian cortex culture system to study the impact of EGF, KL, FSH, and bFGF on follicle development. The results show that KL and bFGF are crucial for the activation of primordial follicles. Moreover, with the extension of culture time, bFGF becomes more essential than KL to this process.

As noted previously, EGF critically participates in the maintenance of intraovarian primordial follicle viability in the prepubertal cat, but not in the promotion of follicle activation in cultured ovarian cortical slices. EGF exerts its actions via stimulation of MAPK and PI3K signaling pathways that, in turn, promote ovarian cell proliferation (Fujihara *et al.* 2014). Regardless of the presence or absence of FSH, EGF vs α -MEM can effectively increase the daily follicular growth rate of secondary follicles during the *in vitro* culture of caprine preantral follicles (Celestino *et al.* 2011). In addition, this study demonstrated that EGF is no more efficient than FSH alone in promoting the activation of primordial follicles, given that we found no significant increase in the fraction of developing follicles in the EF vs FSH treatment group at D18 (Fig. 1). Furthermore, we failed to observe an increase in E₂ and progesterone production in the EF (the combination of EGF and FSH) vs FSH alone treatment group at time points before D18 (Fig. 4). However, EGF apparently contributes to follicle survival in long-term cultures, because higher E₂ and progesterone levels were detected in the EF vs FSH group at D24. Therefore, we conclude that although the actions of EGF and FSH are not additive in the stimulation of follicle development, EGF itself can benefit follicle survival.

KL produced by granulosa cells appears to stimulate oocyte growth and to initiate oocyte development in assorted animal models (Parrott & Skinner 1999, Kezele & Skinner 2003, Skinner 2005). For example, KL induces a primordial-to-primary follicle transition in the rat ovary (Parrott & Skinner 1999), and likewise triggers a reduction in the percentage of primordial follicles with a concomitant increase in the percentage of primary and secondary follicles in the neonatal murine ovary (Gougeon *et al.* 2010). In addition, KL stimulates the activation of primordial follicles in the goat, promotes the survival of antral cavity formation by caprine preantral follicles after *in vitro* culture, and facilitates the growth and meiotic resumption of caprine oocytes in the absence of FSH (Celestino *et al.* 2010, Lima *et al.* 2011). However, according to another report, KL-kit

interactions might not be associated with the growth initiation of primordial oocytes in the pig, although KL through kit does appear to enhance the survival of porcine oocytes (Moniruzzaman & Miyano 2007).

KL/kit signaling through PI3K promotes ovarian follicle maturation, but is dispensable for primordial follicle activation in mice (John *et al.* 2009). We found that KF (the combination of KL and FSH) increased the numbers of developing follicles in the macaque ovarian cortex, especially at D18 (Figs 1 and 2). Therefore, we propose that KL can activate primordial follicle development in the prepubertal macaque ovary in the presence of FSH. Moreover, the KF combination enhanced progesterone and E₂ levels relative to α -MEM. However, no consistently additive effects were observed for KL and FSH actions in hormone production before D18 (Fig. 4).

Studies in mammals demonstrate the expression of bFGF and its receptors in early human ovarian follicles (Yeh & Osathanondh 1993, Quennell *et al.* 2004, Ben-Haroush *et al.* 2005). In addition, bFGF produced by the oocyte during the early stages of follicle development apparently acts on adjacent somatic cells to promote cell growth and maturation. Thus, bFGF, similar to the action of KL, appears to be a primordial follicle-inducing factor. In support of this hypothesis, bFGF treatment dramatically decreased primordial follicle numbers and increased developing follicle numbers in cultured 4-day-old prepubertal rat ovaries, similar to the actions of KL (Nilsson & Skinner 2004). Both bFGF and KL are required to optimally promote the primordial-to-primary follicle transition, and oocyte-derived bFGF functions in part to increase KL expression in granulosa cells. Furthermore, bFGF regulates primordial follicle development to directly influence female reproductive efficiency (Nilsson *et al.* 2001).

Recently, we established that bFGF promotes the development of isolated preantral follicles *in vitro* (Wang *et al.* 2014). However, many infertility patients present with few or no preantral follicles, or even without any visible follicles. Ovarian cortex culture now supplies hope for fertility preservation in these follicle-deficient patients, as well as for older women with declining fertility. In this study, we found a positive role for bFF (the combination of bFGF and FSH) not only in the activation of follicle development in macaque ovarian cortex culture, but also in the growth of small-diameter follicles, including preantral follicles (Figs 1, 3, and 5). Furthermore, the hormone levels of progesterone and E₂ were remarkably higher in the bFF group compared with the other treatment groups at D18, and also reached their peak levels at D18 (Fig. 4). As such, our findings indicate that bFGF can be used in combination with FSH for *in vitro* follicle development during an appropriate time window spanning the primordial to preantral stage.

In this study, EbKF (the combination of EGF, bFGF, KL, and FSH) was no more efficacious than bFF alone in the stimulation of follicle development (Figs 1 and 2),

implying that EGF and KL cannot augment the actions of bFGF and FSH. Moreover, this study used bFGF at a concentration of 100 ng/ml, although we previously employed the growth factor at 200 ng/ml for *in vitro* follicle culture. Whether bFGF is more effective at 100 or 200 ng/ml is unknown in future human ovarian cortex culture and will require further comparative experimentation. Regardless, bFGF almost certainly can be selected to enhance primordial follicle activation and growth to the preantral stage in humans as well as macaques.

Our research group also previously demonstrated that bFGF might substantially improve the quality of transplanted murine ovarian tissues by increasing follicle quantity and by promoting neoangiogenesis (Gao *et al.* 2013). At D24, the percentage of developing follicles and the levels of progesterone and E₂ were lower or similar to those at D18 and D12 (Figs 1, 2, and 4). These findings might be explained by the fact that after long-term culture of ovarian cortex, some of the follicles develop into large preantral follicles or more advanced premature follicles. These latter follicle types require more complicated culture conditions (e.g., a three-dimensional culture system, which we successfully used in an earlier study of preantral follicle growth; Wang *et al.* 2014). We also suggest that after 18 days, the preantral follicle should be isolated from the ovarian cortex and cultured independently due to the absence of adequate nutrients and oxygen in the intact cortical tissue. In conclusion, we showed that the combination of bFGF and FSH is sufficient to activate primordial follicle development in the cultured macaque monkey ovarian cortex. The results of this study may provide a foundation for future human ovarian cortex culture to restore fertility in cancer patients and older women, and other subjects with impaired or degenerating follicles. First, our short-term goals are to obtain preantral or antral human follicles by culturing the human ovarian cortex with bFGF and FSH, and then to isolate the follicles for further maturation *in vitro*; or alternatively, to attain mature human oocytes for fertility preservation or assisted reproduction.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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